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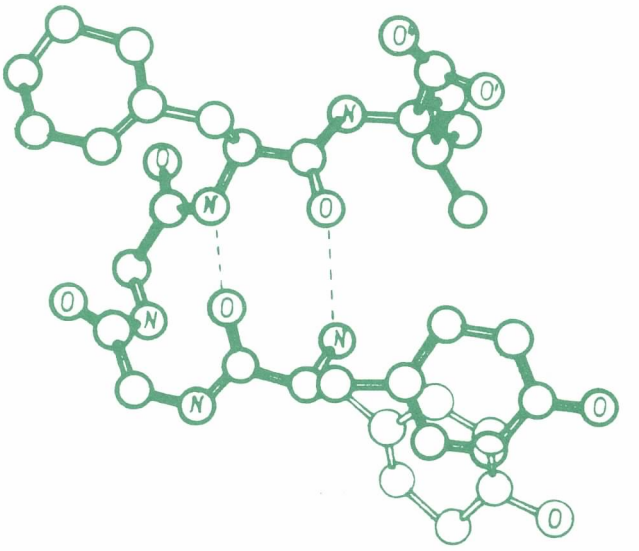
Opioid Peptides: Medicinal Chemistry



DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

Opioid Peptides: Medicinal Chemistry

Editors:

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Division of Preclinical Research
National Institute on Drug Abuse

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Opioid Peptides: Medicinal Chemistry

ACKNOWLEDGMENT

This monograph is based upon papers and discussion from the technical review on the medicinal chemistry and molecular pharmacology of opioid peptides and the opiates which took place on September 4 - 6, 1984. at Bethesda. Maryland. The meeting was sponsored by the Office of Science and the Division of Preclinical Research, National Institute on Drug Abuse. The papers on medicinal chemistry are presented in this volume; those on the molecular pharmacology, biosynthesis, and analysis of opioid peptides appear in NIDA Research Monograph 70.

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Foreword

The search for the molecular basis of drug addiction led, in the mid-seventies, to the exciting discovery of opioid receptors and a variety of endogenous and exogenous ligands. The fact that thousands of publications have appeared already is astounding and attests to the attention attracted by this area. The discovery of multiple opioid receptors brings an additional dimension to research in this area, and medicinal chemists have already succeeded in synthesizing many highly specific ligands to these receptor subtypes.

The interest in this area is not only to develop nonaddictive peptide analgetic substitutes for morphine, but also to understand the role of these endogenous peptides in health and in disease. Although several thousand analogs of enkephalins and other opioid peptides have been synthesized, including some that are stable and can be taken orally, a nonaddicting opioid has yet to be synthesized.

Hence, the National Institute on Drug Abuse felt that it was timely to call together experts in the area to evaluate current research on the development of new opioid peptides with potential value for basic research and therapeutics.

In this monograph, emphasis is placed on structure-activity relationships, conformational analysis using various spectroscopies, computer assisted drug design, and molecular mechanics. It is hoped that the discussions of current research by various experts presented here will helpful in establishing future goals for development of new molecular entities with desired biological activity or receptor specificity. An effort has been made to cover all aspects of medicinal chemistry pertaining to opioid peptides, so that the volume can serve as a broadly useful reference text.

Marvin Snyder, Ph.D., Director
Division of Preclinical Research
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Introduction

Rao S. Rapaka, Ph.D.

The discovery of opioid peptides has opened up new scientific area and posed questions which are still at an early stage of exploration. This area is of major interest to the immediate and long-range goals of the National Institute on Drug Abuse, and in order better to focus the rapidly expanding research areas associated with peptide chemistry and its potential application to drug abuse research and therapeutic treatment, the Institute sponsored a technical review in September 1984.

This monograph presents contributions both from the symposium speakers and from other invited authors in the various aspects of the medicinal chemistry of the opioid peptides and the opiates. Highlights of these reviews are presented here. Biosynthetic, analytical, and molecular pharmacology aspects are presented in NIDA Research Monograph 70.

A number of presentations are on structure-activity relationships (SAR) of the opioid peptides--Drs. Morley and Dutta on the SAR of enkephalins, Dr. Chang on morphiceptins, and Dr. Feuerstein on dermorphins. Conformational restriction has produced, to date, highly active μ- and δ-selective enkephalin analogs. This feature is described extensively by Drs. Schiller and Hruby. In addition, conformational restriction by stereochemically constrained analogs is described by Drs. Kishore and Balaram. Very recent work on the synthesis of dehydro and cyclopropyl amino acids is presented by Dr. Stammer; and a general review on enkephalin SAR, including the design of peptides with predictable μ/δ specificity and the synthesis of enzymatically stable peptides, is presented by Dr. Shimohigashi. An excellent and elaborate account of peptide conformation and biological activity, with general examples from such peptides as gramicidin analogs and elastin analogs, is given by Dr. Urry.

A number of presentations are related to conformational analysis of the opioid peptides, advanced methodologies, and design of peptides. Nuclear magnetic resonance as a tool in determination of conformations, along with a preliminary background on peptide conformations (such as β-turns and β-sheets), 2-D NMR, and NOE, is

presented by Dr. Khaled. An update on X-ray crystallographic research on enkephalins and enkephalin analogs, such as [p-bromo Phe^4]-leucine enkephalin and Tyr-D-Nle-Gly-Phe-NleS, is presented bv Drs. Camerman and Camerman. Applications of the comolementarv techniques FT-IR and laser Rama spectros copies to the determination of conformational states of Leu^5- and Met^5- enkephalins are described by Dr. Renugopalakrishnan and colleagues. An account on computer-aided drug design is given by Dr. Marshall and colleagues, and an investigation on SAR of opioids by an interdisciplinary approach is discussed by Dr. Loew and her associates. An excellent account of conformational analysis and conformational restriction in the design of receptor-selective analogs is given by Dr. Schiller. Great progress in research on opioid peptides has been made possible by simultaneous advances in the techniques of synthesis, purification, and analysis of peptides. In this volume, synthetic techniques are discussed by Dr. Stewart.

In this monograph, one approach to understanding receptor types, by affinity labels, is discussed by Drs. Portoghese and Takemori. Receptor types and analysis of binding data are dicussed at greater length in NIDA Research Monograph 7O.

A review of the historical search for a nonaddicting analgesic--starting from days of opium use to the development of agonists, antagonists, and the advent of opioid peptides--and of future hopes is presented by Dr. Archer.

Through the interdisciplinary exchanges that took place at this conference and the resulting volumes, an effort has been made to summarize the existing information in the area of opioid peptides and to discuss the implications of that information for future research objectives.

Frontiers of Research in the Medicinal Chemistry and Molecular Pharmacology of Opioid Peptides

Rao S. Rapaka, Ph.D.

The conquest of pain has been long sought by monks, medical practitioners, quacks, and laymen, alike, and each of them has attempted to achieve their goal in their own way. Opium was used for centuries by the ancient cultures for its analgetic effects. In 1806, Serturner discovered the analgetic effects of morphine. However, the side effects of the opiates, such as addiction liability and respiratory depression, were soon realized. This further motivated research for other compounds, either from naturally occurring plant drugs or synthetic sources. A number of useful compounds--such as meperidine, methadone, d-propoxyphene, nalorphine, phenazocine, etc.--were synthesized. However, the goal to discover an ideal analgetic without the undesirable side effects has not been achieved. In the meantime, advances in the neurosciences, endocrinology, protein synthesis, molecular biology, and other sciences have resulted in a better understanding of brain function and its modulation. The characterization of opiate receptors (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973) and later the isolation and structure determination of the enkephalins (Hughes et al. 1975) have ignited an explosive interest in the understanding of the role of the endogenous ligands in general, and their role in brain function in particular. Subsequent developments in recombinant DNA technology added an important tool to research on precursors of the endogenous ligands.

The opiate receptor--the site where the opiates interact to produce the biologic effect--was soon to be called the opioid receptor, as the endogenous ligands are peptides. A number of other opioid peptides were soon discovered. Following their discovery, several hundred synthetic analogs were made and tested. Extensive pharmacological testing and studies on binding at the receptor site showed that the opioid receptors are heterogeneous (Martin et al. 1976) and that at least four types of receptors exist (Martin et al. 1976; Lord et al. 1977). As information accumulated on opioid peptide families and their functions, it seemed that it would be useful to discuss the pertinent questions on the medicinal chemistry and molecular pharmacology of opioid peptides.

Research on opioids is progressing in several directions. Some of the important topics that have attracted a great deal of attention by researchers are: biosynthesis, processing of precursors, endogenous antagonists, specificity in drug design, conformational restriction in analog design, inhibitors of enkephalin-degrading enzymes as analgetic drugs, conformational features of peptides, conformation-receptor selectivity, retro-inverse analogs, amphiphilic helices, receptor types and their structure and organization, drug-receptor interaction, the role of lipids in the interaction, and analytical methodology. Some of these aspects are reviewed in a preliminary fashion below. (For additional information, see reviews by Udenfriend and Kilpatrick 1983; Coplov and Helme 1983; Hughes 1983; Rapaka et al. 1985.)

Biosynthesis of opioid peptides is being actively investigated. A number of translational and posttranslational events are involved in the generation of active peptides, and these processes occur in a well-defined order. Processing of the opioid peptides appears to be tissue-specific, and different sets of processing enzymes seem to be responsible for the tissue-specific processing of the neuroendocrine peptides. Pairs of basic amino acids serve as signals for the processing enzymes. The enzymes appear to be trypsinlike and, quite often, carboxypeptidase like activity is also involved (Fricker 1985; Rapaka et al., in press). Other posttranslational modifications include phosphorylation, acetylation, sulfation, methylation, glycosylation, amidation, etc.

Processing of precursor peptides to bioactive peptides is largely explored using synthetic peptides (Marks et al., this volume; Rapaka et al., in press). In addition to the "dibasic residues," X-Arg-Pro- appears to be another processing signal as postulated by Matsuo (NIDA Research Monograph 70), or referred to as the single arginine cleavage (Weber et al. 1983). This is exemplified by the generation of metorphamide (Weber et al. 1983) or adrenorphin (Matsuo et al., NIDA Research Monograph 70) from proenkephalin (corresponding to 210-217). Another example of single arginine cleavage is the generation of dynorphin(1-8) from prodynorphin corresponding to 209-216 residues). Single arginine cleavage was also postulated for the processing of dynorphin B-29 to dynorphin B(1-13) at the Thr-Arg bond (Devi and Goldstein 1984). Here it is to be noted that Arg is followed by Ser (not Pro).

Another interesting processing event is amidation. The mechanism of C-terminal amidation was investigated by Bradbury et al. (1982). C-terminal amidation involves the action of a specific enzyme, and glycine is a mandatory amino acid in the C-terminal position. The amide does not arise from amnonia or by direct amidation or transamidation. The mechanism involves removal of hydrogen from the C-terminal glycine and spontaneous hydrolysis of the imino linkage. Amidation is well known for neurohypophyseal peptides, oxytocin, and vasopressin; and

more and more amidated opioid peptides are being isolated. Metorphamide or adrenorphin Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH_2 is an amidated peptide. Dermorphin, Tyr-D-Ala-Gly-Phe-Tyr-Pro-Ser-NH_2, is another amidated peptide and is isolated from the skin of the South American frog of the phyllomedusa species (Montecucchi et al. 1981). Recently, another amidated peptide, amidorphin, was isolated from bovine adrenal medulla (Seizinger et al. 1983). A very significant aspect in the isolation of this peptide is that it was logically deduced to be a processed product of the known bovine pro-enkephalin A sequence, as it has the necessary processing signals, including a terminal glycine essential for amidation. The other significant aspect of the paper is that the expected product was first synthesized to establish the presence of the proposed endogenous product.

An important aspect of amidation is its effect on receptor selectivity. This is reflected in the decreased δ-receptor affinity of Met^5-enkephalinamide as compared to Met^5-enkephalin. However, amidation of Val-8, as in metorphamide, causes an increased affinity at the k-receptor site with an insignificant change at the μ and δ sites.

Li (NIDA Research Monograph 70) and colleagues have determined the primary sequence for β-endorphins from various species. They all consist of 31 amino acids with Tyr-Gly-Gly-Phe-Met at the N-terminal sequence. Although minor differences are noted in the primary sequence for β-endorphins isolated from various species as compared to the human, it appears that the amino acid sequence in general is highly conserved. These various β-endorphins isolated from several species, due to minor changes in the primary sequence, may be considered as the naturally occurring analogs of the β_h-endorphin. Biological activity of some of these endorphins was evaluated and it was found that the ratio of binding activity to analgetic potency varied for these endorphins. This might be due to the fact that once the peptide was bound to the receptor, the analgetic potency might depend on the efficacy of the agonists.

In addition, smaller fragments of β-endorphin appear to be present in the pituitary glands of several animals. In fact, β-endorphin (1-27) was isolated from porcine tissue (Smyth et al. 1978) and was later synthesized (Zaoral et al. 1981) . This naturally occurring β_h-endorphin (1-27) inhibits the analgetic properties of β_h-endorphin. Other synthetic fragments of β_h-endorphin (1-28) and (1-26) also inhibit analgesia induced by β_h-endorphin (Nicolas and Li 1985). These observations have led Li and colleagues to formulate the following hypothesis: "Inhibition of a peptide hormone by a naturally occurring segment of the same hormone may be a general phenomenon in biologically active peptides."

After the realization that the opioid receptor is heterogeneous and that there are at least three types of receptors (μ, δ, and k) and possibly a fourth type (σ), several groups have attempted

to understand the features essential for receptor selectivity. Several thousand analogs of enkephalins were synthesized and receptor specificity was investigated (see Shimohigashi, this volume; Fournie-Zaluski et al. 1981; Roques et al. 1982). Following rational design, δ-receptor specific enkephalin analogs, such as H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSLET) and H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET), were developed. To summarize briefly, the principal characteristics for δ-receptor binding affinity for noncyclic (excluding the conformationally restricted analogs) enkephalin analogs are:

a) hydrophilic side chain in position 2,
b) aromatic residue (4th position),
c) C-terminal carboxyl group in the fifth position, and
d) the lengthening of the peptide chain by Thr^6 residue. Here, it is to be noted that ($D\text{-}Pen^2$, $D\text{-}Pen^5$) enkephalin, a conformationally restricted analog, is the most selective δ-ligand available (See Hruby, this volume and references therein).

Characteristics of high specificity for μ-binding sites are,

a) D-residue in the second position,
b) shortening of the enkephalin sequence,
c) removal of the terminal carboxyl group,
d) replacement of Phe^4 residue with lipophilic alkyl chain and stereo-orientation of the Phe^4 residue, and
e) N-methylation of Phe^4 residue.

Although these modifications produced significantly improved receptor-specific ligands, alternate approaches were also attempted.

One of the synthetic approaches that has resulted in the development of highly receptor-selective ligands is conformational restriction or constriction. For an excellent minireview on conformational restriction in peptides via amino acid side chains, see Hruby (1982). Linear peptides are highly flexible and exist in a conformational equilibrium. Moreover, the conformational states may be influenced by the solvent and the surrounding environment. Hence, if one of the various conformers is the most active form, then dilution of the active forms can be avoided by reducing the number of inactive forms. Formation of cyclic peptides is one of the main modes. Some other structural modifications include N-methylation of the amide NH, replacement of the C^{α}-H (to C^{α}-CH_3), formation of disulphide bonds, and similar side-chain cyclizations. These conformationally restrained analogs might have increased agonist or antagonist properties due to enhanced conformational integrity, thus possessing increased specificity and biological activity. These analogs may have increased enzymatic stability, as well. This technique has already been utilized for developing analogs with desired properties in the oxytocin and bradykinin series. In the opioid peptides, a number of conformationally restricted analogs were synthesized for the

enkephalin peptides. As the integrity of the Tyr residue has to be maintained at position 1, and as manipulations are permissible at positions 2 and 5, mast of these analogs were modified at positions 2 and 5. One of the earliest syntheses was by Schiller and colleagues using D-α, w-diaminobutyric acid as the second residue and affecting the cyclization of w-amino group to the C-terminal carboxyl group (DiMaio and Schiller 1980; DiMaio et al. 1982). Similarly, other cyclic peptides were synthesized utilizing cyclizations between the side chains of Lys and Orn, with those of Asp and Glu (Schiller et al. 1985). Generally, the cyclic peptides exhibited preference for μ-receptors over δ-receptors and were biologically more active, whereas the corresponding open chain analogs did not show similar preference or enhanced activity. Another type of side-chain to side-chain cyclization was attempted using Cys residues in the 2 and 5 positions.

Using this technique, Hruby and colleagues (this volume) syntesized a series of analogs. Among these analogs, [D-Pen^2, Cys^5]-enkephalin, [D-Pen^2, Pen^5]-enkephalin and [D-Pen^2, D-Pen^5]-enkephalin are some of the very highly selective δ-receptor selective compounds. (D-Pen^2,D-Pen^5)-Enkephalin demonstrates beneficial effects of further conformational restriction of gem-dimethyl groups of the Pen moiety. It also appears that the cyclic analogs and the linear compounds have similar modes of binding to receptor sites.

Other types of sterically constrained enkephalins are those that contain α-amino isobutyric acid and cyclopentane-1-carboxylic acid residues (see Kishore and Balaram, this volume).

Another mode of conformational restriction is the synthesis of peptides incorporating α,β-dehydroamino acids (referred to as the dehydro- or Δ-amino acids) or the cyclopropyl amino acids (▽-amino acids). These amino acid substitutions confer unique chemical and stereochemical properties, as they effect both chemical reactivity and conformation. For reviews, see Noda et al. (1983) and Stammer (this volume, 1982). A substitution at the second position, i.e., Δ-Ala^2-enkephalin, facilitated, interaction at the μ-receptor site (due to enhanced lipophilicity), whereas substitution at 3-position in enkephalin may have facilitated the interaction at the δ-receptor site. Studies on Δ-Phe^4-enkephalins indicated that the Z-compound (phenyl ring and C=O are trans) sterically favors interaction with the δ-receptor site (Shimohigashi and Stammer 1983). An additional advantage of substitution with amino acids is that peptide linkages with α, β-dehydroamino acid residues are highly resistant to enzymatic degradation at the C-terminal side and completely resistant at the N-terminal side (Shimohiqashi et al. 1982). Enkephalin analogs with cyclopropyl amino acid residues seem to be resistant to enzymatic degradation as well (Stammer, this volume).

To date, conformational restriction appears to provide the most successful approach in the design of synthetic analogs with high

receptor selectivities.

Another original approach for improving the selectivity of receptor binding, although less explored, is formation of dimeric peptides (Shimohigashi, this volume; Shimohigashi et al. 1982; Rodbard et al. 1983). Dimeric penta- and tetra peptide analogs of enkephalins were shown to exhibit enhanced affinity and selectivity for the **δ**-receptor.

The biological activities of peptides generally depend on both primary and secondary structure. For some cyclic peptides it was shown that retro-isomers--in which the direction of the amide bonds linking the residues is reversed--retain biological activity. In these cyclic peptides, it may be assumed that biological activity results from the three dimensional topology and not from the peptide backbone. The concept of retro-peptides has also been extended to linear peptides (Goodnan and Chorev 1979). To obtain closeness in three-dimensional structure, a further modification of chirality at each chiral center was also investigated. These modified analogs are named "retro-inverso-peptides." Several partially modified retro-inverso-peptides were synthesized for a number of biologically active peptides. These modifications helped in assessing the relative importance of side-chain versus backbone structure in conferring biological activity. An additional reason for synthesis of these analogs is the expected resistance to biodegradation. This concept was applied in developing analogs of enkephalins (Berman et al. 1983), enkephalinase inhibitors such as retrothiorphan (Roques and Fournie-Zaluski, NIDA Research Monograph 70), and dermorphin (Salvadori et al. 1985).

Prediction of the formation of certain types of secondary structures from the amino acid sequences is very helpful in the rational design of biologically active peptides. The amphiphilic environment of the aqueous solution and lipid bilayar of the membrane could impose a secondary structure on the peptide. If the secondary structure formation is initiated by the membrane, it is likely to be characterized by the amphiphilic distribution of the individual amino acid side chains; amphiphilic helical structures have been hypothesized to be important in determining the biological activity of a number of peptides. This approach was successfully utilized in the design of **β**-endorphin analogs (Blanc et al. 1983). For a review on amphiphilic secondary structures, see Kaiser and Kezdy (1984).

One of the main problems in the bioassay of the peptides and in their use as analgetics has been their ease of biodegradation (NIDA Research Monograph 70: Marks et al.; Shimohigashi; Roques and Fournie-Zaluski). Enzymes involved in the metabolism of opioid peptides, as well as in their isolation, assay, and specificity are described by Marks et al. Enkephalins are easily metabolized; Tyr^1-Gly^2 bond is cleaved by amniopepidases, Gly^3-Gly^4 bond by enkephalinase, and Phe^4-Leu^5 (Met^5) by carboxypeptidases. Although extensive research has been carried out on these enzymes, the specificity

of enzymess and the precise mechanisms of action are still not established. In addition to the specific cleavage at a particular amide bond, sequential processing has also been proposed as an alternate mechanism of action (Fricker 1985; Mains et al. 1983; Rapaka et al. in press).

In order to prevent the degradation of the enkephalins, a variety of approaches were utilized, such as replacement of D-amino acid for L-amino acid, N-methylation of the amide linkages, replacement of amide linkages, or substitution with unusual amino acids (dehydroamino acids, etc.). A closely related but more innovative approach is the rational design of inhibitors for the enkephalin-degrading enzymes (Roques and Fournie-Zaluski, this volume). These compounds will prolong the duration of action of the endogenously released enkephalins and, hence, may be devoid of the major side effects of narcotic analgetics. A number of enkephalinase inhibitors are derived from Phe-Leu bearing carboxyl groups (to chelate Zn required for this metalloenzyme) and are of the general formula: R-CH-($CH_2\phi$)-CONH-CH[$CH_2CH(CH_3)_2$]COOH with R=-COOH,-CH_2COOH, -NH-CH_2-COOH and -NH-CH_2-CH_2-COOH. Highly potent enkephalinase inhibitors were also obtained from L-Phe-β-Ala derivatives. Another series of enkephalinase inhibitors was obtained from compounds that contained a thiol group, R-CH-($CH_2\phi$)CONH-CH[CH_2CH-(CH_3)2]-COOH, where R=-CH_2SH and -SH-CH_2-CO. Although these are excellent enkephalinase inhibitors, they are also angiotensin converting enzyme (ACE) inhibitors. To enhance the specificity, the "retro" compound HS-$CH_2CH(CH_2\phi)$ NH-CO-CH_2-COOH, retrothiorphan, was developed which, while retaining enkephalinase inhibitors property, exhibited significantly less ACE inhibitor property. In summary, this type of rational design has provided researchers with a number of inhibitors, such as thiorphan, retrothiorphan, and kelatorphan. The clinical use of these compounds, however, is not promising, due to the limited bioavailability of some. Development of synthetic analogs that are orally active or have good bioavailability may be of therapeutic use; and this is expected to be achieved in the near future.

Conformational aspects of opioid peptides have been examined extensively. Most of the work has been focused on enkephalins, on morphiceptins, and to some extent on endorphins. The presence of a β-turn in enkephalins was proposed, from x-ray crystallographic data and solution studies (Camerman and Camerman, this volume; Schiller, this volume; Schiller 1984). The number of hydrogen bonds (2 or 3), and the type of β-turn (type I or II or I' or II') are not yet agreed upon universally and much more work is warranted in this area. Recently, Yamashiro and Li (1984) reviewed the conformational features of endorphins.

Some of the other opioid peptides of interest are dermorphin and dynorphin. Few conformational studies are reported on dynorphin in the literature (Schiller 1983; Maroun and Mattice 1981).

From circular dichroism studies on dynorphin(1-13), Maroun and Mattice concluded that dynorphin does not have an ordered structure in water. However, addition of a detergent, such as dodecyl sulfate, enhances helical content to some extent. From fluorescence studies on the biologically active Trp^4-analogs of dynorphin(1-13), Schiller observed that Tyr^1-Trp^4 are at least 15 A^o apart (unlike the enkephalin Tyr^1-Trp^4 residues, which appears to have a folded conformation) and the predominant conformation is where the N-terminal tetrapeptide is almost completely extended. In addition, Schiller proposed that the these conformational differences between dynorphin and Leu^5-enkephalin may play a role in preferences for various opioid receptor subclasses.

To date, few conformational studies have been done on dermorphin (Salvadori et al. 1983; Arlandini et al. 1985; Bhatnagar et al., in press). Salvadori et al. suggested the presence of a linear extended structure from NMR studies and, due to lack of significant dependence on PMR parameters over a concentration range, the results indicated a monomeric peptide. Arlandini et al. (1985), using various spectroscopic techniques, suggested that dermorphin may preferentially exist in an extended, flexible conformation and that an intermolecularly stabilized form exists in DMSO. Recently, from FT-IR, laser Raman, CD, conformational energy calculations, and molecular modeling studies, it was proposed that dermorphin contains two **β**-turns--a type III **β**-turn between residues one and four and a type II **β**-turn between residue five and the terminal amide group. This conformation with two **β**-turns is highly folded and mimics the "morphine-type" conformation; and due to this conformation, it may act as a potent μ-receptor aqonist. This highly folded conformation and the hydrophobic environment may explain its stability as well (Bhatnagar et al., in press).

Although earlier conformational studies mainly addressed the correlation of structure between opioid peptides and rigid opiates, the discovery of multiple opioid receptors and the realization that different opioid receptor types may have differing conformational requirements (Schiller and DiMaio 1982) changed the direction of research in this area considerably. Conformational analysis using theoretical methods has been extensively reviewed by Gorin et al. (1978) and Schiller (1984). In addition, computer-assisted drug design (Marshall, this volume) and multidisciplinary approaches have also played a significant role in the understanding of conformational features. Recently, a computational procedure for determining the energetically favorable binding sites on macromolecules has been reported (Goodford 1985) and this may be of help in druq design.

The role of conformation and its relation to biological activity is not fully established. The peptide may exist in a number of conformations and may continuously interconvert, especially in the case of small peptides such as the enkephalins. It is a question of whether a bioactive conformation preexists, such as

in the hydroxylation of protocollagen by proline hydroxylase (Rapaka et al. 1978) or is adopted (such as the "zipper"-type model, Burgen et al. 1975; See also Schiller, this volume). Similar to the concept of Burgen et al. is the concept of "orbital steering," where large rate accelerations are observed in enzymatic reactions. In orbital steering, the reactive atoms are constrained to react along selected pathways (Dafforn and Koshland 1971). It is conceivable that the opioid receptors have conformational requirements for interaction which differ depending on the receptor type (Schiller and DiMaio 1982). A folded β-turn conformation, similar to morphine, was proposed to be the bioactive conformation for morphine-like or μ-receptor activity (Smith and Griffin 1978). On the contrary, Camerman et al. (1983) proposed for μ-receptor binding that an extended conformation is the preferred conformation. Doi et al. (1984) found that Boc-Tyr-Gly-Gly-(4-bromo)Phe-Met-OH is composed of antiparallel β-sheets, that this structure is stabilized by intermolecular hydrogen bonds, and that the Boc-peptide has considerable analgetic activity (although less than the zwitterionic peptide). From these observations, they proposed that the dimeric extended conformation may be involved in binding to the δ-receptor. This proposal is supported by the finding that dimeric enkephalins exhibit enhanced binding to δ-sites (Shimohigashi et al. 1982). Later, the same group (Ishida et al. 1984) determined the crystal structures of Leu^5- and Met^5-enkephalins and their (4-bromo)-Phe^4-analogs. From these studies they found that Leu^5-enkephalins have type I' β-turn conformation and Met^5-analogs possess an extended dimeric conformation. They proposed that dimeric extended forms interact with the δ-receptor, while the β-turn conformer interacts with the μ-receptor sites. Recently, Renugopalakrishnan et al. (1985), from their conformational studies using FT-IR and laser Raman spectroscopies, proposed that β-turn containing enkephalins interact with δ-receptor sites and β-sheet containing peptides interact with μ-receptor sites. Future studies might help in determining the conformation-receptor selectivity in the opioid peptides.

So far, I have discussed the ligand-receptor interaction as if these were the only components involved. The situation is much more complex. Interaction between the opioid peptides and lipid bilayers might be a very important event in the molecular mechanisms of biological activity of these peptides (Loh and Law 1980; Schwyzer et al. 1982). "The lipid phase of the membrane acts as a matrix for the receptors and is essential for the functionality and biological arrangement of the receptor proteins" (Gysin and Schwyzer 1983). This does not mean that lipids are the peptide receptors, but rather that they are a means to capture peptides (either by hydrophobic or electrostatic interactions or both) and facilitate interaction with the receptor (Berg and Purcell 1977). Gysin and Schwyzer investigated the head group and structure specific interaction of dynorphin and enkephalin liposomes. δ-Agonists interacted more with phosphatidylserine (PS) and a mixture of lecithin and phosphotidic acid (PC/PA), than with cerebroside sulfate (CS).

μ-Agonists interacted more strongly with CS liposomes than with PS and PC/PA, and k-agonist dynorphin (1-13) interacted more strongly and more equally with all the systems. This makes it possible to distinguish ligand types by lipid head group specificity. Several other groups studied the interaction of lipids and detergents with hormonal peptides (Gremlich et al. 1984), methionine enkephalin (Spirtes et al. 1978), and dynorphin(1-13) (Maroun and Mattice 1981). The significance of these investigations has yet to be explored by further research.

An area of growing interest and need is the quantitative analysis of opioid peptides. Although RIA methods are largely being used at present, instrumental methods such as HPLC and LC/MS that are accurate, specific, sensitive, and reproducible are required. These methods are essential to provide specific analysis of low levels of endogenous peptides (active components, precursors, and metabolites) and to understand pathological states mediated through either lower or higher levels of these peptides. These methods should be available in conjuction with immunoassay methods to determine the levels of synthetic peptides if they are used as druqs. These methods should also be available for the pharmacokinetic evaluation of the various bioactive moieties in biofluids.

Current research on opioid receptors has been reviewed extensively (Simon, NIDA Research Monograph 70; Robson et al. 1983). In earlier studies, Pert and colleagues (Bowen et al. 1981; Olgiati 1982;) characterized type I and II receptors. The receptor types are now understood better. For reviews see Simon, this volume; Goldstein and James 1984; Herz 1984; Paterson et al. 1984; and Kosterlitz 1985. Now sub-types are already identified for some receptors. In the near future, the receptor types will be mapped and the subtle differences involved in their structure and organization will be understood. At present, it may be speculated that endogenous ligands might exist for μ-, δ- and k-receptors. A recent report indicated the existence of morphine as an endogenous compound in the bovine CNS and in the skin of the toad and other animals (Oka et al. 1985). This raises the question of whether there exists an endogenous peptide ligand for the μ-receptor and whether it is peptidic in nature. A PCP receptor has been identified (Quirion et al. 1981) and evidence suggests that the endogenous ligand is present and is peptidic in nature (Quirion et al. 1984). One might speculate that this will be an area of research in drug abuse in the near future.

Other developments include synthesis of peptide antaqonists such as ICI 174,864 (Cotton et al. 1984) and photoaffinity agents such as azido DTLET (Garbay-Jaureguiberry et al. 1983; Morley 1983) and [D-Ala^2,Leu^5]-enkephalin N-[(2-nitro-4-azidopheny)-amino]ethylamide [Fujoka et al. 1984).

Attempts are also under way to develop clinically useful druqs and evaluate tnem. One example, FK 33-824, was shown to be useful for detoxifying hard-core heroin abusers (Wen 1982).

Extensive research has been carried out on naturally occurring and synthetic opiates in order to obtain more useful analqetic agents. Opioid peptides offer more promise than the opiates in drug development because:

1. These compounds are endogenous and, on metabolic degradation (unlike the opiates), break down to amino acids. Hence, the metabolites are nontoxic and do not cause kidney and liver damage (unlike the opiates).

2. Since the peptides are made up of several subunits, the amino acid residues, a large number of analogs can be synthesized from a few basic building blocks. Moreover, simple modifications, such as substitution of unnatural amino acids, sterically constrained amino acids, or other modifications, may be attempted to develop analogs with a desired biological activity.

3. The peptide analogs (unlike the opiates) are flexible conformationally and are ideal molecules to investigate relationships between 3-D structure and biological activity. They may be cyclized or subjected to other synthetic modifications, so that analogs of varying and controlled flexibility can be synthesized and studied (unlike the opiates).

4. As these molecules are substantially polar, solution studies can be performed with different solvents and ions (calcium, sodium, etc.) to understand their influences on peptide conformations.

5. Technology for the synthesis of peptides is readily available and solid-phase peptide synthesis is already automated to a considerable extent, so that even complex molecules can be synthesized in large quantities. Even the ultimate goal of synthesizing pro-enkephalin (about 250 residues) and pro-dynorphin to provide scientists with adequate material for studies on processing mechanisms seems possible (see Bodanszky 1985).

6. To date, the compounds that are highly selective for **δ**-receptors have been opioid peptides.

7. Due to the unique character of some of the peptide drugs, i.e., their inability to cross placental barrier (due to placental enzymatic deactivation of the peptides), these drugs have a distinct advantage for being used as analgetics in pregnant females. They are definitely superior to meperidine in this respect.

At present, research in opioid peptides is progressing at a rapid rate. Some of the important aspects follow:

Analgetic peptides are to be designed that will show minimal drug dependence potential; the peptides of potential interest

are enkephalins, endorphins, dynorphins, dermorphins, morphiceptins, and related opioid peptides. Some of the goals are: a) the development of peptide analogs that can be administered orally, that have long duration of action, and that are without the potential for abuse; and b) synthesis of inhibitors of enkephalin-degrading enzymes as potential analgetics. Extensive SAR studies on the opioid peptides may be of great value in the rational design of these analogs. It is further anticipated that SAR studies on opioid peptides might result in the development of antagonists and mixed agonist-antagonists that are clinically useful in the treatment of opiate addiction.

Several hundred analogs of the opioid peptides have been evaluated to aid in delineating the essential features of the μ-and δ-receptors. The synthesis of even more specific analogs for all types of receptors can be anticipated.

The receptor types are well differentiated on the basis of binding studies and/or pharmacological studies. However, considerable information on sites and functions is still lacking. Particularly, the component peptides of the different receptors must be isolated and characterized, and receptors or receptor models must be synthesized or reaggregated to understand mechanism(s) of action. It may be possible to study the conformation of the receptor in response to environment-mediated conformational changes (i.e., lipids, ions, solvents, ionic strength, polarity, etc.) and the conformation of the drug-receptor complex itself.

A number of conformational studies have been conducted on opioid peptides; however, most of them are performed on enkephalin peptides as they are relatively small pentapeptides and are easily accessible. Conformational studies using theoretical solid-state and solution methods have to be extended to all the pertinent opioid peptides and their analogs. The effects of the surrounding environment (solvent, pH, ionic effects, etc.) must be examined; and, finally, the receptor-bound conformation of the drug must be determined. Advanced methods such as X-ray crystallography, FT-IR, laser Raman, nuclear magnetic resonance, C.D., and computer-assisted drug design will play an increasing role in these studies.

Research in the opioid peptides is progressing at a rapid rate. In the near future, we may look forward to still more exciting discoveries, and there is room for optimism to believe that therapeutically useful peptide analgetics may soon be developed.

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AUTHOR'S NOTE

This review is not comprehensive. Only a few selected references and topics are included, as it is impossible fully to review an extremely active area such as this, and other authors have presented pertinent topics and references. For further information on the topics or references, the reader is referred to the following chapters in this monograph, NIDA Research Monograph 70, and The peptides: Analysis, Synthesis, Biology. Vol. 6, Opioid Peptides: Biology, Chemistry, and Genetics (S. Underfriends and J. Meienhofer. eds. New York: Academic Press 1984).

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Synthesis of Opioid Peptide Analogs

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INDTRODUCTION

Elucidation of the structures of the first opioid peptides, the enkephalins, in 1975, stimulatad an unprecedented wave of synthetic activity. Fortunately, peptide synthetic methods had been develoqed to the point where synthesis of opioid peptides was easily accomplished, particularly the small ones. In this chapter, contemporary methods of peptide synthesis will be discussed, especially as they apply to the cpioid peptides.

Peptides may be synthesized either in solution, using classical organic synthetic procedures, or by the newer method of solid phase peptide synthesis introduced by Merrifield. Solution synthesis may be practical for small peptides, particularly if large quantities are to be synthesized. Successful application of solution methods generally requires that the person performing the synthesis be skilled and experienced in peptide synthetic procedures. On the other hard, solid phase peptide synthesis (SPPS), introduced twenty years ago, allows rapid synthesis of a wide variety of peptides and doss not require a high degree of chemical shill on the part of the person doing the synthesis. It has been applied sucessfully to the synthesis of many longer peptides, such as ß-endorphin and its analogs. The availability of commercial automatic peptide synthesizers sakes SPPS a rapid and convenient procedure. It is particularly useful for synthesism of a wide variety of analogs, such as has been done in the case of the cpioid peptides.

PRINCIPLES OF PEPTIDE SYNTHESIS

Successful peptide synthesis rests upon two maajor factors: 1) application of a suitable set of selectively removable blocking groups for the reactive functions of the amino acids to be incorporated into the peptide, and 2) use of suitable activating reagents to perform the coupling reactions involved. Synthesis of a tripeptide is depicted schematically in figure 1. During every coupling step, only those reactive groups which are intended to

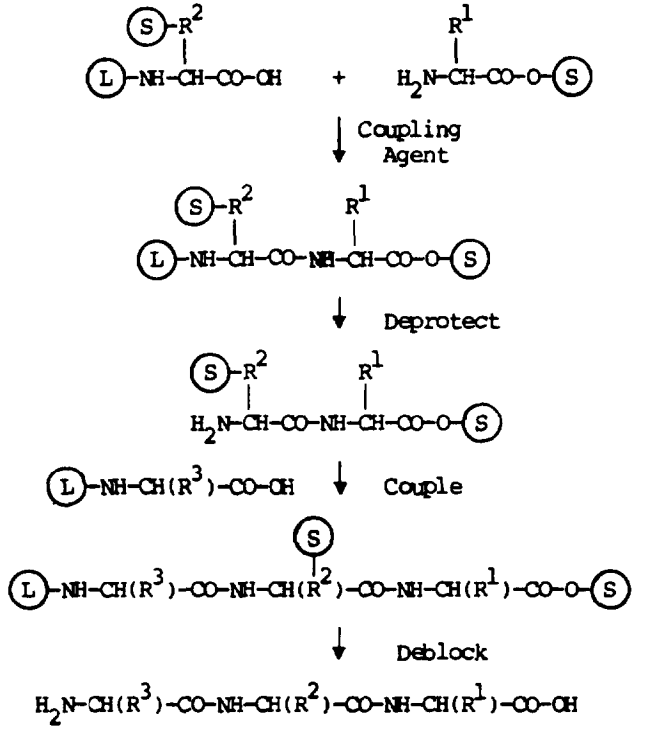

FIGURE 1

Schematic Synthesis of a Tripeptide
S=stable blocking groups; L=labile blocking group

react are left unprotected. Other reactive groups are blocked either by stable blocking groups (indicated with S),which remain intact throuh out the process of assembly of the desired peptide, or by means of a labile blocking group (indiated above as L), which will be removed at each step of the synthesis. Synthesis usually begins at the carboxyl end of the peptide. The most commonly used procedure for synthesis of peptides of the size range of the opioid peptides is that of stepwise synthesis, in which a single amino acid is added at each coupling reaction. It is extremely important that optical purity of all the amino acid residues be maintained throughout the synthesis. For all the naturally occuring amino acids, the L isomer is used. However, in synthesis of enzyme-resistant opioid analogs, D-amino acids are frequently used. Coupling reactions must be chosen with care to avoid racemization of the amino acid residue which is activated during the coupling reaction. The most reliable method for avoiding such racemization is coupling of single amino acids, each having its α-amino group protected with a urethane-type protecting group.The most commonly used such urethane group at the present time is the t-butylaxycarbonyl (Boc) group: its structure is indicated in figure 3. The Boc protecting group is commonly

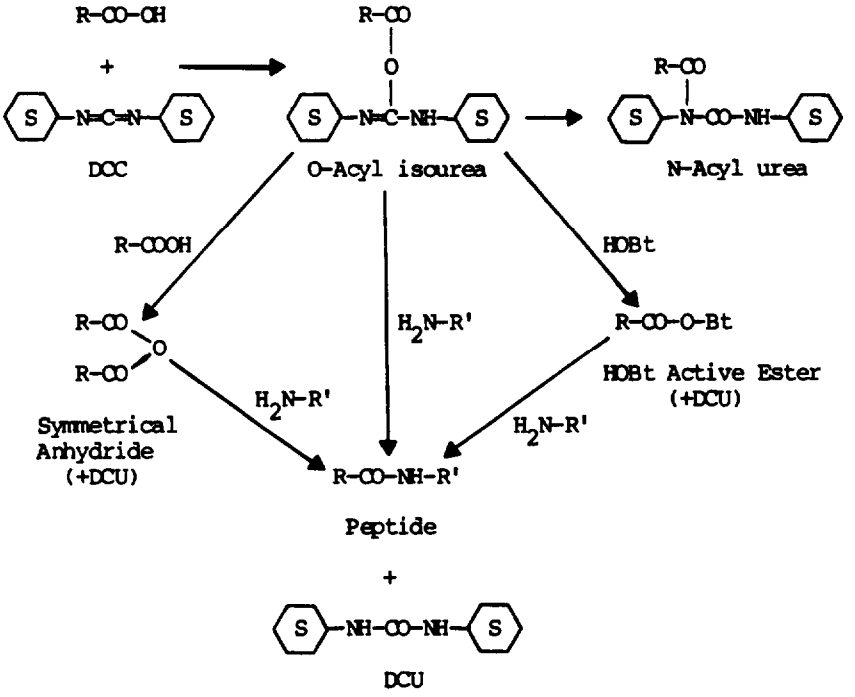

FIGURE 2

Mechanism of coupling Reactions Mediated by DCC

removed at each step of the synthesis by treatment of the protected peptide with anhydrous acid, much as trifluoracetic acid (TFA) in an inert solvent such as dichloramethane (DCM).

When the Boc group is used for the labile α-protecting group, the stable blocking groups are usually derived fran benzyl-related blocking groups. Thus, the stable blocking group for the C-terminal carboxyl of the peptide may be a benyl ester, which can also be used for blocking side chain carboxyls of aspartic and glutamic acid residues. The aliphatic hydroxyl groups of serine and threonine may be blocked as benzyl ethers. The hydroxyl group of tyrosine and the amino group of lysine are usually blocked with a group related to the benyloxycarbonyl blocking group, which has been in use for many years in peptide chemistry. Arginine and histidine side chains are most commonly blocked with the p-toluene sulfonyl (Tos) group. All these stable blocking groups can be removed from the peptide at the end of the synthesis by treatment with a strong anhydrous acid, such as liquid anhydrous hydrogen fluoride.

More labile systems of blocking groups have been developed for peptide synthesis. For example, the labile blocking group can be the biphenylylpropoxycarbonyl (Bpoc) or the phenylisopropyloxycarbonyl (Poc) group. When these very labile groups are used for α-protection, side chains can be blocked with functions related t-butyl groups, such as t-butyl esters and Boc groups. Very dilute TFA is used to remove the labile protecting group at each stage of the synthesis, while more concetrated TFA can be used to remove the stable blocking groups at the end of the synthesis.

These two synthetic schemes outlined above depend for their success on the differential lability to anhydrons acid of the labile and stable blocking groups. Some reaserch has been done on developement of orthogonal systems of blocking groups where the labile and stable blocking groups are removed by completely different types of reagents. Such a systen can use the fluorenylmethoxycarbonyl (Fmoc) α-blocking group; it is removed by treatment with an amine such as piperidine. The stable blocking groups can be t-butyl derived functions, which are stable to the mine used to remove the labile blocking group. This systen has received only limited application to synthesis of opioid peptides, principally due to the greeter cost of Fmoc amino acid derivatives. Furthermore, yields have usually been quite satisfactory using selective acidolysis procedures.

Given a set of blocking groups suitable for the synthesis at hand, ate needs then to choose appropriate coupling reactions. By far the most commenly used coupling reagent is dicyclohexylcarbodiimide (DOC). Use of DOC is particularly convenient, since it can usually simply be added to the solution containing the amine and carboxyl component to be coupled. It reacts rapidly with the free carboxylic acid present to form an "active ester" internediate (the isaurea),which may react with the amine component directly or may proceed through an intermediate symetrical anhydride (see figure 2). Rearrangement of the isourea to the N-acyl urea is an undesired side reaction. Coupling reactions mediated by DOC can also be modified by addiction of other reagents, such as as 1-hydroxybensotriazole (HOBt). This leads to intermediate formation the HOBt active ester, which may minimize racemization and may also facilitate some coupling reactions. If glutamine is being incorporated into the peptide, it can also minimize dehydration of the omega amide group to a nitrile. Mixed anhydrides have also been frequently used to promote coupling reactions, particularly in solution synthesis methods. However, a discussion of such reagents is beyond the scope of this article. A good introduction to peptide synthesis in general is given by the book of Bodanszky et al. (1976).

SYNTHESIS OF PEPTIDES IN SOLUTION

Peptide chmists working during the last fifty years have developed methods suitable for effective synthesis of peptides in

solution, applying the principles outlined in the previous section. Solution methods have been used mainly for synthesis of shorter opioid peptides, such as analogs of the enkephalins. Solution synthesis is usually used for production of large quantities of peptides, particularly if the peptide chain is short. Solution synthesis also offers the opportunity for use of segment coupling methods, in which a small block peptide is synthesized and then coupled to the reminder of the chain in me step. A major problem with segment synthesis methods is that of racemization of the carboxy-terminal residue in the segment when it is activated for coupling. However, this is not a problem with enkephalin synthesis if the fragment is properly chosen, since glycine residues cannot racemize and the presence of two glycine residues in the enkephalin sequence allows for convenient breaking of the chain into segments. This approach can offer practical advantages for synthesis of a series od analogs modified at the carboxy end. For example, variations in the structure of enkephalins at the 4 and 5 positions (phenylalanine amd leucine or methionine) could utilize a blocked fragment consisting of tyrosyl-glycyl-glycine or tyrosyl-D-alanyl-glycine for coupling to various C-teminal dipeptides. In solid phase synthesis (SPPS), the entire sequence must usually be repeated in a stepwise fashion for each of such analogs.

Various combinations of the blocking groups and coupling reagents described in the previous section have been used in the synthesis of opioid peptides. Syntheses of these peptides described in the literature may appear very simple, and in principle they are. However, successful results in solution synthesis of peptides generally requires considerable experience. The novice will frequently waste a great deal of time and money attempting to synthesize even small peptides by these methods, and is advised to use solid phase techniques instead.

Amides ob enkephalin peptides have been found to have high potency, particularly for mu receptors. In solution synthesis, peptide amides are synthesized by beginning the synthesis with the mide of the C-terminal residue to be incorporated into the peptide, e.g., methionine amide. They can also be synthesized fran peptide esters by ammonolysis, provided there are no aspartic or glutamic residues in the peptide (these would be concomitantly changed to asparagine and glutamine residues). Certain enkephalin analogs in which the C-terminal methionine carboxyl group has been reduced to an alcohol have been found to be very potent. Such peptide alcohols can be synthesized by beginning the synthesis with a benzyl ether of the C-terminal alcohol and using HF for final deblocking of the finished peptide. If there are no aspartic or glutamic acid residues in the peptide, the synthesis my be carried out with the methyl ester of the C-terminal residue. Treatment of the finished blocked peptide with lithiumborohydride will then yield the C-terminal alcohol.

SOLID PHASE PEPTIDE SYNTHESIS

Solid phase peptide synthesis was introduced by Bruce Merrifield in 1963 in the hope of overcoming many of the problems inherent in solution methods of peptide synthesis. The experience of the past twenty years has shawn that hope to be amply justified. Autmatic instruments have been develcped and are available ambercially which will carry out SPPS rapidly and efffectively. Many thousands of peptides have been synthesized with SPPS. In contrast to the relatively high degree of technical sophistication required for successful peptide synthesis in solution, successful SPPS of many peptides can be carried out by persons with no prior experience in peptide synthesis and only limited competence as organic chemists. As one imnunolcgist said, "solid phase synthesis is so simple than even an inmunologist can do it!" The comprehensive review of Barany and Merrifield (1980) provides an encyclopedic coverage of the solid phase literature, and the laboratory manual of Stewart and Young (1984) provides specific laboratory directions for synthesis of peptides by SPPS.

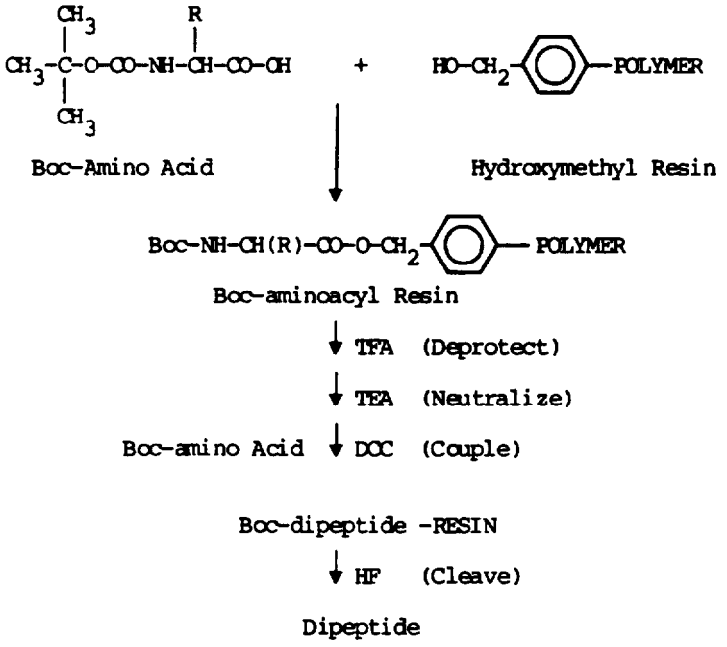

FIGURE 3

The Merrifield Scheme of Solid PHase Peptide Synthesis. The polymer is 1%-crosslinked polystyrene; TFA=trifluoroacetic acid; TEA=triethyl amine; DCC=dicyclohexylcarbodiimide.

Solid phase peptide synthesis is a special case of the general scheme of stepwise synthesis of peptides fran the carboxy end, in which the stable blocking group for the C-terminal carboxyl of the peptide being synthesized is an insoluble polymer (figure 3). In the Merrifield system of SPPS, the C-terminal amino acid residue is esterified to a functionalized cross-linked polystyrene resin, which serves as the carrier. Mast SPPS has been done with Boc amino acids. Follwing attachment of the first Boc amino acid to the resin, the resin is analyzed for the degree of substitution of the amino acid cm the polymer. A batch of Boc-amino acid resin is then placed in the reaction vessel of a manual or automatic synthesis apparatus; the resin need never be rmoved from the vessel until the assembly of the peptide is finished. By avoiding any transfer of material from one vessel to another during the synthesis, much loss of material is eliminated. The Boc group is removed from the amino acid on the resin by treatment with an acidic reagent, usually TFA in DCM. HCL in dioxane has also been used successfully for my syntheses, but is less covenient to use. The amino acid-resin is then treated with a tertiary amine base to neutralize the acid salt and liberate the free amino group for coupling with the next Boc-amino acid. The next Boc-amino acid can be coupled to the amino acid-resin by use of DCC or any other oonvenient method for coupling. Use of DOC: is rapid and convenient, and usually gives satisfactory results. In those cases where complete coupling cannot be achieved in one or two cycles, use of an active ester (either formed in situ by addition of HOBt or used pre-formed HOBt active ester) or use of a Boc-amino acid symetrical anhydride can be tried. Symmetrical anhydrides are very effective for promoting complete coupling reactions, but their use involves additional work and is frequently not necessary. Repetition of the deprotection, neutralization and coupling steps with each Boc-amino acid to be introduced into the peptide sequence will then yield the blocked peptide an the resin. If the peptide being synthesized is to have a free carboxyl group, treatment of the peptide-resinwith liquid anhydrous HF will cleave and deprotective peptide in me operation.

If all deprotection and coupling reactions go fully to completion, a homogeneous peptide will be assembled on the resin. If the cleavage reagent does no harm to the peptide, then the crudepeptidewill be essentially pure. Using the best of modern techniques, this level of success can frequently be achieved in practice. In such cases purification of the final peptide is very simple. After the initial Boc-aminoacylresin is synthesized and analyzed, autamatic synthesizers can carry out assembly of the peptide at the rate of one residue every two to fair hours. Cleavage of the peptide fran the resin with HF will require an additional half day of time. If the synthesis procdures were carried out effectively, purification can then frequently. be a simple short operation like chromatography or countercurrent distritution. The speed of SPPS carried out in this way makes it particularly practical for synthesis of analogs of opioid peptides, especially those analogs in which variation in the sequence will be toward the anino end of the peptide. In that

case, a large batch of aminoacyl-resin can be carried through the synthesis up to the point where the different substitutions will be made. Individual syntheses can then be carried out with the desired amino acids on a smaller scale, using portions of the peptide-resin previously prepared.

Synthesis of peptide amides is also convenient by SPPS. If the peptide chain does not contain aspartic or glutamic acid residues, the sequence can be assembled on Merrifield resin and cleaved by amonolysis. However, if the sequence contains these residues (ß-endorphin contains glutamic acid residues), this approach is not satisfactory, because the glutamic residue will be banged to glutamine if the ammnolysis is conducted on a peptide resin in which the glutamic side-chain carboxyl is blocked with a benzyl ester. A hydroxymethyl resin support can be used for a synthesis, however, in which the labile system of blocking groups or the orthogonal systen (as described above) is used. In those cases, the glutamic carboxyls can be protected as t-butyl esters, which should not be converted to glutamine residues during ammonolysis. A simpler approach uses for the insoluble support a resin which will yield a peptide amide directly upon cleavage of the peptide with anhydrous HF. Such resins are the benzhydrylamine (BHA) and methylbenzbydrylamine (MBHA) resins (see figure 4). If an aminanethyl resin analogous to the hydroxymethyl Merrifield resin is used for synthesis of peptides, HF will not cleave the peptide fran the resin, as that group is not sufficiently labile. If the aminomethyl resin is

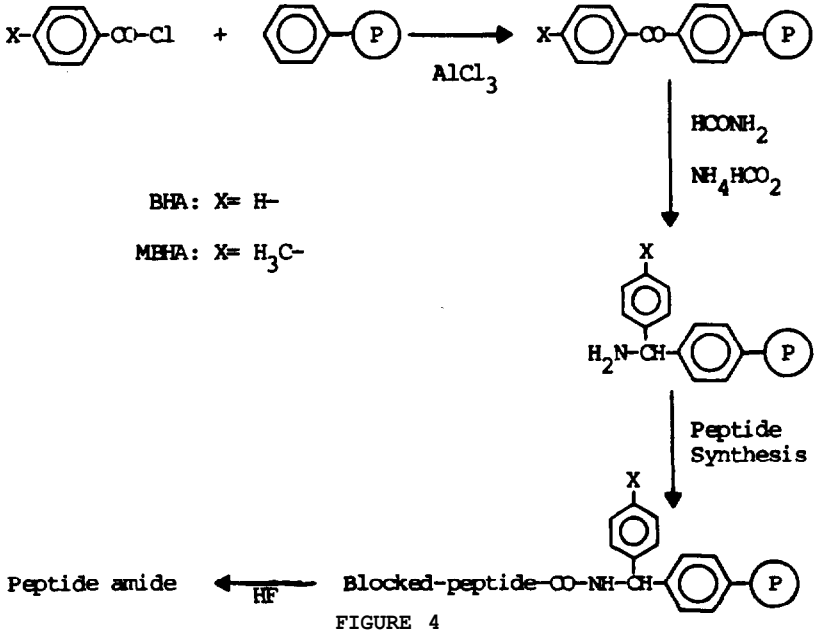

FIGURE 4

Synthesis of peptide Amides via Amine Resins

labilized by addition of another phenyl or p-methylphenyl residue as in the case of the BHA and MBHA resins, the nitrogen-carbon linkage is now sufficiently labile that treatment of peptide-BHA (or MBHA) resins with HF will yield peptide amides directly. The linkage of peptides to the MBHA resin is somewhat more labile than in the case of the BHA resin, and the MBHA is more satisfactory for the synthesis of peptides whose C-terminal residue is me which makes the peptide relatively resistant to acicdolysis, such as phenylalanine or leucine. Miny analogs of [D-Ala^1]-methionine-enkephalin amide have been synthesized on these resins. Directions for synthesis and use of these resins are given in the laboratory manual of Stewart and Young (1984).

Peptide alcohols,whichhavebeen found useful in the enkephalin series, can also be synthesized satisfactorily by solid phase methods if the amino acid sequence does not contain glutamic or aspartic residues. The peptide sequence is assembled on the hydroxymethyl Merrifield resin, and the blodced peptide-resin is treated with lithium borohydride to effect reductive cleavage to an alcohol of the ester linking the peptide to the resin.This procedure is also described in the Stewart and Young manual.

SIDE REACTIONS DURING PEPTIDE SYNTHESIS

If an appropriate set of blocking groups and reaction conditions is chosen for synthesis of peptides, undesirable side reactions during assembly and deblocking of the peptide can be minimized. If the conditions for solid phase synthesis recommended in the Stewart and Young manual are followed, little difficulty can be expected However, if less than ideal condtions and derivatives are used, some problems can be expected. Methionine-enkephalin contains two residues which can cause problems: methionine and tyrosine. The sulfur in methionine residues can be readily alkylated under appropriate conditions (see figure 5). Such conditions can cccur during deblocking of the peptide. When benzyl blocking groups are removed from side-chain functions during HF cleavage, the benzyl oation, if allowed to persist for any significant length of time,will alkylate methionine to the sulfonium derivative. The sulfonium derivative of methionine-enkephalin has very low biological activity. This undesirable alkylation can

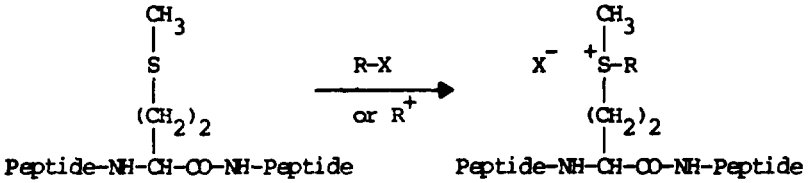

FIGURE 5

Formation of Methionine Sulfonium Peptides

be prevented by inclusion of a large excess of scavenger reagent in the clevage reaction mixture. Such reagents as anisole and methylethylsulfide are commonly used for this purpose.Methionine can also be oxidized easily to the sulfoxide derivative. Indeed, the sulfoxcide has been found highly potent the case of certain methionine-enkephalin derivatives. However, in this case, the synthesis can be done with Boc-methionine sulfoxide, which should be stable throughout the synthesis and HF cleavage. If sulfoxide is not desired, syntheses can usually be carried out with Boc-methionine without significant oxidation to the sulfoxide. However, if it is found that in a particular sequence or under particular reaction conditions being used this undesirable oxidation takes place, any sulfoxide in the peptide-resin can be reduced conveniently to methionine by inclusion of 2-mecaptopyridine in the HF cleavage reaction mixture (see Stewart and Young 1984).

If the hydroxyl group of tyrosine is blocked during synthesis as the benzyl ether, HF treatment can yield some of the undesired 3-benzyl tyrosine (see figure 6). This Friedel-Crafts type reaction is promoted by anhydrous HF. The alkylation can be prevented if large quantities of scavenger are included in the cleavage reaction mixture. Most peptide chemists currently practicing solid phase synthesis do not use O-benzyl tyrosine for this reason. The 2,6-dichlorobenyl and the 2-bromocarbobenzoxy derivatives have been found to yield very little of the undesired alkylated derivate. Bath the 3-benzyl tyrosine and the S-benzyl methionine analogs of enkephalin have been found to have very little biological potency. One should normally take pains, there fore, to suppres these side reactions. Other possible side reactions which may be encountered during solid phase synthesis are disscused in the Stewart and Young laboratory manual.

If the directions given in the Stewart and Young manual are followed even novices could be able to synthesize opioid peptides without serious problems. Only general procedures are given in that manual. If the chemist wishes to follow a set of directions specific fox methionine-ankephalin, such directions

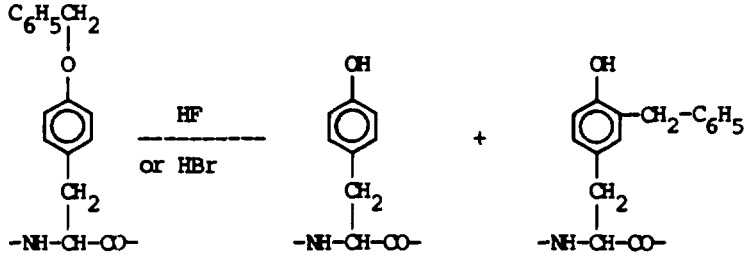

FIGURE 6

Alkylation of the Tyrosine Ring by Benzyl Groups

can be found in the volume of "Methods of Enzymology" dealing with synthesis of neuropeptides (Stewart 1983).

PURIFICATION AND ANALYSIS OF PEPTIDES

Following assembly and deblocking of a desired synthetic psptide, the product must be purified essentially to homogeneity and analyzed for identity before being used. While some ideas of biological potency my be obtained in rapid screening programs by use of new analogs not fully purified, accurate quantitative data can only be obtained with fully purified peptides. Physical studies on peptides also require synthetic wterials of the highest purity.

When peptides are synthesized in solution, the intermediate products are usually pirified frequently during the various steps of the synthesis. In that case, final purification of the peptide is frequently quite simple. However, even if the final protected peptide is fully purified, same by products may be introduced during deblocking, and these must be removed. Some small peptides can be satisfactorily synthesized in solution by repetitive procedures without full purification at each stage. In those casses, final purification of the product can be quite difficult.

In solid phase synthesis there is normally no opportunity for purification ob the peptide at intermediate stages, and the only purification possible cones after removal of the peptide fran the resin support. In order to achieve a homogeneous product, every deprotection and coupling reaction must go fully to completion. If the best available techniques are used for synthesis, this god can be practically achieved, or at least very nearly so. Every assembly of opioid peptides on solid phase resins should finish with a deprotection step, in order to remove the protecting group fran the chain to minimize alkylation side reactions during cleavage. This is equally true if the final cleavage will be done by HP or on labile resin systems by use of trifluorcacetic acid. As noted above, adequate scavengers must always be used during the cleavage to minimize side reactions.

Procedures using partition methods, such as countercurrent distribution, partition chromotography or reversed-phase high performance liquid chromotography (HPLC) are particularly appropriate for purification of opioid peptides synthesized by solid phase methods. The crude synthetic peptide usually contains some of the scavengers used in the cleavage reaction, and these materials are frequently very different in polarity fran the desired peptide. Moreover, if alkylation of methionine or tyrosine residues has occurred, these by products will also differ significantly in polarity and partition coefficiant. The benzyl tyrosine derivative will be found to be sore hydrcphobic while the methionine sulfonium derivative will be more hydrophilic, and thus readily separated fran the desired product by partition. Quantities greater than 100 mg of peptide can usually be purified by an overnight automatic run in a Craig countercurrent

distribution apparatus. Preparative HPLC is even more rapid, but the apparatus is quite expensive and the resins used will also represent a significant expenditure, especially since the packing material can reasonably be used only for one peptide. Peptides frequently adsorb quite tightly to the reversti-phase material and the final traces are gradually eluted in subsequent runs. In commercial or very large-scale operations, where large amounts of peptide or repeated syntheses will be made, this may be very practical. For the investigator synthesizing large numbers of analogs, the economics may be a problem. Partition chromotography has long been used for purification of synthetic peptides, and while very large columns will be needed for purification of significant quantities of peptide, this an be done rapidly. All these procedures are discussed in the laboratory manual of Stewart and Young.

All purifications mist be accompanied by suitable analytical techniques which will reveal the presence of any impurities in the synthetic product and indicate their nature. Paper electrophoresis is uniquely appropriate for this purpose. Thin-layer electrcphoresis can also be used but is not quite as convenient. Gel electrophoresis is not appropriate for small peptides of the size of the opioids because of excessive diffusion of the peptide in the gel matrix. If suitable amino acid markers are included as standards in an adjacent channel, in paper or thin-layer electrophoresis, quite accurate information on the size and charge of the peptide product can be obtained. Inpurities differing in charge and differing significantly in size are readily resolved. Electrophoresis can be done in buffers of different pH in order to enable appropriate jud regarding inpurities to be made. Thin layer chranatcgraphy (TLC) is also a valuable analytical technique. It is particularly useful for indicating the presence of impurities which differ in hydrophobicity from the desired product.

If meaningful infomation on purity of synthetic peptides is to be obtained, it is critical that the procedures used for analysis be based upon different phenomena than the procedures used for purification. For example, if the peptide was purified by a partition method such as countercurrent distribution or reversed-phase partition HPLC, then similar HPLC or TLC should not be used to assess purity. Rather, a method utilizing charge and/or size of the peptide should be used, such as electrcphoresis or ion-exchange TLC. In any event, once homogeneity has been acheived by a suitable purification procedure or procedures, and has been demonstrated by appropriate analytical methods, an aliquot of the peptide should be hydrolyzed with acid and the ratio of constitutent amino acids determined by amino acid analysis. Since all opioid peptides contain tyrosine, the acid hydrolysis mixture should contain phenol to prevent loss of tyrosine by chlorination due to contamination of the HCL by chlorine. If tryptophan-containing analogs are synthesized, reasonable values for this acid-labile amino acid can often be obtained by including marcepto-ethanol in the hydrolysis mixture. Since many opioid peptides

contain methionine residues, anaslysis of the oxidation state of this residue is quite important. The biological potency of a peptide can frequently change drastically depending upon whether the methionine residue is present per se or is oxidized to the sulfaxide. Rydrolysis with 6 N HCL converts methionine sulfoxide largely to methionine, so the original state of that residue cannot be determined in this way. Rather the peptide must be hydrolyzed with methanesulfonic acid, which does not disturb the oxidation state of methionine residues. Precise details of these procedures are given in the Stewart and Young (1984) handbook.

Accurate amino acid analysis also provides a reasonable estimte of the "working molecular weight" of the peptide. Samples of carefully purified and lyophilized peptides contain varying amounts of non-peptide material. Peptides which have a net acidic or basic charge will also contain a counter-ion to neutralize these excess charges. These salts must be taken into account in deriving the theoretical molecular weight. One must also remember that the final salt form will depend upon the acidic or basic strength of the final purificaticn system. Far example, a peptide containing a free amino and carboxyl group in addition to a single arginine residue will be found to contain one mole of acetic acid if it is purified fran an acetic acid-containing system. On the other hand, if the peptide is purified finally from a system conaining trifluorcetic acid, two moles of TFA will be bound to the peptide since TFA is a stronger acid than the carboxyl group of the peptide chain. This same consideration alsoapplies to the side-chain carboxyl groups of aspartic and glutamic acids. The theoretical molecular weight calculated in this way (containing bound salt) is useful for determining the purity of the synthetic peptide. The most practically useful value, however, is the "working molecular weight," which is the reciprocal of the average micromoles per milligram of peptide analyzed. In calculating this ratio, one should not include values found for residues known to be destroyd during hydrolysis, such as marine, theronine, and tryptophan. This "working molecular weight" should be applied by the person synthesizing the peptide to whomever will use the peptide, 80 that accrate molar potencies can be calculated.

STORAGE AND HANDLING OF PEPTIDES

Most synthetic opioid peptides will be found to be quite stable if stored absolutely dry and in the freezer. When the peptide container is removed from the cold, the contianer should be allowed to warm to room temperature in a desiccator before the container is opened; other wise atmspheric moisture will condense on the cold peptide particles. When the peptide is again stored, this moisture will accelerate degradation of the peptide. Peptides should be stored in relatively small aliguots in separate containers, so that a large batch of peptide is not repeatedly warmed and chilled.

Amino acid analysis frequently reveals that lyophilized peptides contain considerable quantities of solvent. If these peptides were lyophilized from acetic acid, as is often the case, cold storage is particularly important to avoid degradation of the peptide by the acetic acid in the sample. Peptide bonds adjacent to aspartic acid residues are particularly labile to this type of degradation.

Amide bonds, of the C-terminal amide if it is present, and of glutamine and asparagine side chains, are also a problem. Glutamine and asparagine amides have been found to be particularly labile when they occur in the peptide sequence adjacent to the basic residues, such as arginine or lysine; asparagine amides are the more labile. This typed hydrolysis can occur during storage of the "dry" peptide as well as after the peptide is dissolved in aqueous solution. This hydrolysis is also more rapid if the "dry" peptide contains excess acid or base, or if it is dissolved in a solution that is significantly removed fran neutrality. The surface of glass is alkaline, particularly if the glass is aged. All glass containers to be used for storage of peptides or peptide solutions should be rinsed with molar acetic acid and dried care fully. For most careful work, they should then be silanized.

Many peptides, particularly those that are basic and/or hydrophobic, adsorb avidly to surfaces of glass and some plastics. The extremely high potency of some opioid peptides causes them to be used in very dilute solutions in biological experiments. In some cases, these experiments may be totally invalid due to adsorption of peptide from solution onto the surfaces of test tubes, pipettes, and syringes. Among the plastics, polystyrehe is particularly bad for peptide adsorption and should not be used. Polyethylene and polypropylene are probably the best plastics for use with peptides,although some peptides will stick to these surfaces. If low concentrations of peptides must be used, adsorption can be prevented by such measures as making all the dilutions in 0.01 M acetic acid, or by including a low concentration of protein in the solution, in addition to silanizing the glass surface. The method chosen will depend upon the requirements of the experiments. In certain cases, a difference of 1,000-fold in potency of a peptide solution has been ovserved when dilute acetic acid was included in the solution.

Oxidation of methionine residues in dilute solutions of peptide is a serious problem. All solvents to be used for dissolving such peptides should be thoroughly bubbled with nitrogen before use. some very potent enkephalin analogs have contained disulfide rings formed from two residues of cysteine or penicillamine in the peptide chain. Such disulfides are extremely labile in solution if the pH is above neutrality. At higher pH, disulfide interchange occurs, and the monomeric peptide may be rapidly convertd to dimers and polymers, which are usually biologically inactive. Experience with oxytocin and vasopressin solutions has shown that they retain their potency best when slightly acidified (pH 5) and stored just above the freezing point. These conditions

may also be most appropriate for storage of solutions of disulfide-containing opioid peptides.

When dissolving peptides for use, only fairly concentrated solutions (such as 0.1 mg/ml) should be frozen for storage. These solutions should be divided into small aliquots containing the amount to be used for one day's experiments. The tubes should be flushed with nitrogen, closed tightly to prevent spillage or lyophilization in the freezer, and frozen. The ease of dissolution of peptides depends strongly upon the physical state of the material. If the peptide was lyophilized from fairly dilute solution, it will be finely divided and will usually dissolve readily, except in the case of some hydrophobic peptides, which are difficult in any case. If the peptide was dried by evaporation or lyophilized fron a very concentrated solution, the material may be in the form of hard particles which dissolve only very slowly. Such particles may have an index of refraction similar to that of the aqueous medium, and their failure to dissolve may not be noticed unless the solution is examined with a magnifier. Very hydrophobic peptides may need to be dissolved first in a mall amount of a powerful solvent such as dimethyl sulfoxide and then diluted with hater or saline. In this case, appropriate controls must be run. Bubbling air through the pipette or vortexing should not be used for mixing if there is any possibility of oxidation of the peptide.

DESIGN OF OPIOID PEPTIDE ANALOGS

NEW analogs of opioid peptides are synthesized for a variety of reasons. A major goal has beeu to find analogs which will have absolute selectivity for the various classes of opioid receptors. When such selectivity is achieved, further modifications may well be undertaken to improve the resistance of the peptide to enzymatic degradation in order to increase potency and prolong activity in vivo and in vitro. Such selective, potent and long-acting analogs will find many practical applications. Additional analogs may need to be designed in order to delineate more precisely the nature of peptide receptor interactions, as well as for certain physical chemical studies. Analogs containing easily detectable "reporter" functions are particularly useful in such studies. Potent selective antagonists are much needed for a variety of reasons, both practical and theoretical. Since there are no universally applicable rules for design of peptide antagonists, this letter search has been particularly frustrating.

Conformational restriction has been a very valuable tool in design of selective opioid analogs. The ancestral opiate compound, morphine, and many of its analogs synthesized by medicinal chemists were the years have led the way for more recent peptide work. Use of N-methyl amino acids in peptide analogs not only restricts the conformtions available to the peptide, but frequently confers enzyme resistance as well. Some α-methyl amino acids have been incorporated into opioids

with startling results. Aditional work needs to be done with other α-alkyl amino acid residues. Conformtional restriction by cyclization of all or part of the peptide chain has also been very useful in opioids.

Examination of the lists of opioid analogs in this volume and in other publications will show the degrees of selectivity, enzyme resistance and antagonist that peptide chemists have been able to incorporate into analogs at the present time. These developments will not be discussed again here. Details of two groups of peptides not covered elsewhere will be discussed in somewhat more detail. Several aspects of the wisdan gaind from three decades of peptide analog work are sumnarized in a recent article (Stewart, 1982).

MINIMUM STRUCTURE ENKEPHALINS

One of the earliest types of structure modification applied to the enkephalin field was shortening of the peptide chain, either by elimination of residues fran the ends or by removal of residues within the sequence. From those experiments it was found that while significant potency remained if the C-terminal methionine or leucine residue was remved, the phenylalanine residue in position 4 was critical for activity. It was also found that activity was reduced to less than 1% of that of the pentapeptide if one of the glycine residues was removed fran within the chain. Fran this last work it was propsed that the spacing between the essential phenolic side chain of the tyrosine in position me and the phenylalanine side chain was critical for biological activity. This last proposal was somewhat difficult to understand in light of the fact that morphine combines well with the opiate mu receptors and that considerable folding of the enkephalin chain was required to make it conform roughly to the morphine structure.

After it had been learned that enkephalin potency could be greatly enhanced by replacing the glycine in position two with a Dalanine residue and converting the C-terminals to an amide (Marley, this volume), we foumd (Chipkin et al. 1981) that the tetrapeptide Tyr-DAla-Phe-Met-amide was more potent than Met-enkephalin and nearly as potent as Tyr-DAla-Gly-Phe-Met-amide. Evidently the problem with the earlier experiments was not that the Tyr-Phe spacing is so critical but rather that the shortened peptide was being degraded very rapidly, even in the in vitro assay system. This result led to a systematic investigation enkephalin analogs having even shorter chains (vavrek et al. 1981) (see table 1). The most surprising result of this investigation was that Tyr-DAla-phenylpropyl amide had nearly the full potency of Met-enkephalin on stimulated guinea pig ileum and produced analgesia in vivo. It is interesting that in this compound the spacing along the peptide chain from the tyrosine to the aromatic ring is not identical to the spacing in Tyr-DAla-Phe-Met-amide, which we had previously found to be quite potent.

TABLE 1.

Relative Potencies on Guinea Pig Ileum of Minimum Structure Enkephalins

Tyr-Gly-Gly-Phe-Met	100
Tyr-Gly-Gly-Phe-Met-amide	160
Tyr-DAla-Gly-Phe-Met-amide	140
Tyr-DAla-Phet-Met-amide	120
Tyr-Pro-Phe-amide	19
Tyr-DAla-Phe-amide	25
Tyr-DAla-phenylethylamide	6
Tyr-DAla-phenylpropylamide	77
Tyr-DAla-phenylbutyl amide	13

The same spacing as in the tetrapeptide is found in Tyr-DAla-phenylethyl amide, which has very low potency (table 1). In our potent dipeptide the separation is me carbon atom further. The reason for this difference is not understood at this time. Perhaps when the mu receptor structure is known will will have answers to these questions. Resistance to enzymatic degradation is doubtless an important factor in the high potency of Tyr-DAla-phenylpropyl amide. The only amide bonds in this compound involve the amino or carboxyl group of D-alanine, and they should be quite stable to most enzymes.

Sane work done with Stammer (see Stamner, this volume) on enkephalins containing α,β-unsaturated amino acids is also of interest in this regard. When a double bond is introduced between the alpha and beta carbons, the resulting amino acid is quite rigid, and the phenyl ring is held in a position toward the carboxyl in the case of the isomer away from the carboxyl in the case of the Z isomer.When these two isomers were incorporated into the potent pentapeptide enkephdlin Tyr-DAla-Gly-Phe-Met-amide in place of the phenylalanine residue, the potencies were dramatically different (see table 2). The analog containing ΔZ-Phe, in which the aromatic ring is held toward the tyrosine, was super-potent, while the analog containing ΔE-Phe, in which the phenyl ring is held away from the tyrosine, had

TABLE 2

Relative Potency on Ileum of Some Altered Enkephalins The symbol indicates the direction of the phenyl ring in the analog .

Tyr-Gly-Gly-Phe-Met	100
Tyr-DALa-Gly-Phe-Met-amide	150
Tyr-DALa-Gly-ΔZPhe-Met-amide	500
Tyr-DALa-Gly-ΔPhe-Met-amide	low
Tyr-DALa-Phe-Met-amide	130
Tyr-DAla-ΔZPhe-Met-amide	low

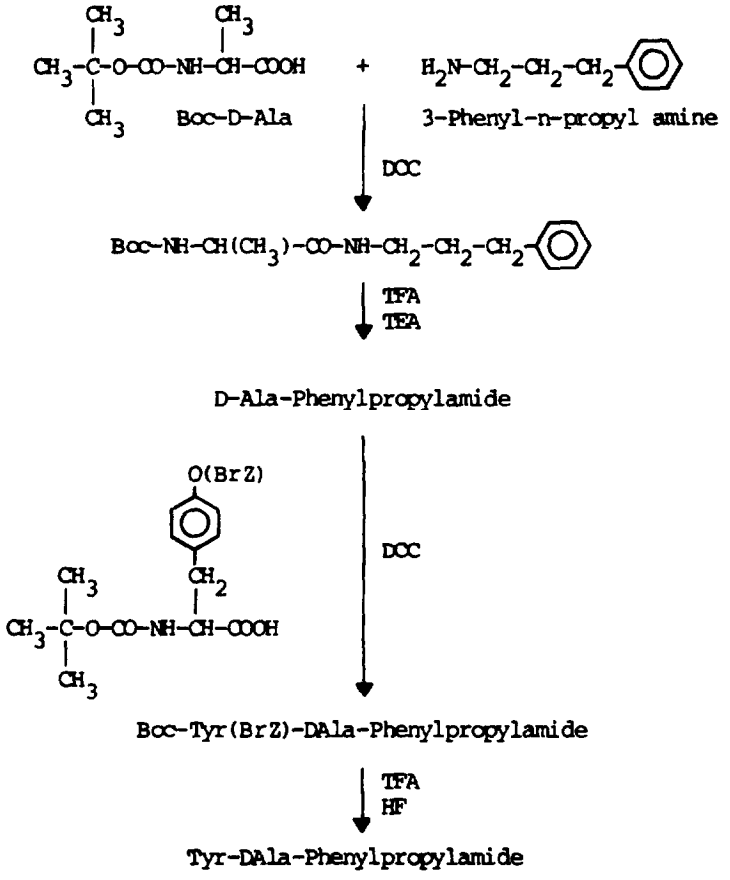

FIGURE 7

Solution Synthesis of the Dipeptide Enkephalin

negligible activity on ileum. This result suggests that the spacing between the binding sites for the aromtic rings on the receptors is rather closely specified, and that the flexibility of the normal pentapeptide allows sufficient bending of the molecule to bring the two aromatic rings into the correct spacing. Since we had found Tyr-DAla-Phe-Met-amide to be very potent, we then examined this analog in which the phenylalanine had been replaced by Z-Phe; this analog was essentially inactive on ileum. unfortunately, the corresponding E-Phe analog was not made, but it probably would have had more potency. Evidently in these compressed analogs the phenyl ring must bend somewhat away fran the tyrosine ring - or at least not be held rigidly toward it.

The dipeptide amide enkephalins are mainly mu receptor agonists; they have quite low potency on the stimulated mouse vas differences and for binding to delta receptors (Vavrek et al. 1983). The receptor selectivity (and relative potency for guinea pig ileum versus muse MS deferens) could be enhanced by addition of a lysine residue to the amino terminus of these short-chain enkephalins. Surprisingly, addition of the lysine residue caused only a alight decrease of ileum potency; the increased selectivity was due to a great loss of vas deferens potency.

The potent dipeptide derivative Tyr-DAla-phehylpropyl amide can be synthesized readily by either solid phase or classical solution methods of peptide synthesis. For solid phase synthesis (Vavrek et al. 1981), the dipeptide Boc-Tyr(BrZ)-DAla was assembled on Merrifield resin. Brz designates the 2-bramobenzyloxycarbonyl protecting group used for the hydroxyl group of tyrosine. The dipsptide-resin was treated for ah extended time with methanol and Methyl amine to remove the peptide as the methyl ester. This ester was then treated with phenyl-n-propyl amine to convert it to the amide. For synthesis of large quantities of the peptide, the solution synthesis scheme shown in figure 7 was used. Boc-D-alanine was converted to the amide with 3-phenyl-n-propyl amine, using DOC as coupling agent. Following renewal of the Boc group with TFA and neutraliation of the salt, Boc-Tyr(BrZ) was coupled, again using DOC. Treatment with TFA for removal of the Boc group and then removal of the BrZ group with HF in the presence of scavengers yielded the desired dipsptide amide.

DYNORPHIN-REALTED PEPTIDES

Of the three major classes of opioid peptides, the dyhorphin group were the last to be characterized (see the chapters in this volume by Chavkin and Kasterlitz). These appear to be the endogenous ligands for the kappa class of opioid receptors. Among the peptides available at this time, dynorphin(1-9) appears to be a very elective ligand for kappa receptors. It has the amino acid sequence Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg. Unfortunately, this peptide is rapidly degraded by enymes, and is thus not practical for in vivo work. Even for receptor binding studies on isolated membranes, a "cocktail" of enzyms inhibitors must be used. One rapid cleavage is at the bond following the leucine residue; this cleavage yields Leu-enkephalin, which is principally a delta receptor agonist. This cleavage thus destroys the kappa selectivity. It would be extremely useful if enzyme resistance could be built into this peptide to yield a stable, selective kappa agonist.

The aualog containing D-alanine in position 2 was synthesized quite soon after the dynorphin sequence was known; that peptide was found to be mainly a delta agonist. It was suggested that the peptide was probably being cleaved to yield [$DAla^2$]-Leu-enkephalin, a potent delta agonist. In order to investigate this question, we synthesized several analogs of dynorphin(1-9) which

TABLE 3

Potencies of Dynorphin(1-9) Analogs for Binding to Mu, Delta and Kappa Receptors in Guinea Pig Brain

Peptide Structure	Relative Potency		
	Mu	Delta	Kappa
Tyr-Gly-Gly-Phe-LeU-Arg-Arg-Ile-Arg	1	1	1
Tyr-Gly-Gly-Phe-Leu-DArg-Ile-Darg	42	1.6	0.2
Tyr-Gly-Gly-Phe-Leu-DArg-DArg-Ile-DArg-NH_2	77	1.3	0.6
Tyr-DAla-Gly-Phe-Leu-Arg-Arg-Ile-Arg	29	1.9	0.1
Tyr-DAla-Gly-Phe-Leu-DArg-DArg-Ile-DArg	43	7.7	0.04
Tyr-DAla-Gly-Phe-Leu-DArg-Ile-DArg-NH_2	81	5.6	0.1

incorporated D-amino acids at various places and in addition examined the effects of making the C-terminal amide of these peptides. All of these changes should impart added enzyme resistance to the peptides. Table 3 gives the structures of the peptides synthesized and compares their binding to guinea pig brain receptors. Fran these experiments (Paterson et al. 1984), done in the cold to minimize enzymatic degradation, it is clear that all the modifications caused loss of potency at the kappa receptor while enhancing binding potency at both mu and delta receptors. Other experiments done at roan temperature or in the presence of enzyme inhibitors showed that the D-alanine substitution conferred enzyme stability on the peptides, while the D-arginine substitutions were not effective. The effect of the D-alanine substitution in dynorphin peptides then is clearly to change receptor specificity. Additional work is needed for develqment of stable, selective kappa agonists.

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Structure-Activity Relationships of Opioid Peptides

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INTRODUCTION

Since our last review (Morley 1980). several new aspects of the structure-activity relationships (SAR) of enkephalinlike peptides have been explored. Most are covered in separate chapters of this volume: SAR of conformationally restricted opioid peptides Schiller; Hruby); SAR of active di-, tri-, and tetrapeptide analogs (Stewart); SAR of dehydro-, cyclopropyl-, and multichain analogs (Stammer); SAR of dermorphin analogs (Feuerstein/Faden), SAR of morphiceptin analogs (Chang, and SAR enkephalin analogs in relation to specificity (Hruby; Roques). We have therefore decided to restrict our own coverage to an update of subject matters contained in our last review--general principles of SAR, the effect of single substitution in [Met/Leu] enkephalin on activity in isolated tissue preparations, and selected in vivo effects--and the outstanding additional topics not covered by other authors.

We discard use of the popular names Met-enkephalin and Leu-enkephalin, since these contravene nomenclature rules (Morley 1981). Instead, the forms [Met]enkephalin and [Leu] enkephalin. now recommended by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB), are employed. Nomenclature proposals for describing backbone-modified enkephalins (see Morley 1981) are also recommended.

GENERAL PRINCIPLES

"Activity," as measured in in vitro, "receptor" binding, and even in vivo studies, continues too frequently to be interpreted as "activity at opiate receptor(s)." This has often led to speculation as to the nature of opiate receptors based on the results of SAR studies. In our last review (Morley 1980), which analyzed the molecular processes involved before activity is expressed, we stressed that such speculation is unjustified. The interplay of two of these processes--the events at the receptor, and proteolysis--has now been convincingly demonstrated by McKnight et al. (1983). By adding inhibitors of peptidases, the observed potency of [Met]enkephalin, for example, in the guinea pig myenteric plexus-longitudinal muscle (GPI), mouse vas deferens (MVD), and rat vas deferens (RVD) tests was increased 18-, 13-, and 200-fold, respectively. The potencies of [Leu]enkephalin, and hexa-, hepta-, and octapeptide C-terminal extensions of [Met] and [Leu]enkephalin, were likewise increased in the presence of peptidase inhibitors, with the greatest effects being seen in the RVD test; whereas the

potencies of more metabolically stable opioid peptides (e.g., beta-endorphin) were little affected.

If we can assume that the combination of peptidase inhibitors used by McKnight et al. (1983) specifically eliminates the capacity of the tissues to inactivate enkephalins, interpretation of results in isolated tissues becomes more secure. For example, the 25-fold greater potency of [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin, as compared with [Met]enkephalin in the GPI test without inhibitors (which some had previously interpreted as reflecting greater activity of the analog at the opiate receptor), reduces to near equipotency when inhibitors are present. If the assumption is correct, we may now conclude that the receptor interaction of the two peptides is very similar. More instructively, two patterns of activity remain on after comparing the activities of metabolically unstable analogs in the 'inhibited' GPI and MVD tests, and more stable analogs in the two normal tests. An enkephalinlike pattern, observed with [Met] and [Leu]enkephalin,[D-Ala^2, L and D-Leu^5] enkephalin, and most endogenous opioid peptides, is characterized by greater potency in the MVD as compared with the GPI test. In contrast, a morphine like pattern, characterized by greater potency in the GPI as compared with the MVD test, is seen with some synthetic peptides, e.g., [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin. This finding is compatible with the evidence for two distinct receptors--predominantly mu in the GPI and delta in the MVD--in the tissues, but the dangers of drawing conclusions as to the nature of receptors based on work with agonists should be recognized.

Use of cocktails of peptidase inhibitors in receptor binding assays has not yet been described, but we can assume that similar shifts of potencies will be observed.

RESULTS USING ISOLATED TISSUE PREPARATIONS

The tables given in our last review (Morley 1980) showing the effect of single substitution (any one amino acid position varied, but not two) on the potency of [Met] or [Leu]enkephalin, or their methyl esters or amides, are now updated (tables 1 to 7). We also include data on peptide backbone modifications (table 8); in these cases, the [D-Ala^2]enkephalin series is included since [D-Ala^2]enkephalin has often served as reference compound.

All potencies have again been related to [Met]enkephalin = 1. When [Leu]enkephalin has been used as standard in the original paper, the conversion factor [Leu]enkephalin = 0.37 x [Met]enkephalin in the GPI test (Morgan et al. 1976) has been applied. When values for neither [Met] nor [Leu]enkephalin have been quoted, the IC_{50} values for analogs have been compared with the following IC_{50} values for [Met]enkephalin (McKnight et al. 1983): 125 nM $(GPI)^{50}$. 17.7 nM (MVD).

Limitation of space permits only cursory discussion of the new results. At the Tyr^1 position, restriction in the degree of conformational freedom of the residue as in the tetralin analog (table 2, third compound listed) leads to a sevenfold to eightfold increase in potency in the GPI test, and a thirtyfold decrease in the MVD test, as compared with the reference compound, [Leu]enkephalin (Deeks et al. 1983). The inactivity of the indanyl analog (table 2, fourth compound listed) illustrates the differential effect of restricting conformational flexibilit on potency in the two tests. Requirement of the p-hydroxy group of Tyr^1 for activity is questioned from the finding that a melphalan analog (in which the p-hydroxyl group is replaced by bis-2-chloroethylamino) of [D-Ala^2, Leu^5]

TABLE 1

N-Terminlly Substituted Derivatives. In Vitro Activity[a] of Analogs of Type X-Tyr-Gly-Gly-Phe-Y

Analog[b]		Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
X =	Y =				
Methyl	Met-OH	1.45	0.23		Summers & Hayes 1981
	Leu-OMe				Di Maio & Schiller 1979
Dimethyl	Leu-OMe				"
Trimethyl ammonium iodide	Leu-OMe				"
Ethyl	Met-OH	0.04	0.0086	0.088	Summers & Hayes 1981
Propyl	Met-OH	0.045	0.0081	0.078	"
Isopropyl	Met-OH	0.013	0.0058	0.004	"
Butyl	Met-OH	0.20	0.0078	0,075	"
Pentyl	Met-OH	0.67	0.011	0.169	"
Hexyl	Met-OH	0.16	0.027	0.44	"
Heptyl	Met-OH	0.11	0.0064	0.122	"
Octyl	Met-OH	0.08	0.0078	0.097	"
Cyclopropylmethyl	Met-OH	0.014	0.0025	0.0095	"
Phenethyl	Met-OH	0.15	0.01	0.125	"
MeCO (acetyl)	Met-OMe	0.005	0.001		Friedmann et al. 1982
H_2N-CO- (carbamoyl)	Leu-OH	0.033	0.001		"
$ClCH_2$-CH_2-NH-CO-	Leu-OH	0.027	0.009		"
Lys-	Leu-OMe	0.38	0.087		"
Lys(CO-NH-CH_2-CH_2Cl)-	Leu-OH	0.53	0.1		"
	Leu-OMe				Suli-Vargha et al. 1981

[a] potencies in the eletrically stimulated guinea pig and mouse vas deferences and receptor binding relative to [Met]enkephal = 1.

[b] Abbreviations for amino acids and their use in the formulation of derivatives follow the various recommendations of the IUPAC-IUB Commision Biochemical Nomenclature.

[c] comparism of data obtained by different investigators seldom justified.

TABLE 2
Variation of Tyrosine[1] Residue. In Vitro Activity[a] of Analogs of X-Gly-Gly-Phe-Y

Analog[b] X =		Y =	Guinea pig ileun	Mouse deferens	Receptor binding [c]	Reference
Phe HO-C_6H_4-CH=CH-CO-		Met-Ome Leu-OH	0.005	0.001		Friedmann et al. 1982 Amar et al. 1983
		Leu-OMe	1.3	0.052		Deeks et al. 1983
		Leu-OMe	0.028	0.00036		Deeks et al. 1983
	(-) isomer	Met-NH_2	inactive	inactive		Ramakrishman & Portoghese 1982
	(+) isomer	Met-NH_2	inactive	inactive		"
	(-) isomer	Met-NH_2	inactive	inactive		Ramkrishman & Portoghese 1982
	(+) isomer	Met-NH_2	inactive	inactive		"

[a]- [c] See footnotes to table 1.

TABLE 3
Variation of Glycine[2] Residue. In Vitro Activity[a] of Analogs of Type Tyr-X-Gly-Phe-Y

Analog[b] X =	Y =	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
4-Aminobutyryl (Gaba)	Met-NH_2				Di Bello et al. 1982
D-4-aminobutyryl	Leu-NH_2	4.13	0.90		Di Maio et al. 1982b
2-Aminoisibutyryl (Aib)	Met-NH_2				Sudha & Balaram 1983
Arg	Met-OH				Kubota et al. 1980
	Met-NH_2				"
	Leu-OH				"
	Leu-NH_2				"
D-Arg	Met-OH				Kubota et al. 1980
					Takagi et al. 1982
	Met-NH_2				Kubota et al. 1980
	Leu-OH				Kubota et al. 1980
					Takagi et al. 1982
	Leu-NH_2				Kubota et al. 1980
des-Gly	Met-OH	0.004			Chipkin et al. 1981
	Met-NH_2	0.02			"
Dehydroalanyl	Leu-OH				Shimohigashi & Stammer 1981, 1982a
D-Norleucyl	Leu-NH_2	4.80	1.63		Di Maio et al. 1982a
D-Norvalyl	Leu-NH_2	5.09	1.96		Di Maio et al. 1982b
Norvalyl	Leu-NH_2	0.002			Di Maio & Schiller 1980
D-Pro	Leu-OEt	0.0001			Reig et al. 1981
-N(Me)-CH_2-CO- (Sar)	Leu-OH	0.024	0.021		Tomatis et al. 1981
-N(CH_2-CH_2OH)-CH_2-CO-	Leu-OH	0.076	0.90		"
-N(CH_2-CO_2H)-CH_2-CO-	Leu-OH	0.0014	0.036		Tomatis et al. 1980

[a-c] See footnotes to Table 1

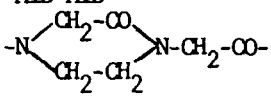
TABLE 4

Varaiation of Glycine[5] Residue. In Vitro Acivity[a] of Analogs of Type Tyr-Gly-X-Phe-Y

Analog[b] X =	Y =	Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
2-Aminoisobutyryl (Aib)	Met-NH_2				Sudha & Balaram 1983
Dehydroalanyl	Leu-OH				Shimohigashi & Stammer 1981, 1982b
-N(Me)-CH_2-CO- (Sar)	Met-NH_2	0.004			Echner & Voelter 1983
Ser	Leu-OH^2				Shimohigashi & Stammer 1982b

TABLE 5

variation of Glycine[2,5] residue. In Vitro Activity[a] of Analogs of Type Tyr-X-Phe-Y

Analog[b] X =	Y =	Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
-NH-$(CH_2)_4$-CO-	Met-OH				Kawasaki & Maeda 1982
-NH-$(CH_2)_5$-CO-	Leu-OH	0.0001	0.0001		Paruszewski et al. 1983
	Leu-NH_2	0.0001	0.0001		"
-Aib-Aib-	Met-NH_2				Sudha & Balaram 1983
	Leu-OH	0.0014	0.01		Tomatis et al. 1981

[a-c] See footnotes to Table 1.

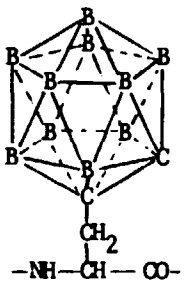
TABLE 6

Variation of Phelylalanine[4] Residue. In Vitro Activity[a] of Type Tyr-Gly-Gly-X-Y

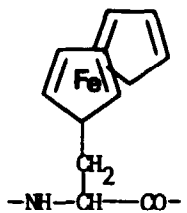
Analog[b] X =	Y =	Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
Car [d]	Leu-OH	0.12			Schwyzer 1979
Car [d]	Leu-NH_2	0.07			"
Fer [e]	Leu-OH				Epton et al. 1981
D-Fer [e]	Leu-OH				"
Dehydrophenylalanyl [f]	Leu-OH				Shimohigashi & Stammer 1981 Shimohigashi et al. 1983

[a-c] See Footnotes to Table 1

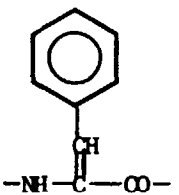
[d] Car =



[e] Fer =



[f] dehydrophenylalanyl =



[a-c] See Footnotes to table 1

TABLE 7

Variation of Met/Leu[5] Residue In Vitro Activity of Analogs of Type Tyr-Gly-Gly-Phe-X

Analog[b] X =	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
Cys(Et)-NHMe				Theodoropoulos et al. 1983
Cys(Et)-NMe_2				"
-NH-C(=CH-Pr^i)-CO- ('dehydroleucyl')			0.47	Shimohigashi & Stammer 1981
-NH-CH(CH_2-CH_2OMe)-CO- (homoseryl methyl ether)				Theodoropoulos et al. 1983
Leu-CH_2Cl	0.81	0.06		Friedmann et al. 1982
Leu-NH-NH-CO-CH_2-CH_2-NH_2	0.35	0.076		"
Leu-NH-NH-CO-CH_2-CH_2-NH-CO-NH-CH_2-CH_2Cl	0.85	0.75		"
Met-OMe	0.97			"
Met(O)-OH	0.15	0.35		"
Met-NH-CH_2-CH_2-CO_2Me	0.55	0.078		"
Met-NH-CH_2-CH_2-CO-NH-CH_2-CH_2-CO_2H	1.53	0.30		"
Met-Lys-Lys-Gly-Glu	0.53	0.29		"
Met-NH-CH(CH_2-S-CH_2-CO-NH_2)-CO_2H (Met-Cys(Cam)-OH)	0.37			Nadasdi et al. 1983
Met-Thr-OH	0.53	2.13		Fournie-Zaluski et al.1981
Nle-OH	1.97	2.94		Bajusz et al. 1980
-NH-CH(CH_2-CH_2-CH_2-CH_3)-PO_4H (NleP)	5.5	26.1		"
-NH-CH(CH_2-CH_2-CH_2-CH_3)-SO_3H (NleS)	12.2	14.11		"
" (D-isomer)	0.197	0.294		"
Phe-NH_2	0.59			Nadasdi et al. 1983
Ser-OH	0.0001			Valencia et al. 1981
Ser(Me)-OH	0.001			"
Ser(Me)-OMe	0.0005			"
Ser-OEt	0.005			"
Ser(Et)-OH				Theodoropoulos et al. 1983

(Table 7 continued next page)

Table 7 (continued)[a]

Analog[b] X =	Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
Thr-OH	0.0001			Valencia et al. 1981
Thr-OEt	0.001			"
Thr(Me)-OH	0.001			"
Thr(Me)-OMe	0.002			"
Trp-NH_2	0.11			Nadasdi et al. 1983
$-NH(CH_2)_3-CO-NH_2$				Di Bello et al. 1982
$-NH(CH_2)_5-CO_2H$	0.028	0.14		Paruszewski et al. 1983
$-NH(CH_2)_5-CO-NH_2$	0.01	0.01		"

[a-c] See footnotes to table 1.

enkephalin methyl ester has high affinity for mu and delta binding sites (IC_{60} 50 and 10 nM respectively) (Szucs et al. 1984), and activity in the GPI test (Medzihradszky 1984). Judgment on this issue must be suspended since cleavage of the bis-2-chloroethyl-amino group to a hydroxy group (via ethyleneimmonium intermediate), under the conditions of the tests, seems possible, offering an alternative explanation of the results. However, in support of the conclusion of Szucs et al., it will be recalled that the (tyrosine) O-methyl ether of [Met]enkephalin, presumably metabolically stable at the p-position, is claimed to have significant activity in the GPI test (see Morley 1980). The $Azgly^3$ (-NH-NH-CO-) analog still remains the only 3-substituted enkephalin with appreciable activity in any test, suggesting exploration of planar 2, 3-substituted analogs. Such analogs have not yet been described (see table 5). At the Met/Leu^5 position, substitution of the carboxyl group of [Nle]enkephalin by a phosphonic or sulphonic acid group causes, respectively, a 5- or 12-fold increase in potency in the GPI test, and a 26- or 14-fold increase in the MVD test (table 7, 16th and 17th compounds listed) (Bajusz et al. 1980). The increased acidity and bulkiness of the phosphonic acid group apparently favors interaction at the delta binding site. In contrast, similar replacements in [$D\text{-}Nle^6$]enkephalin result in substantial loss of activity.

Turning to the backbone modifications (table 8). significant activity in one or more of the tests results from replacement of the $Tyr^1\text{-}Gly^2$ peptide bond by $CH_2\text{-}NH$ or $CH_2\text{-}CH_2$ (Szelke et al. 1979; Hudson et al. 1980), by CH=CH (Hann and Sammes 1980; Hann et al. 1982; Cox et al. 1980), but not by CS-NH (Clausen et al. 1983. 1984); replacement of the bond by an aminoxy linkage (CO-NH-O) provides a weakly active analog (Salvadori et al. 1981). Replacement of the $Gly^2\text{-}Gly^3$ peptide bond by CS-NH leads to a 1.48-fold increase in potency in the GPI test and a 15- to 22-fold increase in the MVD test (Clausen et al. 1983, 1984); other modifications of the bond (CH=CH, CH_2S, $CH_2\text{-}NH$, $CH_2\text{-}CH_2$, CO-NH-O) lead to inactive analogs (see table 8 for references). Replacement of the $Gly^3\text{-}Phe^4$ peptide bond by $CH_2\text{-}NH$ leads to slightly increased potency in the GPI test, and to decreased potency in the MVD test (Szelke et al. 1979); other modifications lead to almost inactive analogs (CH_2-S. CH_2-SO, CO-NH-O). Finally, all replacements of the $Phe^4\text{-}Met/Leu^5$ peptide bond (CS-NH, CH_2-NH, CO-NH-O. CH_2-S, NH-CO, CO-O) lead to analogs having potencies similar to, or slightly greater than. those of the reference compound (analogs in the $D\text{-}Ala^2$ series should be compared with [$D\text{-}Ala^2$]enkephalin).

ENKEPHALIN ANTAGONISTS

A significant development in the past 2 years has been the emergence of antagonists which are structurally related to the enkephalins. They are of two main types--one based on N,N-diallyenkephalin with delta binding site specificity, and the other based on enkephalin (1-4) tetrapeptide dialkyl amides with mu binding site specificity. The former type has probably been explored more extensively, but little of the work has yet been published. All published work on both types is summarized in table 9.

Dealing first with the N,N-diallyl type, it has been known for some years that substitution of the (N) methyl group of morphine and other opiates by certain lipophilic groups (e.g., allyl, cyclopropylmethyl) leads to loss of agonist activity, and may provide analogs with potent antagonist activity (e.g., naloxone, naltrexone). Monosubstitution of such groups at the amino terminus of enkephalins provides only weak agonists or partial agonists, but Shaw et al. (1982) found that substitution of diallyl at the amino terminus provided an

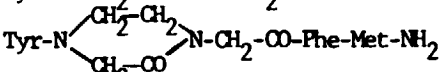
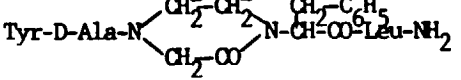
TABLE 8

In Vitro Activities[a] of backbone Modified Analogs of Tyr-Gly-GLy-Phe-Met and Tyr-Gly-Gly-Phe-Leu

Analog[b]		Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
$H_2N-CH(CH_2-C_6H_4OH)-CS-NH-CH_2-CO-Gly-Phe-Leu-OH$		inactive	0.07		Clausen et al. 1983, 1984
$H_2N-CH(CH_2-C_6H_4OH)-CH_2-NH-CH_2-CO-Gly-Phe-Met-NH_2$		0.56		0.5	Szelke et al. 1979 Hudson et al. 1980
$H_2N-CH(CH_2-C_6H_4OH)-CH_2-NH-CH_2-CO-Gly-Phe-Met-ol$		0.83		2.0	Szelke et al. 1979 Hudson et al. 1980
$H_2N-CH(CH_2-C_6H_4OH)-CH_2-CH_2-CH_2-CO-Gly-Phe-Met-NH_2$		0.03		0.09	Szelke et al. 1979 Hudson et al. 1980
$H_2N-CH(CH_2-C_6H_4OH)-CO-NH-O-CH_2-CO-Gly-Phe-Leu-OH$		0.004	0.0036		Salvadori et al. 1981
$H_2N-CH(CH_2-C_6H_4OH)-CH=CH-CH_2-CO-Gly-Phe-Leu-OH$				0.25	Hann & Sammes 1980 Hann et al. 1982
$H_2N-CH(CH_2-C_6H_4OH)-CH=CH-CH_2-CO-Gly-Phe-Leu-OMe$		1.11	0.38		Cox et al. 1980
$H_2N-CH(CH_2-C_6H_4OH)-CH_2-CH=CH-CO-Gly-Phe-Leu-OH$				0.03	Hann et al. 1982
$Tyr-NH-CH_2-CS-NH-CH_2-CO-Phe-Leu-OH$		1.48	15.2 22.1		Clausen et al. 1983 Clausen et al. 1984
$Tyr-NH-CH_2-CHMe-S-CH_2-CO-Phe-Leu-OH$	isomer I isomer II	weak antagonist 0.049			Spatola et al. 1981 "
$Tyr-NH-CHMe-CH_2-S-CH_2-CO-Phe-Leu-OH$ (D)		inactive	0.00005		Clausen et al. 1983

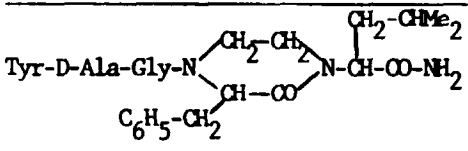
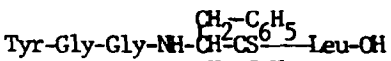
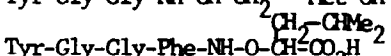
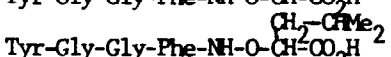
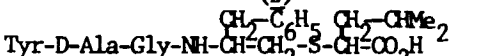
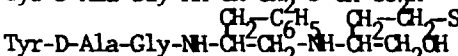
(Table 8 continued next page)

Table 8 (continued)

Analog[b]	Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
Tyr-NH-CH_2-CH_2-NH-CH_2-CO-Phe-Met-OH	0.01		0.05	Szelke et al. 1979 Hudson et al. 1980
Tyr-NH-CH_2-CH_2-NH-CH_2-CO-Phe-Met-NH_2	0.003		0.03	"
Tyr-NH-CH_2-CH_2-NH-CH_2-CO-Phe-Met-ol	0.003		0.03	"
Tyr-NH-CH_2-CH_2-CH_2-CH_2-CO-Phe-Met-NH_2	0.003		0.01	"
Tyr-NH-CH_2-CO-NH-O-CH_2-CO-Phe-Leu-OH	0.004	0.036		Salvadori et al. 1981
				Moon et al. 1981
Tyr-CH_2-CH_2-CO-CH_2-CH_2-CO-Phe-Leu-OH			0.001	Almquist et al. 1984
Tyr-Gly-NH-CH_2-CO-NH-O-CH(CH_2-C_6H_5)-CO-Leu-OH	0.004	0.0036		Salvadori et al. 1981
Tyr-Gly-NH-CH_2-CO-NH-O-CH(CH_2-C_6H_5)-CO-Leu-OH	0.004	0.0036		"
Tyr-D-Ala-NH-CH_2-CH_2-S-(D)CH(CH_2-C_6H_5)-CO-Leu-OH		0.0013		Clausen et al. 1983
Tyr-D-Ala-NH-CH_2-CH_2-SO-CH(CH_2-C_6H_5)-CO-Leu-OH		0.013		"
Tyr-D-Ala-NH-CH_2-CH_2-NH-CH(CH_2-C_6H_5)-CO-Met-ol	1.2	0.1		Szelke et al. 1979
				Moon et al. 1981

(Table 8 continued next page)

Table 8 (continued)

Analog [b]	Guinea pig ileum	Mouse vas deferens	Receptor binding [c]	Reference
 (L,L;D,D)				Moon et al. 1981
(L,D;D,L)				"
	1.23	3.49		Clausen et al. 1983
	0.37		0.36	Szelke et al. 1979
	0.05	0.51		Salvadori et al. 1981
	0.09	0.21		"
	3.24	6.96		"
	2.65	4.18		Clausen et al. 1983
	5.2		2.0	Szelke et al. 1979
	2.0			Chorev et al. 1979

(Table 8 continued next page)

Table 8 (continued)

Analog[b]	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-NH-CO-CH(CH_2-CH_2-SMe)-$CO-NH_2$	1.75			Chorev et al. 1979
Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-NH-CO-(D)CH(CH_2-$CHMe_2$)-NH-CHO	14.65	0.98		Chorev et al. 1979 Berman et al. 1983
Tyr-D-Ala-Gly-NH-CH(CH_2-C_6H_5)-NH-CO-(D)CH(CH_2-CH_2-SMe)-NH-CHO	14.99			Chorev et al. 1979
Tyr-D-Ala-Gly-Phe-NH-CH(CH_2-$CHMe_2$)-NH-CHO	9.45	2.57		Berman et al. 1983
Tyr-D-Ala-Gly-Phe-O-CH(CH_2-CH_2-SMe)-$CO-NH_2$		0.95		Geselichen et al. 1981
Tyr-D-Ala-Gly-Phe-O-CH(CH_2-$CHMe_2$)-$CO-NH_2$		2.0		"
Tyr-D-Ala-Gly-Phe-O-CH(CH_2-C_6H_5)-$CO-NH_2$		0.22		"
Tyr-D-Ala-Gly-Phe-N(Me)-CH(CH_2-CH_2-SMe)-$CO-NH_2$		5.0		"

[a]- [c] see footnotes to table 1.

TABLE 9

Enkephal in Antagonist and Their Potency

Antagonist	K_e (nM) mouse vas deferens vs. LeuE "δ"	vs. MetE "δ"	vs. Normorphine "μ"	"mu/delta"	Reference
Diallyl-Tyr—Gly—Gly—Phe—Leu-OH	204		14100	69.1	Belton et al. 1983
——————————OMe	323		4470	13.8	Shaw et al. 1982
——————D-Ala ——————OMe	1430		3730	2.6	Belton et al. 1983
——————D-Ala ——————OH	1410		8090	5.7	"
——————D-Ala ——————D-Leu-OH	6880		30000	4.3	"
——————D-Met ——————OMe	3602		3030	0.84	"
——————D-Met ——————Pro-NH_2	1590		597	0.37	"
——————$NH-CH_2-CH_2-S-CH(CH_2-C_6H_5)-CO-Leu-OH$ [a]	254	871	7410	29.1	"
——————$NH-CH_2-CH_2-S-CH(CH_2-C_6H_5)-CO-Leu-OH$ (D)	310		5780	18.6	"
H-Tyr-D-Ala-Gly-MePhe-N($CH_2-CH_2-C_6H_5$)($CH_2-CH_2-CHMe_2$)		566	44	0.07	Bower et al. 1981
——————NMe-$CH_2-CH_2-C_6H_5$		2530	725	0.28	"
——————$N(CH_2-CH_2-C_6H_5)_2$		1110	83	0.07	"
——————N($CH_2-CH_2-C_6H_5$)$(CH_2)_3-S-Me$ [b]		724	52	0.07	"
H-Tyr-NH-CH_2-CHMe-S-CH_2-CO-Phe-Leu-OH(isomer I)	'weak antagonist in guinea pig ileum assay'				Spatola et al. 1981

[a] K_e vs. [D-Ala^2, D-Leu^5]enkephalin 246nM, vs. etorphine 4170 nM, vs. ethylketocyclazocine 30,000 nM.

[b] Analogous compounds with Sar or N-phenethylglycine in place of MePhe also described.

analog, N,N-diallyl-[Leu]enkephalin methyl ester (ICI 139462) (table 9, , 2nd compound listed), devoid of agonist activity, which effectively antagonized the action of [Leu]enkephalin in the MVD, but was much less effective in antagonizing the action of normorphine. Based on this lead, the metabolically more stable N,N-diallyl-Tyr-Gly-Gly-psi(CH_2S)-Phe-Leu-OH (ICI 154129) (table 9, 8th compound listed) evolved, which is approximately 30 times more potent against [Leu]enkephalin than against normorphine in the MVD, and which is effective in vivo in antagonizing a response mediated by delta-type opiate agonist (Gormley et al. 1982). No simple relationship was found to exist between the agonist activity of a given enkephalin analog and antagonist potency of its diallyl congener (Belton et al. 1983). Thus, N,N-diallylation of the potent and relatively mu selective agonists [D-$Ala^2$$Leu^5$]enkephalin. [D-$Met^2$, Leu^5]enkephalin methyl ester. and (D-Met^2, Pro-NH_2^5]enkephalin gave rise to weak nonspecific antagonists (table 9; 4th, 6th, and 7th compounds listed). In these analogs, the delta antagonist potencies are severely reduced relative to N,N-diallyl-[Leu]enkephalin. More surprisingly, the N,N-diallyl analog of the potent and relatively delta selective agonist [D-Ala^2, D-Leu^5]enkephalin was found to be a weak delta partial agonist, with little or no mu antagonist activity (table 9, 5th compound listed). Replacement of the Gly^3-Phe^4 peptide bond by a methylenethio (CH_2-S) linkage in [Leu]enkephalin or [D-Phe^4, Leu^5]enkephalin gave analogs (table 9; 8th and 9th compounds listed) which were both moderately potent and highly selective delta antagonists as diallyl congeners. The effect of replacing the Gly^3 residue by D- and L-Ala was also explored by Belton et al. (results not shown in table 9). Replacement of Gly^3 by L-Ala led to sevenfold reduction in potency, but the D-Ala^3 derivative was equipotent with N,N-diallyl-[Leu]enkephalin. This result contrasts markedly the results of 3-substitution in enkephalin agonists.

The tetrapeptide N,N-diallylamide type of antagonists arose from an investigation of structural substituents which may interact with complementary structural features adjacent to the opiate receptor (Bower et al. 1981). In this series, antagonist activity was found to be restricted to a relatively limited range of structures; the nitrogens of Phe^4 and C-terminal amide must be fully substituted, and the latter must bear a 2-phenylethyl group. Greater structural flexibility is tolerated with respect to the other obligatory C-terminal amide substituent; isoamyl, phenethyl, and 3-methylthiopropyl (table 9; 10th, 12th, and 13th compounds listed) substituents provided essentially pure antagonists of approximately equal activity, which reversed the effect of morphine in the MVD at least 10 times more easily than they reversed the effects of [Met]enkephalin. When the second substituent on the terminal amide was methyl (table 9; 11th compound listed), antagonism in the MVD test was weak, and the analog was a weak agonist in the GPI test.

IN VIVO TESTING

Attention continues to be mostly directed to assessment of the antinociceptive activity of analogs, using the mouse tail flick, hot plate, or writhing tests. Results with the most potent analogs, administered by various routes, have recently been summarized (Morley 1983). Results with a more comprehensive range of analogs are given in table 10.

TABLE 10

Molar Potencies of Enkaphalin Analogs Relative to MetE (In Vitro Tests) or Morphine (In Vivo Tests)

Analog[c]	In vitro[a] (MetE=1)		In vivo results (morphine = 1)[b]								Reference
			Tail flick test				Hot plate test				
	GPI	MVD	icv	sc	iv	po	icv	sc	ip	po	
Tyr-D-Ala-Gly-MePhe-NH-NH-CO-Et								1.0			Shinagawa et al. 1981a
MeTyr-D-Ala-Gly-MePhe-NH-NH-CO-Et								2.0			"
Tyr-D-Gln-Gly-Phe-NH-NH-CO-Me								1.0			Shinagawa et al. 1981b
Tyr-D-Gln-Gly-Phe-NH-NH-CO-Et								2.0			"
Tyr-D-Thr-Gly-Phe-NH-NH-CO-Me								1.0			"
Tyr-D-Met(O)-Gly-Phe-NH-NH-CO-Et								4.0			"
Tyr-D-Met(O)-Gly-MePhe-NH-NH-CO-Et								6.0			"
Tyr-D-Ala-Gly-MePhe-Met(O)-ol				3.0	10.0	1.0					Roemer & Pless 1979
MeTyr-D-Ala-Gly-MePhe-Met(O)-ol				4.0	2.0	0.2					"
MeTyr-D-Met(O)-Gly-MePhe-Met(O)-ol				1.0	1.0	0.13					"
Tyr-D-Ala-Gly-MePhe-Met-NHPr^n	22.7						13.7	0.06			Raghubir et al. 1982
Tyr-D-Ala-Gly-MePhe-Met(O)-NHPr^n	18.7						11.5	0.13		0.14	"
Tyr-D-Ala-Gly-MePhe-Met-NH-Pr^i	9.8						407.4	0.06			"
Tyr-D-Ala-Gly-MePhe-Met(O)-NHPr^i	16.6						196.4	0.32		0.16	"
Tyr-D-Arg-Gly-MePhe-Met(O)-ol					1.37						Kubota et al. 1980
Tyr-D-Met(O)-Gly-Phe-ol	2.1			1.4				0.9*			Kiso et al. 1981
MeTyr-D-Met(O)-Gly-Phe-ol	0.68			1.2				1.5*			"
Tyr-D-Met(O)-Gly-MePhe-ol	14562.5			9.1				6.6*			"
MeTyr-D-Met(O)-Gly-MePhe-ol	17125.0			7.1				6.4*			"
Tyr-D-Lys(CHO)-Gly-Phe-Pro-NH$_2$	2.0		15		0.25						Geiger et al. 1983
Tyr-D-Lys(CHO)-Gly-Phe-Pip-NH$_2$	1.0		5		0.15						"

(Table 10 continued next page)

TABLE 10 (continued)

Analog[c]	In vitro[a] (MetE=1)		In vivo results (morphine = 1)[b]								Reference
			Tail flick test				Hot plate test				
	GPI	MVD	icv	sc	iv	po	icv	sc	ip	po	
Tyr-D-Lys(CHO)-Gly-Phe-HCys-	162.5		1		0.5						"
Tyr-D-Ala-Gly-Phe(p-F)-MeMet-NH_2		79.2						5.5			Gesellchen et al. 1981
Tyr-D-Ala-Gly-Phe(p-NO_2)-MeMet-NH_2		95.3						0.75			"
Tyr-D-Ala-Gly-Phe(p-CF_3)-MeMet-NH_2		40.6						0.28			"
Tyr-D-Met(O)-Hfe-NH-Ia	5.45							10*		0.1*	Casiano et al. 1983
Tyr-D-Ala-Gly-MePhe-NH(CH_2)$_2$NO-Me_2	3.31							1000*		2.0*	"

Mouse writhing test (algesic agant phanylquinone or unspecified).

[a]Results obtained using electrically-stimulatad guinea pig myenteric plxus-longitudinal muscle test (GPI), or mouse vas deferens (MVD) teat.

[b]Analog administered intracerabroventricularly (icv). subcutaneously (sc), intravenously (iv), intraperitoneally (ip), or orally (po); usually in mice (SC, iv, ip, po), or rata (icv).

[c]Abbreviation for amino acids and, their use in the formation of deravatives follow the various recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Pip = L-pipecolic acid; HCys = L-homocysteinethiolators; Nfe = (R) 2-amino-4-phenylbutanoic acid; Ia = 3-methylbutyl.

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Design Principles: Enkephalins With Predictable Mu/Delta Receptor Specificity

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INTRODUCTION

Since the discovery of enkephalins in 1975 (Hughes et al.), numerous studies have been done during the last 10 years to elucidate the structure-activity relationships (SAR) of enkephalins and related peptides by scientists belonging to various disciplines. A number of questions have been raised, such as, What determines the receptor affinity? What determines the biological activity? What determines mu/delta receptor selectivity? etc. Considerable activity has been directed toward answering these questions, and by now a reasonable amount of infomation is available to answer the above questions.

Table 1 shows the number of publications in enkephalin and related areas, as listed in Biological Abstracts and chemical Abstracts. It is interesting to note that publications in this area of research have shown a steady growth both in biological and chemical abstracts; however, there appears to be a major growth in chemical abstract publications, particularly in the medicinal chemistry area. This indicates the interest of chemists in important research topics such as structure elucidation. conformational similarity of enkephalin to morphine, the finding of multiple types of opiate receptors, and the recent finding of various enkephalin-containing peptides in precursor proteins and mechanisms for processing. Among them, the most important turning point on the SAR studies of enkephalins is the postulation Martin et al. 1976; Lord et al. 1977) and confirmation (Robson and Kosterlitz 1979; Chang and Cuatrecasas 1979) of a multiplicity of opiate receptors: mu receptors to which morphine binds preferentially, and delta receptors which prefer natural enkephalina. Although before that point most of the synthetic efforts were centered on obtaining highly bioactive peptides (Coy and Kastin 1980; Morley 1980), it is obvious that the accumulated information by such SAR studies has greatly helped us to better understand the receptor specificity to design more specific and more selective ligands for each receptor.

Recent research highlights in the genetic structure elucidation of protein precursors have suggested the presence of a number of

TABLE 1

Numbers of Enkephalin Publications Listed in Biological and Chemical Abstracts

Year	Biological Abstracts	Chemical Abstracts
1975	1	3
1976	14	35
1977	143	174
1978	224	291
1979	412	201
1980	580	535
1981	692	326
1982	752	494
1983	522	587

(Searched by NIH Library Medline)

enkephalin like pept ides having the enkephalin sequences in their N-terminal portion. These include elongated-enkephalins (Noda et al. 1982; Gubler et al. 1982; Comb et al. 1982), neo-endorphins and dynorphins (Kakidani et al. 1982), and endorphins (Nakanishi et al. 1979). It is not clear yet whether these peptides have different functions and receptors. On the other hand, sane studies have suggested that the opiate receptors designated as "delta" may not be so closely related in the central and peripheral nervous systems (Brantl et al. 1982; Shimhigashi et al., unpublished). The elucidation of such a further heterogeneity of the receptors and their functions must be one of the research directions of future SAR studies on enkephalins, and thus it becomes more important to have an insight into the interaction between the enkephalins and their receptors.

A new generation of the SAR studies has also been developed recently by the bioevaluation of various conformationally restricted analogs such as dehydro- (Shimohigashi et al. 1981a. 1982b). cyclopropyl- (Kimura et al. 1983). cyclic- (Schiller and DiMaio 1982; Mosberg et al. 1983). and thioamide- (Clausen et al. 1984) enkephalins, and of dimeric enkephalins (Shimohigashi et al. 1982b, 1982c). Such more dynamic structure-activity studies are certainly useful as probes of specific receptor interactions.

In this article, the classical structure-activity studies of enkephalins will be reviewed briefly and reevaluated in order to better interpret the new SAR data specific for delta and mu opiate receptor Structural elements in the enkephalin molecule essential to the specific interaction with these receptors will be analyzed as character istics for the predictable receptor specificity.

ENZYMOLYSIS OF ENKEPHALINS

Enzymatic Degradation of Enkephalins

The in vivo inactivation of opioid peptides is due to their susceptibility to enzymatic degradation during passage to their receptors (Schwartz et al. 1981). Studies of ^{3}H-enkephalins in mice and rats have shown that their half-life is extremely short (Meek et al. 1977; Stine et al. 1980). Enzymatic hydrolysis is possible at all the peptide linkages in the eknkaphalin sequence. A shown in the figure 1. i.e., Tyr^{1}-Gly^{2} by amniopeptidases (Hambrook et al. 1976; Marks et al. 1977): Gly^{2}-Gly^{3} by dipeptidylaminopeptidase (enkephalinase B) (Gorenstem and Snyder; 1979), Gly^{3}-Phe^{4} by enkephalinase (Malfroy et al. 1978), and Phe^{4}-Leu^{5} (or Met^{5}) by carboxypeptidases (Marks et al. 1977; Meek et al. 1977). The most important cleavage is the removal of the Tyr^{1} residue by aminopeptidases, which have been found, besides in blood (Coletti-Previero et al. 1981; Turkall et al. 1982), as a membrane-bound form in brain (Schnebli et al. 1979; Hui et al. 1983). neuroblastoma x glioma hybrid cells (NG-105-18) (Deslauriers et al. 1980), and guinea pig ileum (GPI) (Geary et al. 1982). Enkephalinase, a membrane-bound carboxydipeptidase; seems responsible for the metabolism of enkephalins, and its distribution in brain has been demonstrated to be in good agreement with that of enkephalin and opiate receptors (Schwartz et al. 1981).

For the prevention of these enzymatic degradations, potent and specific inhibitors of enkephalin-degrading enzyms have been developed especially to protect the peptide in the bioassay systems. Puromycin (Barclay and Phillipps 1978; Vogel and Altstein 1979) and bestatin (Wagner and Dixon 1981) have been shown to be potent inhibitors of aminopeptidases. Thiorphan (Roques et al. 1980), some hydroxmic acid derivatives of peptides (Hudgin et al. 1981) and amino

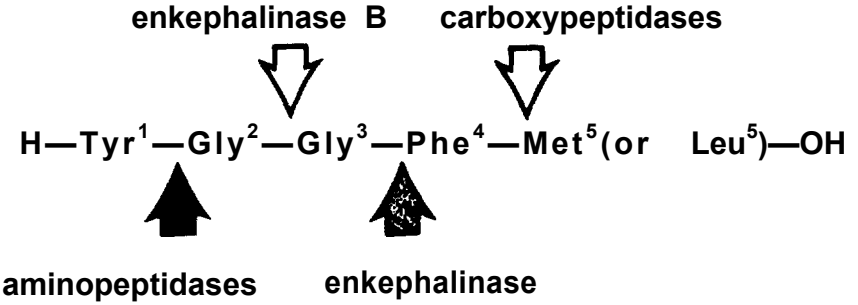

FIGURE 1

Enzymatic Degradations of Enkephalin

acids (Coletti-Previero et al. 1982), and phosphoryl-Leu-Phe (Altstein et al. 1982) strongly protect the enkephalins fran the action of enkephal inase. Bacitracin, a nonspecific inhibitor of a variety of peptidases, inhibits the aminopeptidases and enkephalinase equally well (Schwartz et al. 1981; Hudgin et al. 1981) and it is utilized most frequently for protection of enkephalin assay systems.

Blood-Brain Barrier

Another important key to in vivo biological activities after systemic administration of the enkephalin is their passage through the blood-brain barrier. After administration of enkephalin analogs by both the intracranial and intraperitoneal routes and then monitoring the general activity in goldfish, Olson et al. (1978) suggested that enkephalin analogs might cross the blood-brain barrier. Rapoport et al. (1980) have also shown, by using radioactive opioid peptides, that they have a moderate and significant cerebrovascular permeability in rats. However, Pardridge and Mietus (1981) have suggested that a high-affinity receptor or transport mechanism for Leu-enkephalin does not exist in the blood-brain barrier, and the barrier contains active aminopeptidase and enkephalinase components. Thus, the interaction of enkephalins with the barrier have been characterized by both a very low permeability and a high enzymic degradative activity. Such a low permeability (2% to 3%) was also demonstrated by Conford et al. (1978). The reported results regarding the enkephalin transport through the blood-brain barrier are somewhat conflicting.

HIGHLY BIOACTIVE ENKEPHALIN ANALOGS RESISTANT TO ENZYMOLYSIS

Effect of General Modifications on Enkephalin Enzymolysis

Since enkephalins are immediately degraded by a variety of peptidases before they reach their receptors, one of the synthetic approaches to more active analogs of enkephalins has been directed toward the prevention of these enzymatic degradations to prolong their circulatory life. This has been done by various chemical modifications of the peptide molecule. In general, the replacement of L-amino acids by D-amino acids and N-methylation of amide linkages are ordinary procedures to prevent the enzymatic hydrolysis of the peptides. The results of these modifications of enkephalins are clear, as shawn in table 2. Enkephalin has three optically active amino acid residues: Tyr, Phe, and Leu (or Met). The Tyr and Phe residues cannot tolerate the L- and D-amino acid substitution, destroying almost all of its activity (Coy et al. 1976; Beddell et al. 1977a). Even the combination of D-Tyr and D-Phe completely destroys the receptor affinity (Shimohigashi et al., unpublished data). These results may be related to the importance of the stereo-orientation of aromatic groups in enkephalins to opiate receptor recognition (Shimohigashi et al. 1984b). However, the Leu^5 residue is quite insensitive to the replacement by D-Leu, which retains almost complete activity.

The N-methylation of the Gly^2 and Gly^3 residues interferes with the interaction of the peptide with the receptors (Beddell et al. 1977b). while the N-methylation of the Tyr^1 and Phe^4 residues preserves considerable activity (Beddell et al 1977b; Dutta et al. 1977). The substitution of MeLeu for the Leu^5 residue has been shown to enhance the activity (Dutta et al. 1977). It should be noted that such small structural modifications alter dramatically the activity of enkephalins.

Classical Structure-Activity Studies of Enkephalins

Numerous chemical modifications for each amino acid residue in the enkephalin sequence have been done as a structural variation for the elucidation of dynamic structure-activity relationships. A number of SAR studies have clarified the mine acid residues that are essential to biological activities. These include the Tyr^1, Gly^3 and Phe^4 residues, as shown in figure 2. In general, chemical modification of these residues resulted in loss of activity. In order to obtain a highly bioactive analog, other residues like Gly^2 and Leu^5 or Met^5 have therefore been the main target of modifications for prevention of enzyrmolysis and for increased affinity with the receptors.

Tyrosine-l. For the Tyr^1 residue, all of its structural elements are required for the biological activity (table 3). The p-hydroxy group (Morgan et al. 1976; Beddell et al. 1977a), aromatic ring, with only one methylene spacer between the ring and α-carbon (Beddell et al. 1977b; Dutta et al. 1977). asymetry of the α-carbon (Coy et al. 1976). and amino group (Morgan et al 1976; Gacel et al. 1979) are all structural requirements of the Tyr^1 residue for the

TABLE 2

Relative Activitieis of Leu-enkephalin Analogs Raving D-Amino Acid or N-Methylamino Acid Substitutions[a]

Substitutions	Relative Potency (M V D)
D-amino acid	
$D\text{-}Tyr^1$	0 . 4
$D\text{-}Phe^4$	0.03
$D\text{-}Leu^5$	118
N-methylamino acid	
$MeTyr^1$	63
$Metyr^1$	0.3
Sar^3	0.3
$MePhe^4$	0.3
$MeLeu^5$	150[b]

a) Calculated relative to Leu-enkephalin (=100) using data of Beddell et al. (1977a) and Dutta et al. (1977).
b) Relative activity in GPI.

FIGURE 2

Chemical Structure of Enkephalin and Its Structural Elements Essential for Biological Activities (With Shadowing) Leu-enkephalin: R = $CH(CH_3)_2$, Met-enkephalin: R = CB_2SCH_3

TABLE 3

Relative Activities of Met-enkephalins Having Chemical Modifications on Tyrosine-1 Residue

Modifications	Relative Potency[a] GPI	MVD	References
Amino Group			
des-amino	Inactive	inactive	Morgan et al. 1976
NH_2CH_2CO-	19	30	Gacel et al. 1979
$NH_2(CH_2)_{2-5}CO$-	1.3	0.3	Gactl et al. 1979
CH_3CO-	0.1	---	Dutte et al. 1977
CH_3-	145	23	Somers & Hayes 1981
$CH_3(CH_2)_{1-7}$-	4-67	0.8-2.7	Summers & Hayes 1981
p-Hydroxy Group			
des-hydroxy	0.2	0.03	Morgan et al. 1976
CH_3O-	---	2.3	Beddell et al. 1977b
Tyr-substitution			
des-Tyr	inactive	0.1	Dutta et al. 1977
D-Tyr	---	0.1	Coy et al. 1976
homo-Tyr[b]	---	3.7	Beddell et al. 1977b
p-OH-pheGly[c]	0.2	---	Dutta et al. 1977

a) Relative to Met-enktphalin (-100)
b) Substituted by Leu[5]
c) Substituted by Leu-OCH_3[5]

fundamental activity of the enkephalin. The removal of the residue, per se, eliminates canpletely the activity (Hambrook et al. 1976). Thus, tyrosine is an essential constituent of the enkephalin, and there appears to be no way at present to modify it chemically for an increased receptor affinity. The only exception, however, is N-alkylation by methyl or pentyl of the amino group, which gives a fairly good activity in an isolated and electrically stimulated smooth muscle preparation of the GPI (Sumners and Hayes 1981).

Glycine-2. Since any chemical modifictaion of the Tyr^1 residue destroys the activity, the prevention of degradation by aminopeptidases should be achieved by modification of the Gly^2 residue. Fortunately, the replacement of this residue with various D-amino acids leads to a preservation or even enhancement of potency. For example, D-Ala (Coy et al. 1976; Beddell et al. 1977b; Walker et al. 1977), D-Met (Dutta et al. 1977; Shaw et al. 1978), D-Ser (Shaw et al. 1978), and D-Thr (Dutta et al. 1977) in position 2 give a fairly active enkephalin analog. Surprisingly, Montecucchi et al. (1981) isolated a novel opioid peptide "dermorphin" fran the skin of frogs, which contains the $D\text{-}Ala^2$ residue in its sequence: H-TYr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2.

Glycine-3. The Gly^3 residue is essential for the enkephalin activity. Almost all the modifications of this residue eliminate the activity. The exceptions are substitutions with azaglycine (Dutta et al. 1977) and dehydroalanine (Shimohigashi and Strammer 1982b) which exhibit a considerable potency in the GPI and in receptor binding, respectively.

Phenylalanine-4. In order to overcome the enzyme barrier by enkephalinase at the Gly^3-Phe^4 linkage, N-Methylation of the Phe^4 residue results in retention of some activity (table 2) and seems synergistic with further modifications in other positions for a higher activity. Whilst, placement of D-Phe or any other D-amino acid in position 4 results in a total loss of activity. The bulkiness or hydrophobicity of the side-chain may be one of structural requirements at this position, since a considerable retention of potency has been observed when the aromatic ring is saturated (Dutta et al. 1977) or when it is substituted by carboranylalanine containing boron atoms (Eberle et al. 1977) or by tryptophan (Schiller et al. 1978). The stereo-orientation of the phenyl group is apparently important in receptor recognition, as evidenced by synthesis of dehydrophenylalanine^4-enkephalins (Shimohigashi et al. 1982d, 1984b) and cyclopropylphenylalanine^4-enkephalins (Kimura et al. 1983). in which the phenyl ring is conformationally restricted to each particular arrangement.

Leucine or Methionine-5. The native enkephalins, Leu- and Met-enkephalins. possess a difference only at this position in their amino acid sequence. Leu-enkephalin is slightly more active in isolated smooth muscle of the mouse vas deferens (MVD) and less active (20% to 40%) in the GPI (Beddell et al. 1977b; Dutta et al. 1977) (table 6). Although it is generally accepted that the MVD contains predaninantly delta receptors, and that the GPI contains

predaninantly mu receptors, it is not clear yet that even these two natural enkephalins have different receptors and functions.

For prevention of degradation by carboxypeptidases, modifications of the C-terminal COOH group by esterification, amnidation, or reduction to alcohol increase the activity considerably in the GPI, but not in the MVD. This fact is one of the important lines of evidence for postulation of a multiplicity of opiate receptors and is also important information regarding the structural requirements of this residue for synthesis of the receptor selective analogs (Ronai et al. 1979, 1981). Replacement of leucine by an amino acid having a relatively smaller side chain, like alanine, leads to a drastic drop in potency, while use of a similar sized mine a id, like isoleucine or norleucine, results in fair activity. $Met(O)^5$-enkephalin exhibits 40% to 70% activity of Met-enkephalin (Beddell et al. 1977b; Schiller et al. 1977). Even the removal of the terminal COOH group or the Leu^5 (or Met^5) residue itself results in the retention of considerable activity when compared with the removal of the Tyr^1 residue (Beddell et al. 1977b; Dutta et al. 1977). The elongation of mine acid residues fran the carboxyl end of the enkephalin sequence does not alter potency and sometimes even elicits a higher potency (Beddell et al. 1977b; Terenius et al. 1976). This observation seems rather reasonable after the discovery of various potent elongated-enkephalins in nature.

Highly Bioactive Enkephalin Analogs

Ineffectiveness due to short duration of action of Leu- and Met-enkephalins in most physiological and pharmacological systems is mainly a function of their susceptibility to enzymatic degradation. The bioevaluation Of the enkephalins has thus required same special steps to be taken in assay systems: the direct injection into a target tissue, temperature adjusmmt (0°C). or addition of proteolytic enzyme inhibitors. It was evident that further elucidation of their biological roles and functions would be greatly facilitated by the availability of opioid peptides resistant to enzymatic hydrolyses and having greater activity. This line of study has, of course, aimed at the development of more effective analgesics than at the usual alkaloid opiates. The term "highly bioactive" must be satisfied by the following requirements: (1) an increased receptor affinity, (2) an increased enzyme stability, (3) an increased transportability, and (4) an increased permeability of the blood-brain barrier. It is important for the SAR study to evaluate the extent to which the above requirements play a role in the molecular mechanism of action of enkephalin analogs. Classical structure-activity studies have clarified some such components essential to enkephalin activity (figure 2). The design for the synthesis of highly potent analogs has been carried out by a canbination of chemical modifications of portions not included in the essential structural elements.

Pentapeptide enkephalin analogs. Table 4 lists sane representative examples of highly bioactive enkephalin analoga and their activities in vivo and in vitro. The first reported analog was [$D\text{-}Ala^2$,$Met\text{-}NH_2^5$]-enkephalin,having modifications at positions 2 and 5

TABLE 4

Relative Potencies of Highly Bioactive Pentapeptide Enkephalin Analogs

Enkephalins	Inhibitory Activities[a])			Analgesic Activities[b])		
	GPI	MVD	Reference[c])	I.C.V.	I.V.	References[c])
D-Ala^2,Met-NH_2^{5}	780	380	Shav 1978	100	3	Pert 1978
D-Ala_2,MePhe^4,Met(0)-ol^5	2,120	94	Kosterlitz 1980	1,000	640	Roemer 1977
D-Ala^2,D-Leu^5	330	2,720	Rosterlitz 1980	15	8	Belluzzi 1978
D-Ala^2$_r$MeMet-NH_2^{5}	---	500	Frederickson 1981	23,800	---	Rederickzon 1981
D-Ala^2,F_5Phe^4,Met-NH_2^{5}	---	500	Coy 1978	1,000	---	Coy 1978
D-Met^2,Pro-NH_2^{5}	2,920	97	Shav 1978	7.800	590	Bajusz 1977
D-Met^2,Thz-NH_2^{5}	---	---	---------	2,700	480	Yamashiro 1977
Leu-enkephalin	20	119	Kosterlitz 1980	weak	Inactive	Beddell 1977a
Met-enkephalin	100	100	---------	0.01	Inactive	Beddell 19778
morphine	220	3	Kosterlitz 1980	100	100	---------

a) Relative to Met-enkephalin (-100)
b) Relative to morphine (-100)
c) Only shows top authors listed in references.

simultaneously (Pert et al. 1976b; Coy et al. 1976; Shaw et al. 1978). This analog binds to opiate receptors with almost the same affinity as Met-enkephalin and causes profound and long-lasting analgesia when injected into rat brain (Pert et al. 1976b). After a systematic SAR study, Roemar and coworkers (Roemer et al. 1977; Roemer and Pless 1979) achieved the synthesis of [D-Ala^2, MePhe^4 Met(O)-ol^5]enkephalin (FK 33-824), utilizing a series of modifications with D-amino acid at position 2, N-ethylation or p-nitration of Phe and with amino acid alcohol at position 5. This compound is extremely bioactive and produces analgesia even when administered orally. Many other analogs, some of which are listed in table 4, have been reported as highly bioactive enkephalins (see previous reviews by Coy and Rastin 1980; Morley 1980). However, their receptor affinity is not necessarily improved drmatically and most of the analogs invariably lose the receptor specificity for the delta and mu receptors to result in nonselective or mu-preferential 1 igands . Thus, their high potency has been suggested to be due to the combined effects of enzyme stability in the circulation and tissues and due to favorable transport properties.

Enkephalin analogs with shortened sequence. McGregor et al. (1978) have shown that the tetrapeptide amide resulting from the removal of the amino acid in position 5 is as potent as morphine in vivo and suggested that the tetrapeptide sequence would be a minimal structural requirement essential for the enkephalin activity. Such reported highly active analogs with shortened sequences are shown in table 5. Some of tetrapeptide acyl-hydrazide analogs were reported to be several times more active than morphine in the in vivo assay (Fujino et al. 1979). One of the most successful synteses of an analog with potent and long-lasting peripheral and central opioid activity is that of H-Tyr-D-Met(O)-Gly-MePhe-ol by Kiso et al. (1981), which is 23,300 times and 9 times as active as morphine in the GPI and tail-flick analgesic assays, respectivaly. It should be noted that the removal of the C-terminal Leu^5 or Met^5 followed by chemical modifications enhances the opioid agonist activity in the GPI and in vivo, but leads to a considerable drop in activity in the MVD. This fact indicates at least that the high potency of shortened enkephalins is due to their retention or increase in affinity for mu receptors, accompanied by a decrease in affinity for delta receptors.

Enkephalin Analogs Containing Unusual Amino Acids

Modifications of peptide bond with regard to the direction or electrical properties have also been attempted for prevention of biodegradation by proteolytic enzymes. These include the retroinverso isomers (Chorev et al. 1979), isosteric analogs (Cox et al. 1980; Hudson et al. 1980; Kawasaki and Maeda 1982), and depsi-analogs (Gesellchen et al. 1981). Analogs containing so-called fatty amino acids, such as carboranylalanine and t-butylglycine, in position 4 or 5 were reported to be almost as active as Met-enkephal in in the isolated muscle assays (Schwyzer et al. 1981; Do and Sohwyzer 1981; Fauchere 1982).

TABLE 5

Relative Potencies of Enkephalin Analogs Having Shortened Sequences

Enkephalins	Inhibitory Activities[a])		Analgesic Activity[b])	References[c])
	GPI	MVD	i.c.v. or i.v.	
H-Tyr-D-Ala-Gly-Phe-NH_2	---	---	250	McGregor 1978
	800	7	---	Fournie-Zalueki 1981
H-Tyr-D-Ala-Gly-EtPhe(pF)-NH_2	---	550	$1.2x10^6$	Shuman 1981
H-Tyr-D-Ala-Gly-$NHCH_2CH_2C_6H_5$	440	2	100	Morgan 1917
H-Tyr-D-Met(O)-Gly-MePhe-ol	$5.1x10^6$	---	900	Kiso 1981
H-Tyr-D-Met(O)-Gly-MePhe-NHNHCOEt	---	---	400	Fujino 1981
H-Tyr-D-Met-Gly-Phe-NH_2	3,760	12	1,810	Ronai 1979
H-Tyr-Ala-Phe-Met-NH_2	120	---	50	Chipkin 1981
Met-enkephalin	100	100	0.01	Beddell 1977a
morphine	220	3	100	Kosterlitz 1980

a) Relative to Met-enkephalin (=100)
b) Relative to morphine (=100)
c) Only shows top authors listed in references.

Shimohigashi et al. (1981a) have synthesized a series of dehydro-enkephalins containing α,β-dehydrogenated amino acid residues (-NHC(=CHR)CO-) in each position of [D-Ala^2, Leu^5]enkephalin and have shown that dehydrogenation of the enkephalin molecule can afford a peptide with full receptor and biological activity having unchanged delta-selectivity. Thus, simple dehydrogenation of an amino acid residue in a peptide sequence may elicit at least one or all of three changes in the properties: (1) physicochemical and conformational, i.e., rigidity, increased hydrophobicity restricted orientation of ß-substituents, and possible ß-turn type folding; (2) chemical, i.e., a new site of electrophilicity at the α,ß-double bond; and (3) biological, i.e., an increased resistance to enzymatic degradation providing a longer in vivo lifetime. Some of these properties have been demonstrated for dehydro-enkephalins (Shimohigashi et al. 1981b. 1982d. 1983, 1984b).

Enzyme Stability of Synthetic Enkephalin Analogs

Stability of enkephalin analogs synthesized for prevention of proteolytic breakdown has been examined by means of brain hanogenates and various purified ezymes. and it was demonstrated that the enhanced in vivo activity of some analogs can be explained by their greater resistance to enzvmatic hvdrolysis. Met-enkephalin is completely degraded after 30 minutes by incubation with mouse brain homogenates, while [D-Ala^2, Met-NH$_2^5$]enkephalin analog is stable for at least 3 hours (Marks et al. 1978) The Tyr^1-D-Ala^2 linkage in FK 33-824 is also quite stable, since 50%. of the compound can survive similar conditions even after 6 hours (Huguenin and Maurer 1980). Fauchere and coworkers (Fauchere and Petermann 1980; Eberle et al. 1977) observed a considerable resistance of carboranylalanine-containing enkephalin analog to various purified enzymes such as aminopeptidase M, α-chymotrypsin, and pepsin. When mined with carboxypeptidase Y, the saturated Leu-enkephalin is rapidly hydrolyzed from the COOH-terminus. However, it was demonstrated that peptide linkages with α,β-dehydromino acid residues placed in the enkephalin molecule are strongly resistant to the enzyme at the carboxyl side and completely resistant at the amino side of the dehydro residue (Shimohigashi et al. 1982a).

RECEPTOR-SPECIFIC ENKEPHALIN ANALOGS

Multiple Opiate Receptors

Following the postulation of three distinct types of opiate receptors (mu, kappa, and sigma) (Martin et al. 1976), the possible existence of another type of opiate receptors was proposed for the endogenous peptides of enkephalins and endorphins (Lord et al. 1977). Since then, several lines of pharmacological and biochemical evidence have confirmed the heterogeneity of opiate receptors. In the assay to evaluate the inhibitory activity for the electrically stimulated contraction of isolated smooth muscle preparations, it was demonstrated that morphine is more potent than enkephalins in the GPI by interacting with the mu receptors. However, enkephalins are more potent than morphine in the MVD, and the receptor

responsible for the opiate action in this tissue was designated as "delta." Although these tissues contain predominantly mu and delta receptors, it was found that the GPI contains additional kappa and sigma receptors (Su et al. 1981), and the MVD contains additional mu and kappa receptors (Lord et al. 1977).

The presence of multiple opiate receptors was also demonstrated in brain by examining the independent receptor binding affinity using low concentrations of radiolabeled mu and delta ligands (Chang and Cuatrecasas 1979; Robson and Kosterlitz 1979). The ratio of receptors in the guinea pig brain was calculated as 25% (mu), 45% (delta), and 30% (kappa) (Kosterlitz et al. 1981). Radioautographical studies have also elucidated regional differences in the distribution of the three receptor subtypes in the brain (Pert et al. 1976a; Goodman et al. 1980; Goodman and Snyder 1982).

The fact that the specific receptor for enkephalins is different fran that for morphine dramatically altered the research direction of the structure-activity studies of enkephalins. The classical SAR studies based on somewhat opportunistic bioevaluations must thus be reevaluated to find the structural characteristics for discriminative recognition of delta and mu receptors by enkephalin analogs. Also, a new direction in the synthesis of analogs has been undertaken to obtain more specific and more selective ligands for each receptor (Ronai et al. 1981; Wüster et al. 1980).

Delta-Receptor Specificity: Enkephalinlike Characteristics

The substantiation of opiate receptor multiplicity has inevitably required the reevaluation of the specificity and selectivity of enkephalins for at least two distinct receptors: mu and delta. Eventually, it became clear that most of the enkephalin peptides, which bear various chemical modifications for enzyme stability and favorable transport in the circulation, are nonspecific or mu-preferential and that their pattern of activity resembles that of morphine rather than that of the naturally occurring peptides. There was an indication that Met-enkephalin is mu-preferential, while Leu-enkephalin is delta-preferential , as evidenced by both uncle and binding assays (Lord et al. 1977; Kosterlitz et al. 1980). Especially for the elucidation of functional roles of the delta receptors, it becomes urgent to unravel the important determinants of delta specificity and selectivity and then to synthesize more enkephal inlike peptides.

The Tyr^1 residue, per se, is essential to the display of enkephalinlike activity via delta receptors.

Replacement of the Gly^2 residue with D-alanine increases the potency 5 to 10 times in the muscle assays of both GPI and MVD. When the delta selectivity is calculated, however, Met- and Leu-enkephalins show a different profile due to their different extent of potency increments in each tissue. [$D\text{-}Ala^2$]-Met-enkephalin shows almost unchanged selectivity of Met-enkephalin because of simultaneous gain in mu and delta activities, whereas delta-preferential Leu-enkephalin

drastically loses its delta selectivity by D-Ala^2-substitution due to a marked increase in mu activity (table 6). These results indicate that a hydrophobic subsite for the specific interaction with the D-Ala^2 residue may exist in the mu receptors (Fournie-Zaluski et al. 1981). α,β-Dehydrogenation of D-Ala^2 in Leu-enkephalin provides further evidence that a hydrophobic character in position 2 facilitates the interaction with the mu receptors (Shimohigashi and Stammer 1982a). On the other hand, a nonhydrophobic character in position 2 appears favorable for interaction with the delta receptors.

As for the Gly3 residue, it seems that the presence of this amino acid itself in enkephalin is important for delta, but not mu, receptor interaction. The evidence supporting this postulate are as follows: (1) Chipkin et al. (1981) reported that the tetrapeptide analogs of des-Gly^3-enkephalin are potent agonists in the GPI; (2) dermorphins , having an N-terminal amino acid sequence very similar to enkephalin but missing the Gly3 residue, also display high mu activity (Broxardo et al. 1981); and. similarly, (3) morphiceptin or beta-casomorphin-4-amide (H-Tyr-Pro-Phe-Pro-NH_2). which lacks Gly^3, is highly selective for the mu site in rat brain membranes (Chang et al. 1981; Brantl et al. 1982). These results suggest that the presence or absence of glycine at position 3 in these enkephalin like peptides influences the interrelationship of the Tyr^1 and Phe residues, and such a relationship between the two aromatic groups appears to be of great importance in discriminative receptor recognition (Chang and Cuatrecasas 1981). Thus, the delta receptors may require the Gly^5 residue for enkephalins as an adequate spacing element between the Tyr^1 and Phe residues, as one of the conformational determinants of the enkephalin molecule, or as a structural factor to interact directly with a particular subsite located in the delta receptors.

The phenyl ring of the side chain in Phe^4 plays a crucial role in delta-receptor recognition. The importance of its existence in position 4 was demonstrated by Rogues et al. (1979) by replacement of the phenyl ring by an aliphatic hydrophobic moiety, which causes a total loss of activity in the MVD but retention of some activity in the GPI. Whilst, the importance of the stereo-orientation of aromatic groups (Tyr and Phe) in enkephalins to opiate receptor recognition has been emphasized by the synthesis of dehydrophenylalanine^4-enkephalins having the Z- (phenyl and C=O, trans) and E- (phenyl and C=O, cis) configurations (figure 3) (Shimohigashi et al. 1982d, 1984b). The Z-isomer exhibits an enhanced or unchanged receptor affinity over the saturated analogs, showing a greater delta selectivity. However. the E-isomer is dramatically decreased in its affinity for both receptors, which is almost equal to that of inactive [D-Ala^2, D-Phe^4]-Leu-enkephalin. Due to the difference in the orientations of the phenyl groups in the Z- and E-isomers of Phe, the phenyl ring in the Δ^zPhe^4-enkephalin is held rigidly toward the amino end of the enkephalin sequence, to which the other aromatic amino acid (Tyr^1) residue appears. In contrast, the phenyl group in the E-isomer is restricted to the opposite arrangement. These results clearly show that the molecular shape, restricted and

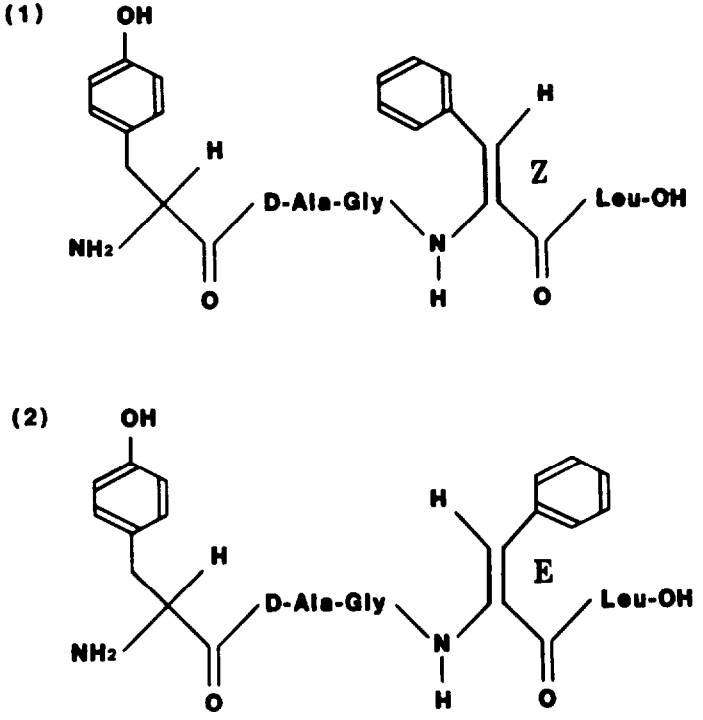

FIGURE 3

Chemical Structures of Dehydrophenylalanine[4]-enkephaline Having the Z- (1) and E- (2) Configurations

stabilized by the Z-configuration of Phe[4], enhances discriminative recognition between the opiate receptors. Recent work leading to the incorporation of cyclopropylphenylalanines into enkephalin has shown similar results (Kimra et al. 1983; unpublished data). Collectively, a subtle discriminative recognition between delta and mu receptors by these conformationally restricted analogs may be due to the different stereochemical requirements of each receptor at the binding regions corresponding to the two aromatic groups in the enkephalin ligand.

Leu-enkephalin is delta-preferential (table 6), indicating that an isobutyl side-chain of Leu[5] is an appreciable receptor-discriminating component. Placement of D-Leu in position 5 results in a marked gain in delta selectivity in muscle assays, as shown in table 6. The COOH-terminal modifications, such as amidation and esterification, invariably cause a change in delta versus mu selectivity, arid at least partially eliminate interaction with the delta receptors. Therefore, the COOH group can be recognized as one of the most important enkephalinlike characteristics. It should be noted, however, that the mechanism of a selectivity shift by COOH modifications may

TABLE 6

Receptor Selectivity of Enkephalin Analogs with Substitutions at Position 2 and 5[a])

Amino Acids		Inhibitory Activities[b])		Delta Selectivity[c])
position 2	position 5	GPI	MVD	
Gly	Met-OH	100	100	10.7
D-Ala	Met-OR	560	500	9.5
D-Ala	D-Met-OH	180	590	35.5
D-Ala	D-Met-OCH_3	300	300	10.7
D-Ala	D-Met-NH_2	130	60	4.9
Gly	Leu-OH	35	170	51.6
D-Ala	Leu-OH	550	900	17.6
D-Ala	D-Leu-OH	330	2,720	88.5
D-Ala	D-Leu-OCH_3	990	160	1.8
morphine		220	3	0.14

a) Calculated using data of Beddell et al. (1977a) and Kosterlitz et al. (1980).
b) Relative to Met-enkephalin (=100).
c) Selectivity ratio defined as IC_{50}(GPI)/IC_{50}(MVD).

be different between the binding assays in rat brain and the isolated muscle assays (Shimohigashi et al. 1982d). For the muscle assays, the existence of the COOH group is distinctly effective for obtaining a high delta potency, while it causes a decrease in the mu affinity in the receptor binding assays. Sane possibilities may account for this difference: (1) in the muscle assays a degradation of the carboxyl-free peptides may occur; (2) in the MVD both mu and delta receptors are present; (3) the ligands may have same agonist-antagonist properties; and (4) the delta receptors in rat brain and in the MVD may have a structural subtlety in interacting with enkephalin peptides. By characterizing the acidic function of the COOH group, Bajusz et al. (1980) synthesized a series of analogs containing amino sulfonic acid and amino phosphonic acid residues at position 5. Those analogs had little effect on their receptor selectivity.

In summy, structural elements for each residue that result in greater specificity and selectivity for the delta receptors in the enkephalin sequence, or the so-called "enkephalin like characteristics," are as follows (figure 4):

(i) the Tyr^1 residue,
(ii) non hydrophobicity in position 2,
(iii) the Gly^3 residue,
(iv) existence of the phenyl group and its stereo-orientation in position 4,
(v) the isobutyl side-chain of Leu^5, and
(vi) the C-terminal COOH group.

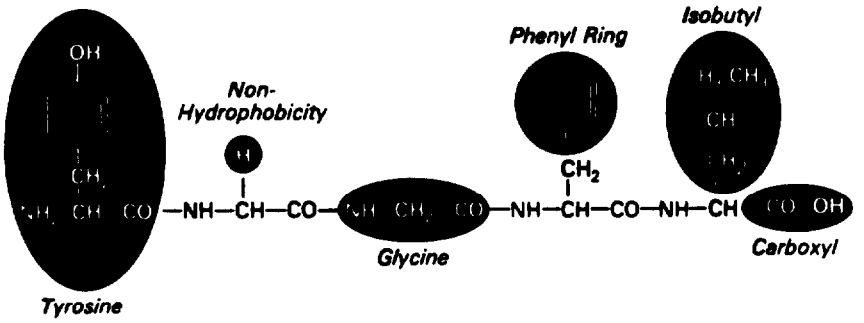

FIGURE 4

Enkephalin like Characteristics
Essential for Specific Interactions with Delta Opiate Receptors

Leu-enkephalin, per se, which possesses all of these characteristics, can be an ideal ligand for the delta receptor under physiological conditions. Among these characteristics, (i), (iii), (iv), and (vi) appear to be particularly important. However, the existence of Tyr^1, Gly^3, and COOH group is inevitable in Leu-enkephalin, and thus factor (iv), which is related to the phenyl ring in position 4, seems a very important determinant for eliciting and enhancing delta specificity and selectivity.

The most utilized delta ligand to date is [$D\text{-}Ala^2$, $D\text{-}Leu^5$]enkephalin (DADLE), which exhibits a selectivity ratio, defined as the ratio of (IC_{50s} in mu/IC_{50} in delta), of 50 to 100 in muscle assays (GPI/MVD), and of about 10 in binding assays in using rat brain (^{3}H-naloxone/^{3}H-DALE). Taking a nonhydrophobic character at position tion 2 into consideration, Roques' group has synthesized Leu-enkephalin analogs containing ß-hydroxyamino acid, D-Ser, or D-Thr at position 2, with a Thr extension at position 6 (Gacel et al. 1980; Zajac et al. 19831. Their selectivity ratio was reported to be 1,000 to 3,000 in muscle assays, but only 7 to 20 in rat brain binding assays. A selectivity ratio of 170 to 330 in similar muscle assays, however, has been reported by another group (Mosberg et al. 1983), and thus the design and synthesis of a highly delta-specific ligand still appear to be of great importance and urgency. It is noteworthy that these reported delta ligands possess little restriction or rigidity around the Phe^4 residue.

Mu-Receptor Specificity

One way to increase mu-receptor specificity is to eliminate sane of the enkephalinlike or delta characteristics from the peptide molecule without introducing any chemical modifications at the Tyr^1 residue. It should be noted that such a synthetic sequence seems to coincide with the design for highly bioactive enkephalins as described above. In fact, the peptides having these types of modifications have been proved to be nonselective or mu-preferential due to their decrease in delta specificity. Highly potent FK 33-824 is mu-selective with a selectivity ratio of about 7 in both muscle and binding assays, mainly due to a pronounqced gain in mu potency (Kosterlitz et al. 1980). Similarly, a shortened peptide analog synthesized by Kiso et al. (1981) was reported to be several thousandfold more selective for mu than for delta receptors in binding assays (Quirion et al. 1982). This extreme mu selectivity is apparently caused by a drastic loss of delta affinity, but not by a great gain in mu affinity. This may be related to the removal of the amino acid residue at position 5 followed by a possible conformational alteration. The most utilized mu-specific ligand to date is [$D\text{-}Ala^2$, $MePhe^4$, $Gly\text{-}ol^5$]enkephalin (DAGO), an analog of FK 33-824. This peptide lacks only the side chain of $Met(O)\text{-}ol^5$ residue in FK 33-824, and its mu selectivity ratio is almost 100 in rat brain binding assays Wanda et al. 1981).

Ligand characteristics for mu-receptor specificity can be summarized as follows: (1) the Tyr^1 residue, (2) hydrophobicity in position 2, and (3) the phenyl ring arranged in a specific array in position 3

or 4. It seems that N-methylation of Phe[4], for example, in FK 33-824 and DAGO, may elicit a favorable interaction with mu receptors which is presumably due to restriction of sane conformational parameters for suitable spacial arrangement of the phenyl group, together with an increased resistance to enkephalinase.

Conformationally Restricted Enkephalin Analogs

For the elucidation of more dynamic and definitive structure-activity relationships, the incorporation of conformational constraints into enkephalin peptides has been recognized as a useful structural variation to permit an assessment of the stereochemical consequences on the resulting biological profiles, especially on receptor specificity and selectivity. Since a different conformational feature required for interaction with different subtypes of opiate receptors can be obtained fran conformational flexibility, the restriction of this flexibility may lead to an analog having the conformation to interact preferentially with only one class of receptors. One approach toward such a restriction involves cyclization of enkephalin peptides, and it has proven to be adequate to increase mu-or delta-receptor selectivity (Schiller and DiMaio 1982; Mosberg et al. 1983).

A potent cyclic enkephalin was first reported by DiMaio and Schiller (1980); cyclfzation was performed between the COOH-terminal carboxyl group of Leu and the γ-amino group of D-α, γ-diamino butyric acid placed in position 2. The resulting cyclic peptide was found to be mu-preferential in both muscle and binding assays, while its open-chain analog was almost nonselective (Schiller and DiMaio 1982). By canparison of the potency and selectivity of cyclic and open-chain peptides, it is suggested that different receptors have different structural preferences for each complementary ligand. Schiller et al. (1981) also synthesized cyclic analogs containing a cystine bridge between positions 2 and 5, which exhibit moderate selectivities for the mu receptors (figure 5a). Quite interestingly, extremely high delta selectivity has been obtained for analogs of cystine enkephalin only by virtue of substitutions of cysteine by penicillamine (ß,ß-dimethyl cysteine) at both positions 2 and 5, as shown in figure 5b (Mosberg et al. 1983). Its delta selectivity is almost 3,200 in GPI/MVD assays and 175 in ^{3}H-naloxone/^{3}H-DADLE rat brain binding assays. The structural difference between these two S-S bridged cyclic enkephalins lies in the existence or absence of a dimethyl group on the ß-carbon at positions 2 and 5 and amide modification at the COOH terminus. The effect of dimethyl substitutions seems to cause a dramatic selectivity shift in receptor recognition. This indicates that the mu and delta receptors have a more subtle conformational variation for discrimination and optimal interaction with each specific ligand. It is interesting to note that the Phe[4] residue, which impacts an important enkephalin like character, exists within the constraint of cyclic structure. Another interesting cyclic analog, synthesized by Siemion et al. (1981). is a semi-rigid Leu-enkephalin peptide having an azo-bridge between Tyr[1] and Phe[4] residues (figure 5c). By observing a long-lasting morphine like analgesic effect in vivo, they suggested that the biologically

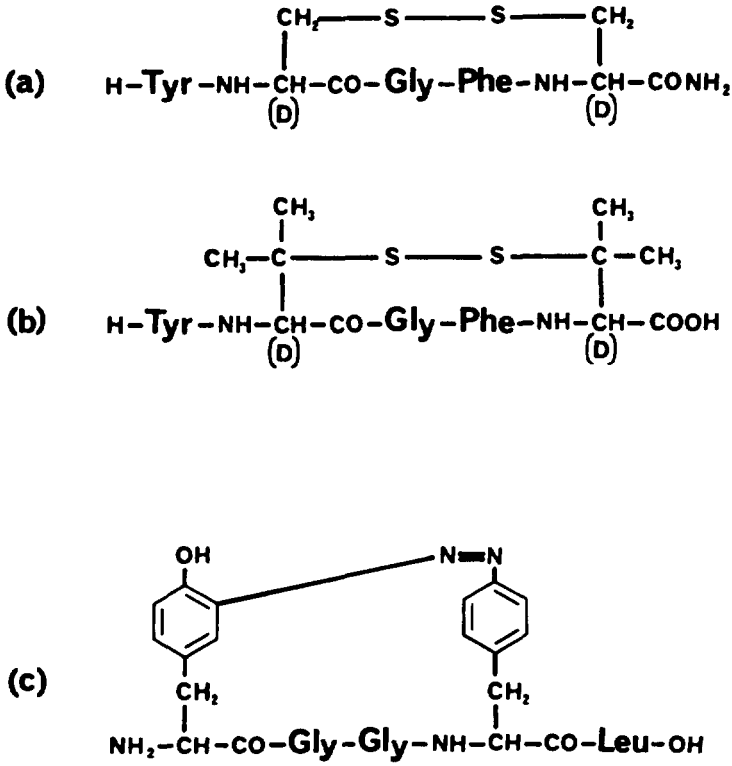

FIGURE 5

Chemical Structures of Conformationally Restricted Cyclic Enkephalins (a) Mu-preferential [D-Cys^2-D-Cys^5]enkephalin Amide, (b) Delta-Selective [D-Pen^2-D-Pen^5]enkephalin (Pen: Penicillamine), and (c) Azo-bridged Enkephalin

active conformation of enkephalin interacting with the opiate receptor must be such that both aromatic rings of Tyr and Phe would appear close to each other.

Local conformational restriction by incorporation of such unusual amino acids as dehydro or cyclopropylamino acids, can also affect strongly the overall molecular conformation of enkephalins and their receptor interactions. This has been well demonstrated by synthesis of the Z- and E-isomers of dehydrophenylalanine^4-enkephalins, as mentioned previously.

Camerman et al. (1983) and Karle et al. (1983) have recently elucidated the crystal structure of Leu-enkephalin, in which four

conformers with extended backbones form an infinite antiparallel ß-sheet. An intriguing feature is the interrelationship between the aromatic rings of Tyr^1 and Phe^4 residues located on opposite sides of the backbone. Two of the four conformer are similar and somewhat puckered, having a distance of 8.2-8.9Å between the two aromatic rings, while the other two conformers are also similar in having a flat backbone (13.3-13.9Å). Such a steric difference in Leu-enkephalin might be related to the differential recognition of mu and delta receptors (Shimohigashi et al. 1984b).

Receptor-Specific Enkephalin like Antagonists

For the agonist-antagonist profile, the structure-function relationships of alkaloid opiates are well established. Substitution of the methyl group by a typical antagonist-inducing pharmacophore, allyl or cyclopropylmethyl, on the mine nitrogen of opiates usually increases narcotic antagonist activity. However, application of these modifications to the enkephalin peptides does not result in generation of analogs having an appreciably pure antagonist property (Hahn et al. 1977; Morgan et al. 1977; Pert et al. 1977) Unfortunately, the structure-activity studies of enkephalins have shed little light on how to prepare peptidic antagonists. Specific enkephalin antagonists that have been reported possess very weak receptor affinity in toto.

Shaw et al. (1982) reported a delta-selective pure enkephalin antagonist having modifications such as diallylation of the amino group of Tyr^1 and replacement of the peptide bond of Gly^3-Phe^4 by a CH_2-S linkage (figure 6a). Its antagonist activity in reversing the effects of normorphine or Leu-enkephalin on the MVD indicates that the peptide acts predominantly on the delta receptors. However, its potency seems considerably poor (Shaw et al. 1982), and its extremely weak receptor affinity has also been found to be 340 times less than that of agonist DADLE in rat brain binding assays (Zajac et al. 1983). On the other hand, Bower et al. (1981) synthesized mu-selective enkephalin antagonists by furnishing additional groupings to the enkephalin molecule, based on the idea that antagonists would bind to the receptor enviromnent adjacent to the agonist receptor (figure 6b). Their binding to opiate receptor labeled by ^{3}H-naloxone was almost unaffected by sodim ion addition, indicating a pure antagonist property, although their receptor affinity was 10 to 70 times less than that of naloxone.

MIF-I, H-Pro-Leu-Gly-NH_2, the alleviative of Parkinson's disease, has been described as a blocker of the analgesic effects of enkephalins and morphine in the tail-flick test in vivo (Kastin et al. 1979). Furthermore, Dickinson and Slater (1980)demonstrated that MIF-I weakly and selectively inhibits mu receptors and suggested its possible role as an endogenous antagonist. Maurer et al. (1982) showed that a somatostatin analog, H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-NH-$CH(CH_2OH)CHOHCH_3$, is an opiate mu antagonist having a receptor affinity of 10% that of naloxone.

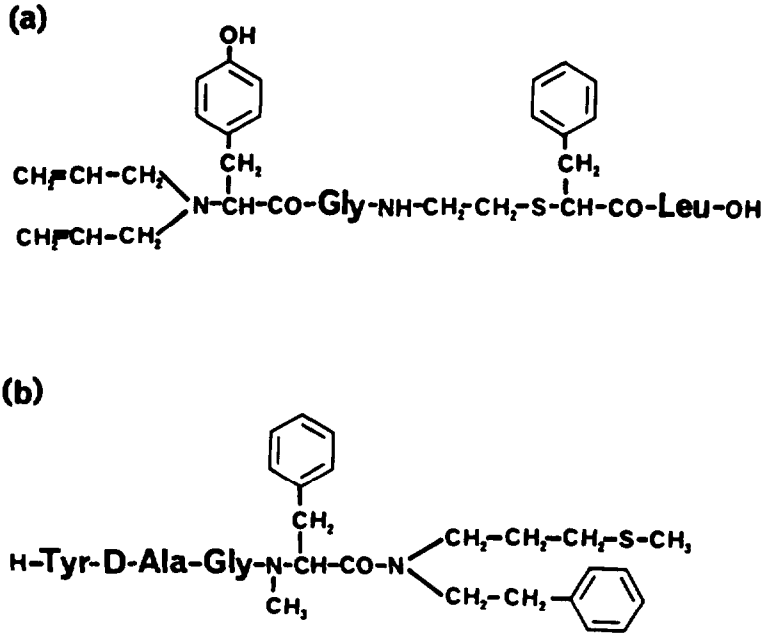

FIGURE 6

Chemical Structures of Enkephalin like Antagonists (a) Delta-selective N,N-Bisallyl-[Gly^3(CH_2S)Phe^4]-Leu-enkephalin, and (b) Mu-selective Des-Leu^5- D-Ala^2, MePhe^4 enkephalin-(3-methylthiopropyl)-(2-phenylethyl)amide

DIMERIC ENKEPHALINS AND THEIR RECEPTOR SPECIFICITY

Delta Receptor-Specific Dimeric Enkephalins

A comprehension of the molecular mechanism of interaction between the receptor and its complementary ligand would facilitate the better design of more specific and more selective ligands in receptor action. Efforts in clarifying receptor organization and dynamics on the membrane have somewhat affected the synthetic and biochemical studies of enkephalins. Among them, the suggestion by Hazum et al. (1979) that delta receptors might be extremely closely clustered in the membrane has provided anideato synthesize a newtype of delta-specific ligand. It is likely that the overall macroscopic affinity

of a ligand for its receptor is due to multiple interactions between a nunber of chemical elements in the ligand and corresponding subsites present in the receptor. Structural variations by incorporation of modified groupings or constraints may enhance such interactions, thereby increasing the receptor affinity. Also, increased affinity can be attained by an increase in the number of interactions or in binding energy obtainable with the resulting multiple attachements to the receptors. In this rationale, multivalent ligands would offer a fair possibility, provided each peptide unit in a ligand sustains parent active interactions with receptors. Even a bivalent ligand in which two active molecules are cross-linked by a spacer is expected to produce at least a twofold enhancement in affinity, because of the statistical advantage in association. Furthermore, if the connecting spacer is long enough to permit simultaneous binding or bridging between two adjacent receptor molecules, the magnitude of enhancement in affinity should be much larger. It is likely that such bridging of receptors might be selective for only a single type of receptor.

A systemic study on dimerization of enkephalin molecules has been performed by Shimohigashi et al. (1982b, 1982c) on the basis of such a subsite-interaction hypothesis (Delean et al. 1979) and of the suggestion of receptor topological organization on the membrane (Hazum et al. 1979). Earlier studies on enkephalin dimers has involved their synthesis by Coy et al. (1978), Hazum et al. (1982), and Lipkowski et al. (1982). In our case, the general strategy for the synthesis of a series of enkephalin dimers was based on traditional SAR studies as described (figure 7): (1) Tyr-D-Ala-Gly-Phe-Leu sequence was chosen as parent backbone for stability; (2) dimerization was carried out at the COOH-terminal, since the NH_2-terminal has been established as essential for activity; and (3) methylene bridges of varying length, $(CH_2)_n$ with n = 2-22, was utilized as spacers to provide simplicity, flexibility, and chemical stability. Dimerization was performed at the carboxyl side of each amino acid residue successively from the COOH terminus, producing dimeric pentapeptide- (DPE_n), tetrapeptide- (DTE_n), and tripeptide- ($DIRE_n$) enkephalins (figure 7). The examenation of the optimal spacer length among these series would help to characterize receptor organization.

In the first series of DP the monamer for dimerization was a pentapeptide H-Tyr-D-Ala-Gly-Phe-Leu-NH_2 (DALEA), which cannot discriminate well between delta mu receptors. In the mu assays (GPI and binding assay using ^{3}H-naloxone) , the dimer with spacer of n = 2. DPE_2. showed no significant changes in relative potency compared with the monaner DALEA (Shimohigashi et al. 1982c; Costa et al. 1985). Increases in the chain length resulted in a progressive loss of potency In contrast, in the delta assays (MVD and binding assay using ^{3}H-DADLE) the pentapeptide dimers were considerably more potent than monomer DALEA. The most active compound was DPE2, which was eightfold and twentyfold more potent than DALEA in the binding and MVD assays, respectively. Spacers of 4, 6, and 8 methylene units caused a consistent increase in potency. Further extension of the methylene bridge to n = 10 and 12 resulted in a progressive loss of activity. These results reveal several distinct

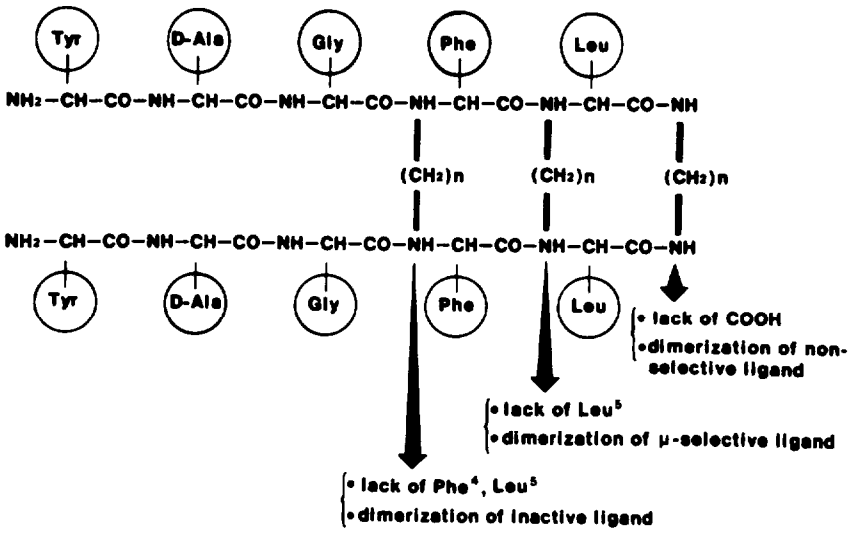

FIGURE 7

Chemical Structure of Dimeric Enkephalins Strategy and Expected Variation Following Dimerisation

features about the receptor action of dimers: 1) Nonselective monomer becomes "delta-selective" by dimerization, and the selectivity ratio of the most specific DPE_2 is compatible with that of most utilized delta ligand,DADLE, in both binding and muscle assays. It should be noted that DPE dimers lack the importantenkephalinlike characteristic of the COOH-terminal free carboxy group, yet they exhibit delta selectivity. 2) Relative potencies are higher in the MVD biological assay than expected on the basis of binding affinities in rat brain or muroblastoma-glioma hybrid cells. 3) The GPI activity and mu-receptor affinity, however, are highly correlated: as the methylene chain length increases from 2 to 12, there is a progressive drop in potency in both muscle and binding assays. The fact that the linkage of two molecules of a nonselective ligand (DALEA) results in a very delta-selective compound with enhanced biological activity has elicited an attractive hypothesis that the dimeric enkephalins might cross-link the delta receptors, presumably clustering to ether with appropriate intermolecular distances (Hazum et al. 1979) or they might cross-link copies of several enkephalin binding sites in the delta receptors (Costa et al. 1985). Several lines of evidence supporting these postulations have also been presented by various kinds of kinetic studies (Costa et al. 1982; Krumins et al. 1982).

The second series involves dimeric tetrapeptides of DTE_n. The monomer, H-Tyr-D-Ala-Gly-Phe-NH_2 (DAPHA), showed little difference in potency in the GPI and MVD assays, but had a tenfold increase in selectivity for the mu receptor in the binding assays. A comparative study between muscle and binding assays has also been performed for the DTE dimers (Costa et al. 1985). DTE_n with spacers of n = 2, 4, 6, 8, and even 10 methylene units were nearly equipotent with monaner DAPHA in both GPI and binding assays. In contrast, a spacer of n = 12 produced a dramatic drop in GPI activity and mu-binding affinity, having only 8% to 9% of monaner activity (table 7). This is in contrast with the marked enhancement of potency by DTE 2 in the MVD and delta-binding assays. Thus, DTE_{12} becomes a "delta-selective" ligand with a significant selectivity ratio of 20 to 90 (Shimohigashi et al. 1982b; Zajac et al. 1983; Costa et al. 1985). Again, it should be noted that dimerization causes a striking selectivity shift, although DTE dimers "lack" two enkephalin like characteristics: the carboxyl group and Leu^5 side-chain. In the delta assay, the DTE dimers are consistently more potent than the DAPHA monamer . However, the increase in MVD activity is significantly less than that expected on the basis of delta-binding affinity (Costa et al. 1985). Such a discrepancy has been seriously discussed as a function of the spacer length, which produces hydrophobicity (Costa et al. 1985; Shimhigashi et al. 1984a).

Bifunctional interaction of the most potent dimers of DPE_2 and DTE_{12} with delta receptors has also been demonstrated by evaluation of activities of alkylmidated monamers having corresponding spacer molecules: DALEA-C_2 and DAPHA-C_{12} (Shimhigashi et al. 1984a; Costa et al. 1985). Ethylamide substitution of DALEA, the spacer addition which creates DALEA-C_2, produces a threefold increase in MVD activity, but an insignificant (1.3 times) increase in delta binding (table 7). When another enkephalin molecule is attached to DALEA-C_2,

TABLE 7

Relative Activities of Enkephalin Monmers and Dimers for Delta and Mu Opiate Receptors

Enkephalins	Delta-activity		Mu-activity	
	MVD	^{3}H-DADLE	GPI	^{3}H-NAL
Penta-series				
DALEA	100	100	100	100
DALEA-C_2	300	130	160	82
DPE_2	2,300	800	75	75
Tetra-series				
DAPHA	100	100	100	100
DAPHA-C_{12}	6	32	8	8
DTE_{12}	320	1,300	9	8

DALEA-C_2 : H-Tyr-D-Ala-Gly-Phe-Leu-NH-CH_2CH_3
DAPHA-C_{12}: H-Tyr-D-Ala-Gly-Phe-NH-$(CH_2)_{11}$-CH_3

creating a dimer DPE_2, the increment in potency is about sevenfold in both the muscle and binding assays. This increment is clearly the net increase due to the bivalency of DPE_2. Such an effect of bivalency, however, cannot be observed in the mu assay systems. Similarly, the net relative effect attributable to bivalency of the DTE_{12} dimer is calculated as a fortyfold to fiftyfold increase in delta, but not in mu, assays (table 7). It is now evident that most of the increase in delta activity and affinity obtained by dimers is the result of the simultaneous presence of the two enkephalin peptides in one molecule.

In the third series, dimerization was performed using a nearly inactive tripeptide, H-Tyr-D-Ala-Cly-NH_2, with the same synthetic strategy. The resulting dimeric tripeptide enkephalins, DTREn (n = 2-22), exhibited up to a several hundredfold increase in binding to delta and mu receptors (Lutz et al. 1985). Delta affinity increased progressively fran n = 2 to a broad plateau at n = 16. It is interesting to note that if one amino acid residue were approximately as long as a methylene chain with n = 3, then chain lengths of n = 2-6 for DPEn dimers--the most delta-active dimers in the series--would correspond to n = 8-12 for DTE_n, and to n = 14-18 for $DTRE_n$, dimers. Such optimal spacer lengths have been found and must be related to a characteristic feature of membrane organization of the delta receptors. Although all the DTRE dimers are mu-preferential or nonselective, the almost 200-fold increase of $DTRE_{16}$ in delta affinity is quite notable, since it "lacks" the additional enkephalin like characteristic of the Phe^4 residue.

Several possible mechanisms have been proposed to explain theoretically the gain in delta activity and selectivity by enkephalin dirmers (Costa et al. 1985). Also, much effort has recently been centered around the isolation and structure elucidation of the opiate receptors. In combining these research efforts, it will become feasible to obtain a novel receptor-specific enkephalin ligand, and, clearly, the study of the dimeric enkephalins appears to be a guidepost.

Mu Receptor-Specific Dimeric Enkephalins

Unexpectadly, the dimeric tripeptide enkephalin with a spacer length n = 2, $DTRE_2$, showed a dramatic increase in mu-selectivity (Lutz et al. 1985). Its selectivity ratio of, mu versus delta receptors was almost 150-fold, with a fair mu affinity (IC_{50} = 29 nm). $DTRE_2$ is 10 times as potent as its monomer in delta receptor binding, but almost 400 times in mu binding. Its agonistic activity was also demonstrated in the GPI assay (Shimohigashi et al., unpublished data). Although the mechanism of interaction between $DTRE_2$ and mu receptors is not clear at this manent, a considerable synthetic study is in progress in our laboratory to elucidate the receptor action of such shortened enkephal in dimers.

CONCLUDING REMARKS

With numerous structure-activity studies, it is now becoming more

evident what determines mu and delta receptor specificity and selectivity. Conspicuous structural elements necessary for the delta receptor interaction have been analyzed and defined as enkephalin-like characteristics. The characteristics of peptides for the mu receptor interaction are also praninent in part. Emphasizing or reinforcing these characters by chemical modifications would offer a kind of "fully" receptor-specific and selective enkephalin-ligand. At this moment, two distinct synthetic sequences seem promising for the design of such ligands: the synthesis of conformationally restricted peptides and the synthesis of multivalent peptides.

Little has been done in the way of SAR studies on elongated-enkephalins and even in kappa receptor-specific dynorphins (Corbett et al. 1982). The natural ligand(s) for the mu receptors is still in question. It is a fact that newly elucidated biological or biochemical aspects of opiate and opioid peptides have affected dramatically the structure-activity studies of enkephalins. If the characteristic membrane feature of each opiate receptor, and then the structural picture of binding sites, were specified for each corresponding ligand, even a graphical &sign of the receptor-fitted ligands would be possible. With burgeoning interest in elucidating heterogeneous receptor functions of the enkephalin peptides, the design and synthesis of fully receptor-specific ligands will unequivocally became an increasingly important requisite.

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Structure-Activity Relationship and Pharmacology of the Highly Selective μ-Opioid Agonist, Morphiceptin

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Morphiceptin is an amidated tetrapeptide with the sequence of Tyr-Pro-Phe-Pro-NH_2 (Chang et al. 1981). This sequence is contained in ß-casein, which is a major milk protein. Morphiceptin is structurally related to the opioid peptide ß-casomorphin, a heptapeptide (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) originally isolated from bovine casein peptone (Brantl et al. 1979). The amino acid composition and receptor selectivity of morphiceptin differ from those of enkephalins. Morphiceptin is a highly selective opioid peptide agonist for μ-receptors (Chang et al. 1981; Zhang et al. 1981). This property is contrasted to that of enkephalins which are selective for δ-receptors (Lord et al. 1977; Chang and Cuatrecasas 1979). Because of its unique high selectivity for μ-receptors, a number of morphiceptin analogs have been synthesized in order to gain insight into the structural requirements for μ-receptor activity and to obtain more potent and metabolically stable μ-opioid peptide. Structure-activity relationship (SAR) of morphiceptin analogs will be first discussed below in detail. Several potent analogs have also been obtained. Some pharmacology of morphiceptin will be summarized here. One morphiceptin analog, [NMePhe^3,-D-Pro^4]morphiceptin (PL017), shows better receptor affinity than morphiceptin and is the most potent μ-opioid morphiceptin analog in in vivo studies (Chang et al. 1983). The pharmacology of this morphiceptin analog will be discussed in detail and compared with classical μ-agonist, morphine, as well as δ-agonist, [D-Ala^2,D-Leu^5]enkephalin (DADLE).

Structure-Activity Relationship

The SAR is based on the μ-receptor binding affinity determined by the IC_{50} value, the concentration of a compound required to decrease 50% of the specific

binding of ^{125}I-labeled μ-agonist, FK33824 [Tyr-D-Ala-Gly-NMePhe-Mel(O)ol], to rat brain membranes (Chang et al. 1979). The bioactivity of μ-receptors was also examined to determine their ability to inhibit the electrically stimulated muscle contraction of isolated tissues.

TABLE 1

Relative Receptor Binding Activity of Morphiceptin Analog with Various Substitutions at Fourth Amino Acid Residue

	μ-Receptor Affinity[a] (IC_{50}, nM)	Relative Affinity[b] (%)
Tyr-Pro-Phe-X-NH_2		
X = Pro (Morphiceptin)	63	100
D-Pro	4.3	1,470
Thz	12	530
Thz(o)	40	160
Tyr-Pro-NMePhe-Y		
Y = D-Pro-NH_2 (PL017)	5.5	1,150
D-Pro	186	30
D-Pro-ol	5	1,260
-OH	1,500	4
D-Pro-$NHNH_2$	2,000	3
D-Pro-Gly-NH_2	8.7	720

[a] μ-Receptor binding affinity was determined by the concentration which inhibited the binding of ^{125}I-FK33824 to the μ-binding sites of rat brain membranes by 50% (IC_{50}) (Chang et al. 1979).
[b] The relative affinity is expressed as percent of the parent compound (100%). Thz = thiazolidine-4-carboxylic acid; Thz(o) = thiazolidine-4-carboxylic acid sulfoxide; Pip = pipecolinic acid; Pro = 3,4-dehydroproline; Hyp = 4-hydroxyproline.

Morphiceptin contains two prolines at the second and fourth positions. Levorotary configuration of proline at the second position, but not the fourth position, is essential for μ-receptor activity (Chang et al. 1981) Replacement of L-proline at second position with D-proline produced an analog with little activity. In contrast, replacing fourth L-proline with D-proline, or various cyclic analogs such as thiazolidine-4-carboxylic acid and thiazolidine-4-carboxylic acid sulfoxide, yielded peptides of greater

activity (table 1). The D-proline[4] substitution is most effective. This analog, [D-Pro[4]]morphiceptin, is about 15 times more active than morphiceptin (Chang et al. 1983).

As shown in table 1, methylation of amino group of [D-Pro[4]]morphicept in produced an analog, [NMePhe[3],-D-Pro[4])morphiceptin (PLO17), of comparable μ-receptor binding affinity but greater in vivo activity (Chang et al. 1983). Removal of thea amide group of PLO17 attenuates activity. However, the activity is retained when the amide is changed to the carbinol. Deleting the fourth amino acid or replacing the amide with the hydrazine markedly reduces activity. Activity is almost fully retained when glycineamide is added to the fifth residue on the carboxyl terminus.

TABLE 2

Relative Receptor Binding Activity of Morphiceptin Analogs with Various Amino Acids at Third Position

	μ-Receptor[a] Affinity (IC_{50}, nM)	Relative[b] Affinity (%)
Tyr-Pro-X-D-Pro-NH_2		
X = Phe	4.3	100
N-Me-Phe	5.5	78
D-Phe	200	2
Trp	100	4
Pheg	1,500	0.3
Tvr	4,000	0.1
0-Me-Tyr	5,000	0.08
His	25,000	0.017

[a]μ-Receptor binding affinity was determined by the concentration which inhibited the binding of ^{125}I-FK33824 to μ-binding sites of rat brain membranes by 50% (IC_{50}) (Chang et al. 1979).
[b]The relative affinity is expressed as percent of the parent compound (100%).

Phenylalanine at the third position is essential for activity (table 2). Substitution of phenylalanine by histidine, phenylglycine, tyrosine, or 0-methyl-tyrosine dramatically reduces the affinity. Replacement of phenylalanine with D-phenylalanine or tryptophan yielded peptides of considerably lower affinity. Schiller et al. (1983) have also shown that the addition of a nitro-group to phenylalanine of

morphiceptin produces a drastic potency drop which is sharply contrasted to the enkephalin analogs. The Phe(pNO_3) substitution of enkephalins always yields more potent enkephalin analogs.

The second amino acid can be substituted with other cyclic amino acid analogs, such as thiazolidine-4-carboxylic acid, pipecolinic acid, or 3,4-dehydroproline, with only a slight reduction in activity (table 3) (Chang et al. 1983). Substitution with other hydrophilic cyclic amino acid analogs, such as thiazolidine-4-carboxylic acid sulfoxide or 4-hydroxyproline, greatly abolishes the activity. Unlike enkephalins, the insertion of a glycine between second and third amino acid virturally abolishes activity.

TABLE 3

Relative Receptor Binding Activity of Morphiceptin Analogs with Various Amino Acids at Second Position

	μ-Receptor Affinity (IC_{50}, nM)	Relative Affinity (%)
Tyr-X-NMePhe-D-Pro-NH_2		
X = Pro	5.5	100
Thz	25	22
Pip	16	34
ΔPro	21	34
Thz(O)	80	6.9
Hyp	800	0.69
Pro-Gly	12,000	0.06

All peptide analogs of morphiceptin that have been synthesized so far exhibit extremely low affinity toward **δ**-binding sites. The IC_{50} values determined in the binding assay against the binding of ^{125}I-labeled **δ**-ligand, DADLE, to **δ**-sites are greater than 10 μM (Chang et al. 1981, 1983). All active morphiceptin analogs mentioned above retain very high selectivity for μ-sites. The μ-site selectivity of (NMePhe^3,-D-Pro^4]morphiceptin is superior to that of other μ-agonists, such as morphine, fentanyl, FK33824, and [D-Ala^2,NMePhe^4,gly-ol^5]enkephalin (DAGO) (Chang 1984). However, Brantl et al. (1982) have shown that substitution of D-Ala at the second position leads to an increased binding affinity to **δ**-sites, whereas the affinity to μ-sites is unchanged; thus, the selectivity is greatly reduced. Neither **κ**-receptors

nor **ε**-receptors exhibit significant affinity for morphiceptin and its analogs.

IN VITRO PHARMACOLOGY

Morphiceptin and most analogs can inhibit the electrically stimulated smooth muscle contraction of the guinea pig ileum (GPI), a μ-receptor dominated tissue, as well as the mouse vas deferens (MVD), a **δ**-receptor dominated tissue. The potency in MVD is about five- to tenfold lower than that in GPI (Chang et al. 1983). The correlation studies show that the bioactivities in both tissues are highly correlated with their μ-site binding affinities. Further studies measuring the antagonist potency of naloxone revealed that morphiceptin and analogs interact with μ-receptors in the MVD despite the predominance of **δ**-receptors in this tissue (Chang et al. 1983). Naloxone competitively antagonizes PL017 in the MVD. Schild plots yield a pA_2 value of 8.7 for naloxone against PL017, which is very similar to the pA_2 value of naloxone against μ-agonist morphine in the μ-receptor dominated tissue of the GPI and is of an order of magnitude greater than that against DADLE in MVD (Chang et al. 1983). This result raises the question on the validity of using the relative potency ratio in GPI and MVD as a means to assess the μ- and **δ**-receptor selectivity.

It was also recently shown that morphiceptin and PL017, like enkephalin, can induce excitatory effect on CA1 neurons in superfused hippocampus slices, suggesting that μ-receptors can participate in the excitatory effect in the hippocampal formation (Dingledine et al. 1983 Bostock et al. 1984).

As a highly selective μ-opioid, morphiceptin was shown to decrease the somatic calcium-dependent act ion potential of cultured mouse dorsal root ganglion (Werz and MacDonald 1982, 1983), suggesting that μ-receptors are involved in the regulation of Cal^{2+}-dependent action potential in dorsal root ganglion.

It is interesting to note that although the affinity of PL017 for μ-sites is lower than that of morphine, PL017 is more potent than morphine in all in vitro studies (table 4), suggesting a difference in the way in which peptide and alkaloid activate μ-receptors. Another difference between alkaloid morphine and peptide agonist is that desensitization occurs to. the excitatory effect of morphiceptin but not to morphine in the hippocampus slices (Dingledine et al. 1983).

TABLE 4

Summary of the In Vitro Studies of [NMePhe^3,-D-Pro^4]-Morphiceptin and Comparison with Morphine

	[NMePhe^3, D-Pro^4]-Morphiceptin	Morphine	References
Receptor binding			
Ki value, nM	5.5	0.4	Chang et al. 1983
GTP sensitivity	+	+	
Na+ sensitivity	+	+	
Isolated tissues, ED_{50}			
Guniea pig ileum	34	134	Chang et al. 1983
Mouse vas def erens	240	1,300	Chang et al. 1983
Hippocampus slices, ED_{50} µM	0.4	3	Bostock et al. 1984

IN VIVO PHARMACOLOGY

Morphiceptin produces transient bradycardia intravenous (i.v.) administration (Wei et al. 1980) and elicits analgesia after intracerebroventricular (i.c.v) injection (Brantl et al. 1982; Chang et al. 1982; Zhang et al. 1981: Barrett and Vaught 1982). The analgesic activities of morphiceptin and ß-casomorphins correlate well with the µ-site binding activities (Chang et al. 1982). [D-Pro^4]Morphiceptin also produces analgesic action (Matthies et al. 1982). Morphiceptin produces a pressor response following third, but not fourth, ventricular injection in unanesthetized rats (Holaday 1982). In pentobarbitone anesthetized rats, morphiceptin caused hypotension and bradypnea after injection into the periventricularis hypothalamus (Feuerstein and Faden 1982); it also caused tachycardia at the lower doses, as well as hypotension and bradypnea at the higher doses, after injection into hypothalamic nucleus preopticus medialis (Faden and Feuerstein 1983). Morphiceptin produces catalepsy by i.c.v. administration (Chang et al. 1982; Holaday 1982). All of these effects are reversible by naloxone.

In vivo studies have also been carried out for PL017 and compared with the classical μ-agonist morphine as well as δ-agonist DADLE. These are summarized in tables 5 and 6.

TABLE 5

Summary of the In Vivo Pharmacology of [NMePhe^3,-D-Pro^4]Morphiceptin and Comparison Comparison with Morphine

	(NMePhfe^3, D-Pro^4]-Morphi-ceptin	Morphine	References
Analgesia, ED_{50}, nmole/animal			
i.c.v. route	0.23	8.4	Chang et al. 1983
i.t. route	1	~30	Han et al. 1984
Tolerance & physical dependence	+	+	Chang et al. 1983
Depressor & bradycardia			
i.t., nmole/animal	~5	>100	Li and Han 1984
Respiratory depression			
↓ V_t/T_{tot} nmole/kg/dog	~50	~800	Haddad et al. 1984
Abolition of conditioned responses	+	+	Mauk et al. 1982 ; Lavond et al. 1983

PL017 injected centrally produces analgesia (Chang et al. 1983), respiratory depression (Haddad et al. 1984), and abolition of conditioned responses (Mauk et al. 1982; Lavond et al. 1983. By intrathecal (i.t.) injection, PL017 potently produces analgesic (Han et al. 1984), depressor, and bradycardia effects (Li and Han 1984). After chronic administration, PL017 produces tolerance and physical dependence (Chang et al. 1983). Like the above in vitro studies, PL017 is far more potent than morphine by the same route of administration in inducing these effects

(table 5). Since similar results are obtained from the in vitro studies in which the metabolism is not a likely explenation for the higher potency for PL017, we propose that the efficacy of peptide morphiceptin or its analogs for activating the μ-receptor system is higher than that of alkaloid morphine.

TABLE 6

Comparison of the Respiratory Effects of [NMePhe^3,-D-Pro^4]Morphiceptin and [D-Ala^2,-D-Leu^5]-Enkephalin

	[D-Ala^2,-D-Leu^5]-Enkephalin (8)	[NMePhe^3,D-Pro^4]Morphiceptin (μ)
Instantaneous minute ventilation V_t/T_{tot}	↓	↑
Tidal volume, V_t	↓then normal	↓
Respiratory cycle time, T_{tot}	↑	↓
Expiratory duration, T_e,	↑	↓
Inspiratory duration, T_i	No change	↓
Respiratory duty cycle T_i/T_{tot}	↓	Slight ↑

Like morphine, PL017 can induce respiratory depression after intracisternal injection (Haddad et al. 1984). In conscious dogs, strikingly different effects on the ventilation and ventilatory pattern were observed between PL017 and DADLE (table 6). PL017 induced a fast and shallow breathing pattern, while DADLE produced deep breathings separated by a period of aprea (Haddad et al. 1984). Detailed analysis reveals that DADLE induces a prolongation in expiratory time (T_e) and a decrease in instantaneous minute ventilation (V_t/T_{tot}), which is a result of a decrease of tidal volume (V_t) and an increase of respiratory cycle time (T_{tot}) In contrast, PL017 induces a decrease in. the inspiratory time (T_i), T_e, and V_t, with a net increase in V_i/T_{tot}. The respiratory effects of μ- and δ-agonists can be reversed by naloxone, but larger doses are required after DADLE than after PL017.

These results suggest that μ- and δ-receptors in conscious dogs participate in different regulatory functions in breathing, and involve different neuronal pathways.

CONCLUSION

Morphiceptin analogs with various substitutions at positions 2, 3, and 4 have been synthesized and studied for their receptor activities. Substitutions with D-proline at fourth position and N-Me-Phe at third position yield a potent and highly selective μ-peptide agonist, PL017. PL017 and morphiceptins interact with μ-receptors in GPI and MVD. They elicit analgesia by i.c.v. and i.t. injection, and evoke depressor and bradycardia by i.t. injection. PL017 produces tolerance and physical dependence after chronic administration. PL017 is more potent than morphine in all pharmacological tests in spite of its lower μ-receptor binding affinity. In conscious dogs, PL017 and DADLE produce a profound difference in regulating ventilation.

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Dermorphin: Autonomic Pharmacology and Structure-Activity Relationships

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INTRODUCTION

In recent years, several biologically active peptides, such as neurotensin, thyrotropin-releasing hormone, bombesin, and other tachykinins, have been identified in the gastrointestinal tract as well as in central and peripheral elements of the nervous system (Dockray and Gregory 1980). Many of these neuroendocrine peptides (e.g., bradykinin, CCK-8, substance P, bombesin) were originally isolated from the skin of various amphibian species (Erspamer 1981) . Dermorphin is a hepta tide recently isolated from the skin of the south American frog of the genus Phyllomedusae (Brocardo et al: 1981; Montecucchi, et al 1981) and has the following amino acid sequence: H-Tyr^{1}-D-Ala^{2}-Phe^{3}-Gly^{4}-Tyr^{5}-Pro^{8}-Ser^{7}-NH_2. In addition to demorphin, Hyp^{6}-demorphin was also isolated from the skin of same of these frogs. Dermorphinlike inmmoreactivity was also demonstrated in rat brain, but the final identification of dermorphin in mammalian tissue (central or peripheral) has not yet been made (Negri et al. 1981). The structure of these new tides is unique since they contain a D-amino acid residue in molecule which is critical for the biological activity (L-Ala^{2}-demorphin is only 1% as active as dermorphin). The structure proposed for the extracted dermorphin from the natural source has been confirmed by synthesis (de Castiglione et al. 1981a).

Dermorphin has already been tested extensively for a variety of opiate like activities. Moreover, analog of dermorphin were already synthetized in order to clarify structure-activity relationships for the multiple biological activities of these opioid peptides.

This chapter summarizes the biological effects of dermorphin, its site of action, the opiate receptors mediating its effects, and the structure-activity relationships of this unique opioid peptide.

DERMORPHIN--OPIOID PEPTIDE

In Vitro Tests

Dermorphin displays a potent depressive action on electrically stimulated contractions of the guinea pig ileum (GPI) and mouse vas deferens (MVD)--two classical reparations for opioid activity assay. In these actions, dermorphin is much more potent than the endogenous enkephalins, β-endorphin, and the opiate prototype, morphine. In the GPI preparation, the only peptide which is more potent (three-to- five-fold) than dermorphin is dynorphin A-(1-13) (Erspamer et al. 1981). In the MVD, dynorphin[6] A-(1-13) enkephalins are eqiupotent to dermorphin, Hydro -dermorphin is 80% to 90% as potent as dermorphion in these tests. These preferential actions of dermorphin on GPI suggest preferential u-receptor mediated action as also indicated from in vitro binding studies (Glaser et al. 1981).

Analgesia

Dermorphin is an extremely potent anal analgesic agent in most species tested so far (rat, mice, rabbit). Surprisingly, dermorphin does not produce analgesia in the chick at low doses (Erspamer et al. 1981). Analgesia can be produced by micramole doses injected ripherally. No known opiate or opioid peptide can compete with dermorphin in analgesia tests (hot-plate, tail-flick and even ß-endorphin is only one-twentieth as potent as dermorphin. Actually, replacement by dermorphin or the N-terminal sequence 1-7 of ß-endorphin results in increased analgetic potency of the [denrmorphin1-7]-ß-endorphin as compared to ß-endor phin (Yamashiro et al. 1983). In the hot-plate test (rat, intracerebroventricular injection), dermorphin is 2000-fold more potent than morphine (Erspamer 1981; Erspamer et al. 1981; Broccardo et al. 1981; Yamashiro et al. 1983).

GASTROINTESTINAL EFFECTS OF DERMORPHIN

Sensory input from the gastrointestinal (GI) tract is conveyed to several brain regions by afferent vagal nerves. Many of these regions, e.g., the solitarius nucleus in the brain stem, limbic areas, contain high levels of opiate receptors as well as opioid peptides (Mar 1978; Snyder 1977). In addition, opiates and opioid peptides were shown to have potent effects on various GI functions.

Dermorphin was also tested for its effects on the GI tract by both peripheral and central routes of administration. Dermorphin was shown to inhibit basal gastric secretion or to stimulate gastric secretion induced by various stimuli-- e.g., water distension of the stomach and insulin-induced gastric acid secretion; however, histamine-induced gastric acid secrction was resistant to inhibition by dermorphin (Improta et al. 1982). In addition, dermorphin was also shown to affect the motility of

both the stomach and intestine. Gastric emptying and intestinal transit time are substantially increased by dermorphin (Broccardo et al. 1982.; Rossi et al. 1983). Intestinal transit, however, is only partially blocked by dermorphin (Broccardo et al. 1982).

The subcutaneous (s.c.) or intracerebroventricular administration of dermorphin produces all of these GI effects; however, the dose of dermorphin necessary to produce GI inhibition by the central route of administration was one-hundredth of the dose necessary to produce comparable effects by peripheral administration of dermorphin, indicating that the opioid peptides act through a centrally mediated mechanism

All of the GI effects of demorphin could be completely prevented by naloxone, suggesting an opiate receptor mediated effect. Furthermore, the inhibition of intestinal propultion induced by central administration of dermorphin can be blocked by a quaternary naloxone analog injected into the cerebroventricular system in a dose which has no effect when administered peripherally (Parolaro et al. 1983); thus., it seems that the GI effects of demorphin are primarily medicated through a centrally located opiate receptor.

Since the studies cited above were conducted on rats, which differ from guinea pigs and other species in the type and distribution of opiate receptors in the central nervous system, it also would be important to investigate the GI effects of dermorphin in other species. Moreover, the type of opiate receptor and the specific sites (nuclei) in the brain where GI regulation by dermorphin commences remain to be explored.

CARDIOVASCULAR AND RESPIRATORY EFFECTS OF DERMORPHIN

Opiates and opioid peptides were long known to affect blood pressure, cardiac functions, and the respiratory system. Therefore, it has been postulated that dermorphin, a potent opioid peptide, might also significantly affect the cardiovascular and respiratory systems. Indeed, administration of dermorphin to human subjects causes a brief increase in blood pressure and heart rate without affecting urinary electrolyte excretion (Degli-Uberti et al. 1983b, 1983c). In the anesthetized dog, systemic administration of demorphin elicits pressor and cardiac acceleration responses (gander and Giles 1982).

In the conscious rat, intracerebroventricular injection of dermorphin causes complex cardiovascular responses; while low doses (picomoles) cause elevation of blood pressure and heart rate, higher doses will cause hypotension and bradycardia (Feuerstein and Faden 1983a). The cardiodepression produced by higher doses of demorphin injected into the cerebroventricular system is accompanied a respiratory depression as evident by hypoxemia, elevated carbon dioxide, and reduction of blood pH (respiratory acidosis) these cardiorespiratory changes are

accompanied by a marked increase in plasma catecholamines (Feuerstein and Faden 1983a). However, the cardiac responses to central administration of dermorphin are more complex: vagal activation seems to underly the bradycardia produced by high doses of dermorphin since the peripheral acting muscarinic blocker, N-methyl-atropine, can prevent or reverse the bradycardia produced by dermorphin (Feuerstein and Faden 1983a. Thus, dermorphin activates both the sympathetic and parasympathetic components of the autonomic nervous system. Also, naloxone reverses all of the central cardiovascular effects of dermorphin.

The anterior hypothalamus is probably the site for the central cardiovascular effects of dermorphin since the pressor/cardiac acceleration effects can be produced by picomole doses of dermorphin microinjected into discrete nuclei of the hypothalamus (Diz and Jacobowitz 1984; and Diz, Vitale, and Jacobowitz 1984; Feuerstein and Faden 1983a). The role of the sympatho-adrewmmdullary system in mediation of the cardiac acceleration and pressor responses elicited by hypothalamic injections of dermorphin is also indicated by the blockade of the cardiac responses with the β-adrenergic blocking agent, propranolol. Yet, it should also be pointed out that other brain nuclei besides the hypothalamus are involved in dermorphin effects since dermorphin induces bradycardia, probably by activation of efferent vagal neurons in the nucleus ambiguus and the dorsal motor nucleus of the vagus. This suggestion, however, awaits further clarification.

The cardiovascular and respiratory effects of dermorphin resemble those of [D-Ala^2-MePhe^4-Gly-ol^5] enkephalin (DAGO), an enkephalin analog which is 200-fold more selective for μ- versus δ-opiate receptors (Kosterlitz et al. 1980; Pfeiffer et al. 1982). Since all of the dermorphin effects on the cardiovascular system are produced at a much lower concentration than DADLE, [D-Ala2-D-Leu3] enkephalin, a relatively selective δ-receptor agonist, it is pertinent to suggest that μ-opiate receptors mediate the central cardiovascular responses to dermorphin. These conclusions are summarized in table 1.

ENDOCRINE AND METABOLIC EFFECTS OF DERMORPHIN

Many opiates and opioid peptides have been shown to affect the regulation of glucose metabolism and the release of pituitary hormones (Dupont et al. 1977; Van-Vugt and Meites 1980; Locatelli et al. 1978). Opioid peptides and opioid receptors are found in both central (brain, pituitary) and peripheral organs involved in endocrine and metabolic regulation (Grandison et al. 1980; Rivier et al. 1977).

TABLE 1

Central Cardiorespiratory Effects of Dermorphin

1. Complex Cardiovascular Responses

   low doses: presser/cardiac acceleration
   high doses: depressor/cardiac deceleration
   site and receptor specific

2. Respiratory Depression

   respiratory acidosis (low pH, low $p0_2$, high $pC0_2$)

3. Activation of the Sympatho-Adrenomedulary System

   increases circulating epinephrine and norepinephrine

4. Activation of the Vagus (High Doses)

   atropine reverses the bradycardia

5. μ-Opioid Receptors Mediate Dermorphin's Effects

   similarities to DAGO (highly selective μ-agonist)
   highly sensitive to naloxone

Prolactin

When dermorphin (0.1 to 5 mg/kg, s.c.) is administered to the rat, it produces a dose-dependent increase in plasma prolactin (Rossi et al. 1983). This effect can also be produced by subnanomole doses of dermorphin injected into the cerebroventricular space, and can also be seen in castrated male rats. However, in vitro experiments show no direct effect of dermorphin on prolactin release from rat pituary cells, suggesting a hypothalamic site for dermorphin-induced prolactin release (Giudici et al. 1984).

dermorphin was also shown to increase plasma levels of prolactin in human subjects when infused systemically (Degli-Uberti et al. 1983a). The increase in prolactin response was more pronounced in females than males. In both the rat and human studies, naloxone effectively blocked the dermorphin effects, suggestion an opiate receptor mediated response. However, Güllner and Kelly (1983) failed to increase plasma prolactin by

low doses of dermorphin in jected intravenously into the anesthetized rat. This discrepancy might be related to the different rat species, the type of anaesthesia (Ketamine), and/or the relatively low doses of dermorphin which might have failed to reach sufficient levels in the brain.

Growth Hormone

Opiates and opioid peptides have been shown to release growth hormone (GH) in rat and man (Von Graffenried et al . 1978; Stubbs et al. 1978; Van-Vugt and Meits 1980). Dermorphin was also shown to produce an increase in GH levels within minutes after intravenous administration to the anesthetized rat (Gullner and Kelly 1983) or to human subjects (Degli-Uberti et al 1983a). The increase in plasma GH by dermorphin could be blocked by naloxone (Degli-Uberti et al. 1983a).

Calcitonin

Dennorphin administration to normal human volunteers (5µg/kg/min over 30 min) does not change the plasma levels of calcitonin over a 3-hour period. In addition, the circulating levels of this hormone are not changed by naloxone infusion (Degli-Uberti et al. 1983b). These data suggest that opiates may not play a significant role in regulation of calcitonin release. However, the role of opiates and dermorphin was not examined during state where endogenous calcitonin release is stimulated (e.g., hypercalcemia).

Thyroid Stimulating Hormone (TSH)

Intravenous injection of dermorphin to rats caused a dose-dependent increment in plasma TSH levels (Gullner and Kelly 1983). The increase in plasma TSH produced by dermorphin in opposite to the effect of endogenous opioid peptides on this anterior pituitary hormone (Bruni et al. 1977).

Luteinizing Hormone (LH)

Opioid peptides were previously shown to reduce plasma levels of LH (Bruni et al. 1977). Dermorphin administration to adult male rats causes a marked decrease in plasma LH at extremely low doses (Gullner and Kelly 1983). The mechanism of this effect has not been studied as yet.

ENDOCRINE AND METABOLIC EFFECTS OF DERMORPHIN

Several neuropeptides, (e.g., somatostatin, ß-endorphin) and peptides isolated from frog skin (bombesin, substance P) have been shown to affect glucose metabolism (Gullner et al - 1983; Brown and Vale 1976). Injection of dermorphin to the rat at doses up to 10 g did not effect plasma insulin or somatostatin; however, plasma glucose was slightly reduced while plasma glucagon was significantly increased (Gullner et al. 1983).

dermorphin's effect of reducing blood glucose is opposite to the increase in plasma glucose induced by ß-endorphin. Also, it is still unclear whether dermorphinexists in the endocrine cells of the pancreas or in any of the peripheral tissues involved in these endocrine functions.

dermorphin Effects on the Renal-Adrenocortical Endocrine System

Opiates and opioid peptides have been shown to affect the pituitary-adrenocortical functions in several species (Smythe et al. 1980; Gaillard et al. 1981; Volavka et al. 1979). Dermorphin infusions to normal volunteers caused elevation of plasma renin activity (PRA) and a small decrease in plasma cortisol which was accompanied by a similar decrease in plasma adrenocorticotropic hormone (ACTH). Plasma aldosterone levels were also slightly increased by dermorphin infusion (Degli-Uberti et al. 1983c). The increase in PRA by dermorphin is in accord with the known effect of other opioid peptides to increase PRA (Cantalamessa et al. 1982) and such an increase in PRA might generate sufficient angiotensin II to account for the pressor response observed shortly after dermorphin administration (Degli-Uberti et al. 1983c). The reduction in plasma cortisol and ACTH by dermorphin is also in accord with the same response of plasma cortisol to enkephalin analog and other opiates (Gold et al. 1980; Stubbs et al. 1978; Gaillard et al. 1981). A role for opioid peptides in regulation of ACTH and cortisol release is also indicated by the elevation of the circulatory levels of these hormones by naloxone administered to normal human subjects.

Catecholamines

Several opioid peptides were shown to stimulate the sympatho-adrenomdullary axis and increase the circulatory levels of norepinephrine, epinephrine, and dopamine (Feuerstein et al. 1983; Pfeiffer et al. 1982; Van-Loon and Appel 1981). Intracerebroventricular administration of dermorphin to conscious rats was likewise shown to produce a marked increase in plasma levels of epinephrine (over twentyfold) and norepinephrine (sixfold) (Feuerstein et al. 1983). The site of the sympatho-adrenal activation seems to lie within the hypothalmus, since microinjection of dermorphin to the anteroventral region of the hypothalamus produces similar incraments in plasma catecholamines and, primarily, epinephrine (Feuerstein and Faden 1983a). The increase in peripheral sympathetic activity is considered to underlie the increase in heart rate produced by dermorphin since adrenalectomy or propranolol (a ß-adrenoceptor blocker) prevents the increase in heart rate by dermorphin (Diz and Jacobowitz 1984). In addition, the increase in sympathetic tone is probably the main stimulus for the observed increase in PRA (Degli-Uberti et al. 1983c).

Table 2 summarizes the endocrinological effects dermorphin obtained from animal and clinical research.

TABLE 2

Endocrine and Metabolic Effects of Dermorphin

	Increase	No Change	Decrease
Epinephrine Norepinephrine	(+++)		
Renin	(++)		
Aldosterone	(+)		
Glucagon	(++)		
TSH	(++)		
Growth Hormone	(++)		
Prolactin	(++)	(Ø*)	
Galcitonin			
Insulin		(Ø)	
Sumtostatin		(Ø)	
Glucose	(+)		(-)
Cortisol			(-)
ACTH			(-)

TSH = thyroid stimulating hormone; ACTH = adrenorticotropic hormone; * anesthetized rat; number of (+) or (-) denotes intensity of increase or decrease in plasma levels, respectively.

BEHAVIORAL EFFECTS OF DERMORPHIN

Effects of dermorphin on the Electroencephalogram (EEG)

Injection of low doses (few nanomoles) of dermorphin into the cerebroventricular system of the rabbit produces synchronous patterns in the cortical EEG and disruption of hippocampal theta waves. Higher doses reduce spike wave lexes (Aloisi and Scotti De Garolis 1981). In the chick, dermorphin administration into the third ventricle also caused total voltage power increase and an EEG shift toward lower frequencies (Erspamer et al. 1981). The EEG changes are all reversed by naloxone.

In rats, doses of dermorphin ranging from 5 to 2,500 ng produced electroencephalographic seizures associated with grand mal motor

manifestations: twitches and tremor with low doses; tonic-clonic convulsion by higher doses. The convulsive patterns were followed by high voltage slow waves and other abnormalities (Aloisi et al. 1982).

Demorphin produces behavioral responsesn known for many opiates, e.g., catatonia, rigidity, sedation. In the chick, "squating" behavior was also observed. In mice, doses as low as 10 ng produce the "Straub Tail Reaction" (tail elevation 90^6), restlessness , and increased motor activity (runing fits); hi her doses produce catalepsy. All these effects are also produced by morphine at higher dose range. In the rat, higher doses of dermorphin result first in reduced spontaneous movement, and later in rigidity and catatonia. Exophthalmos, meiosis, and hypothermia are also observed. In the rabbit, muscular rigidity, catatonia, and loss of righting reflexes are always seen at higher doses along with hypothermia, exophthalms, and mydriasis. In the rabbit, high doses of dermorphin will result in flaccid paralysis of the hindlimbs and, ultimately, death (Aloisi et al. 1982). Topical administration of dermorphin over the sensorimotor cortex in this species results in highly disorganized EEG activity, characterized by exclusively slow waves but no motor or EEG convulsive manifestations. Thus, it appears that in the rat, dermorphin causes EEG; and behavioral epileptic phenomena but is devoi of convulsant activity in the

STRUCTURE-ACTIVITY RELATIONSHIPS OF DERMORPHIN

The minimal peptidic sequence of dermorphin which possesses biological activity is respresented by the N-terminal H-Tyr-D-Ala-Phe-Gly-NH_2 tetrapeptide (Broccardo et al. 1981; de Castiglione et al. 1981b; Salvadori et al. 1982). However, dermorphin (1-4) is only 2% to 5% as active as dermorphin in inhibiting GPI or MVD contraction in response to electrical stimulation and is practically devoid of analgetic and cataleptic activity. dermorphin (1-3) has less than 0.5% of any of the biological activities of dermorphin

However, modifications of the C-terminal of the dermorphin by lipophilic substituents, e.g., Tyr-b-Ala-Phe-Gly-NH-CH_2-C_6H_5, markedly increase the opioid activity of the dermorphin tetrapeptide, and the biological activity; H-Tyr-D-Ala-Phe-Gly-NH-Adamantyl results in over twentyfold increased potency in GPI and MVD preparation, which strongly suggests a μ-receptor rather than δ-receptor mediated effect (Broccardo et al. 1981; Kosterlitz et al. 1980). A quantitive dependence of biological activity on the lipophylic character of the C-terminus was also reported for a series of enkephalin like peptides (Do et al. 1978

Profound changes in the biological activity of dermorphin can also be seen by relatively minor changes in position 1 (H-Tyr).

Replacement of Tye by D-Phe[1] or Ala[1] causes a 6-order decrease in potency on the GPI; substitution of au Acetyl Tyr[1] also profoundly decreases the relative potency in GPI inhibition test. The analog also display reduced capacity to displace naloxone from binding sites on striatum homogenates (Darlak et al. 1983). However, the capacity to displace naloxone from opiate receptors might not alwayslcorrelate with the biological activity of the analog; Tyr (Me)[1]-dermorphin for example, is not less active in displacing naloxone from rat striatun homogenates as compared to dermorphin but is >thirty fold less potent than dermorphin in inhibiting of GPI contraction and almost a hundredfold less active in producing analgesia in mice (Darlak et al. 1983). Modifications of position 1 of dermorphin analog also reveal some differential changes in the various tests of biological activities. Phe[1]-dermorphin is only about 0.0001% as active as dermorphin on GPI, but has thus still 5% analgetic activity as compared to the natural peptide. It is also noteworthy lthat the introduction of a sulphate group on the hydroxyl of Tyr[1]-residue of dermorphin completely destroyed all of the opioid activity of dermorphin (de Castiglione and Perseo 1983).

Modification of enkephalins at position 2 by introducing D-Arg[2]-residue produces analog with potent analgetic (Takagi et al. 1982; Kubota et al. 1980). Similar modification in the dermorphin tetrapeptide has also proved to produce potent opioid tide analog (Sasaki et al. 1984). Thus, D-Arg[2]-dermorphin (1-4) is tenfold more potent than dermorphin tetra peptide in the mice tail-flick test; replacing Gly[4] by Sar[4]-residue in the D-Arg[2]-dermorphin (1-4) further increases the analgesic potency fourfold. Thus, it is clear that D-Ala[2]-residue in dermorphin is not always necessary for the opioid activity, as originally thought. Further modifications at the C-terminus which increase the lipophilic character of the peptide also increase the potency. This is due, in part, to an increased resistance a against carboqieptidases. Resistance against enzymatic breakdown might account for the prolonged (several hours) action at relatively low doses injected subcutaneously.

Several other modes of amino acid substitutjons yield various potencies of the dermorphin molecules. Substitution of Tyr[3] by aromatic amino acid is well tolerated, but Gly dermorphin is devoid of must activities. Substitution of Pro[8] has little effect on the overall opioid activity of dermorphin and this applies also for position 7 (de Castiglione et al. 1981b). An interesting finding is that the deaminated dermorphin maintains its peripheral opioid activity but is devoid of effect on the central nervous system.

Of special interest in the structure-activity relationships of dermorphin analog are the differential activitiy displayed by some analog. Thus, dermorphin (1-5) and Leu[5] dermorphin (1-5) demonstrate augmented potency (about twofold) in the GPI

bioassay test but reduced activity (-80%) in the MVD bioassay test; these dermorphin pentapeptides are virtually inactive in the common analgesia and catalepsy tests. Another example of differential preservation of opioid activity is demonstrated by the benzylated-Tyr^5-dermorphin which presents about 50% of its inhibitory action on MVD bioassay but only 5% in the GPI bioassay. In no case so far (of over 100 dermorphin analog synthesized) has analgesia been preserved as a primary property of a dermorphin analog. The main structure-activity relationships of dermorphin are presented in table 3.

TABLE 3

Differential Opioid Activity of dermorphin Analog

	GPI	MVD	ANALGESIC	CATALEPSY
Dermorphin	100	100	100	100
Bzl-Tyr^5-Dermorphin	<5	50	<1	<0.5
Des-Tyr^5-Dermorphin	<0.1	<0.1	<1	<1
Dermorphin (1-6)	50	40	20	<1
Dermorphin (1-5)	20	200	<5	<5
Leu^5-Dermorphin (1-5)	15	175	<1	--
Dermorphin (1-4)	<2	<5	--	--
Dermorphin (1-3)	<0.3	<0.5	--	--

Numbers represent percent activity as compared to dermorphin (100%). GPI = guinea pig ileum bioassay test; MVD = mouse vas deferens bioassay test.

DERMORPHIN AS A μ-RECEPTOR AGONIST

The type of opiate receptor which mediates the biological actions of dermorphin is still obscure. However, several pharmacological properties of dermorphin effects in vitro and in vivo strongly suggest the μ-opiate receptors are primarily involved in dermorphin effects. The evidence underlying this suggestion is: 1) Dermorphin is much more potent in blocking the contraction of the electrically stimulated GPI rather than the MVD; this bioassay test is considered to be a prototype for morphine (μ) rather than enkephalin (δ) effect. 2) Dermorphin is less potent than enkephalins in inhibiting the PGE_1-stimulated adenylate cyclase (accumulation of c-AMP) in neuroblastoma x glioma hybrid cells (Glaser et al. 1981). 3) Dermorphin

effects were shown to be easil and completely reversed by low doses of naloxone administered peripherally or centrally. 4) Dermorphin effects on the cardiorespiratory system are similar to those of DAGO, which is a highly selective μ-receptor agonist; the cardiovascular effects of dermorphin (pressor/ cardiac acceleration) are produced by doses which are one-hundredth of those necessary to produce the same responses by the relatively selective δ-receptor agonist DADLE (Feuerstein and Faden 1983a, 1983b; Feuerstem et al. 1983).

SUMMARY

The biological activities of dermorphin summarized in this chapter indicate that dermorphin possesses all known effects of opiate/opioid peptides. It seems that dermorphin effects are primarily mediated through a μ-receptor of high affinity. Since none of the known endogenous opioid peptides displays high potency and selectivity for μ-receptors, dermorphin like peptides might be the endogenous selective ligands for such μ-receptors. This hypothesis, however? awaits further investigation. The structure-activity relationships of dermorphin and the unique D-alanine at position 2 clearly indicate that the N-terminus of the first four amino acids is crucial for the complete biological activity. Systematic modifications of the dermorphin sequence also reveal the potential for developing selective agonists (or antagonists) for discrete opioid functions by mdifying receptor selectivity.

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Design of Conformationally Constrained Cyclic Peptides With High Delta and Mu Opioid Receptor Specificities

Victor J. Hruby, Ph.D.

INTRODUCTION

Efforts to understand the conformational structure-biological activity relationships for the endogenous opioid peptides, such as methionine enkephalin, leucine enkephalin, and dynorphin, have been difficult. Despite the preparation of at least a few thousand peptide analogs (for reviews, see Miller and Cuatrecasas 1978; Morley 1980; Bajusz 1982; Olson et al. 1982, 1983), highly receptor-selective and potent peptide analogs have been obtained only recently. The reasons for these difficulties are many, but basically they arise fran the intrinsic physical-chemical properties of most endogencus opioid peptides, the comlexity of the biochemical events related to their biological activities, and the existence of several different classes of receptors for these molecules, each of which my mediate different biological responSeS.

Most of the endogenous cpioid peptides that have been isolated thus far are small or moderate size linear peptides which have a high degree of conformational flexibility. These properties do present certain advantages (table 1), many of which are undoubtedly utilized by the cpioid peptides in vivo. However, fran the standpoint of understanding the physical-chemical basis for information transfer and biological activity, these properties pose several problems for many peptide hormones and neurotransmitters which we (Meraldi et al. 1977; Hruby 1981a, 1982, 1984; Hruby et al. 1983) and others Narshall et al. 1978; Veber 1979) have discussed. For the purpose of this chapter, suffice it to say that the high conformational flexibility of the opioid peptides such as methionine enkephalin, H-Tyr-Gly-Gly-Phe-Met-OH, makes it difficult to determine which of the multitude of possible conformations are of biological significance.

A similar problem exists with regard to the complexity of biological information transfer in the hormone-receptor complex. We have pointed out Hruby 1981a, 1981b, 1984; Hruby et al. 1983; Hruby and Hadley, in press) that binding, transduction, and reversal

TABLE 1

Some Advantages of Conformational Flexibility in Biologically Active Peptides

1.	Many Conformations Accessible at kT.
2.	Different Conformational Structures Can Readily Be Utilized at Different Receptors.
3.	Easily Fit into Enzymes; Biological Degradation Facile.
4.	"Zipper Model" Is Possible, Providing a Thermodynamically Accessible Route to Peptide-Receptor Interaction.
5.	Different Conformational Structures Available for Binding, Transduction, and Reversal at Receptors.

probably each utilize different conformational structure properties of a peptide hormone or neurotransmitter. Thus, appropriate ligands and biological assays are needed to help sort out this complexity

Finally, as with most peptide hormones and neurotransmiters, the endogenous opioid peptides appear to interact with several different receptors at different cell types. These include the delta **(δ),** mu (μ), kappa **(κ),** epsilon **(ε),** and possibly other receptors (Martin et al. 1976; Lord et al. 1977; Chang et al. 1979). These different receptor types have biological significance, and in several cases there is good evidence that they have different structural requirements for cpioid peptides. Thus, the pharmacophore for each receptor type is different, a fact which has been largely ignored in previous attempts to understand the "biologically active conformation" for opioids.

In an effort to resolve some of these problems, we have sought in our studies to develop receptor-specific peptide analogs. Our approach to obtaining such analogs has been via conformational restriction (Marshall et al. 1978; Veber 1979; Meraldi et al. 1977; Hruby 1981a, 1982, 1984). In this chapter, we review how this approach has led our laboratory to the design and synthesis of cyclic, conformationally constrained peptides with high delta and mu opioid receptor specificities.

CYCLIC DELTA RECEPTOR-SELECTIVE PEPTIDES

Oue starting point in 1981 for the development of **δ** receptor-specific enkephalin analogs was several observations related to enkephalin structure-function studies. First, substitution of Gly^2 in methionine- or leucine-enkephalin by several D-amino acid residues was compatible with high biological activity (e.g., see Beddel et al. 1977). Second, substitution of Met^5 or Leu^5 by a wide variety of L or D amino acid residues was compatible with high potency (e.g., see Morley 1980). Third, the compact

structural nature of many nonpeptide opioids suggested that, on interacting with a receptor, the opioid peptides may assume more compact conformations involving a turn conformation. With this in mind, we decided to apply the concept of pseudoisosteric cyclization which we had applied successfully in the development of superpotent cyclic melanotropins (Sawyer et al. 1982). In this regard, the Gly^2, Met^5 residues in enkephalin were highly reminiscent of the Met^4, Gly^{10} residues in **α**-MSH. Hence, substitution of Gly^2, Met^5 by D-Cys^2, D(or L)-Cys^5 to produce a 14-membered cyclic structure appeared to be a reasonable starting point for conformational restriction.

On examining the literature, we found that Sarantakis (1979) had already prepared a [D-Cys^2, L-Cys^5]enkephalin analogue, and though the compound was active, the bioassay results provide no data on receptor specificity. We, therefore, decided to prepare even more rigid analogs by utilizing geminal dimethyl groups for restricting the conformation of these medium-sized peptide rings, which we had demonstrated to occur in oxytocin antagonist analogs (Meraldi et al. 1975, 1977; Mosberg et al. 1981; Hruby 1981b). Thus, we prepared the enkelinamide (EA) analogs[D-Pen2, Cys^5]EA and [D-Pen^2, D-Cys^5]EA, and examined (Mosberg et al. 1982) their delta versus mu receptor selectivity using the mouse vas deferens (MVD) and guinea pig ileum (GPI) as the putative delta and mu receptor-selective assays (Kosterlitz and Watt 1968; Henderson et al. 1972). Fortunately, as we began our studies, Schiller et al. (1981) also prepared the enkephalinamide compounds of Sarantakis and examined their biological activities in the GPI

TABLE 2

Biological Activities (IC_{50}) of Cyclic Enkephalinamide Analogs in the Guinea Pig Ileum and Mouse Mouse Vas Defrens (MVD) Assays

Compound[a]	IC_{50}(nM)[b] GPI	MVD	IC_{50}(GPI) / IC_{50}(MVD)
[D-Pen^2, Cys^5]EA[c]	118 ± 19	3.6±0.67	32.4
[D-Pen^2, D-Cys^5]EA[c]	117 ± 21	16.8±3.1	6.9
[D-Cys^2, Cys^5]EA[d]	1.51 ± 0.03		
[D-Cys^2, D-Cys^5]EA[d]	0.78 ± 0.01		
[D-Ala^2, Met^5]EA[c]	2.20 ± 0.40	3.75±0.04	0.59
Normorphine[c]	91 ± 19	540±113	0.17

[a]EA = enkephalinamide
[b]Values are the mean (± s.e.m.) of 3 to 4 determinations.
[c]Mosberg et al. 1982
[d]Schiller et al. 1981

assay and membrane binding assay system. The results from the studies of Schiller and our studies are given in table 2. An interesting result was observed. While the cyclic [D-Cys^2, D(or L)-Cys^5]EA analogues of Schiller et al. and Sarantakis are slightly μ receptor-selective (schiller et al. 1981), the [D-Pen^2, D(or L)-Cys^5]EA analogs we prepared were somewhat delta selective (Bosbsrg et al. 1982). Examination of the data in table 2 indicates that this delta receptor selectivity is primarily a result of continued high potency of the D-Pen^2 analogs at the MVD receptor system (δ), but much lower potency at the GPI receptor system (μ). Conformational analysis of the [D-Pen^2, D(or L)-Cys^5]EA analogs and the D-Cys^2, D(or L)-Cys^5]EA analogs(Mosberg and Schiller 1984) suggests that, whereas the D-Pen^2 analogs are the more conformationally constrained analogs, as expected, the differences in biological activity may result from conformational difference at the C-terminal portion of the peptides (possibly a transanular effect of the geminal dimethyl groups of D-Pen^2 in a 14-membered ring).

These interesting results led us to examine this receptor specificity of the carboxylate terminal analogs of D-Pen^2-substituted cyclic enkephalins (E) since previous structure-activity studies (Morley 1980; Kosterlitz et al. 1980; Ronai et al. 1981) had indicated that carboxylate terminal enkephalins were relatively more reactive in the MVD assay (δ receptor), while carboxamide terminal enkephalins were relatively more reactive in the GPI assay (μ receptor). Indeed, as shown in table 3, [D-Pen^2, L-Cys^5]E and [D-Pen^2, D-Cys^5]E are considerably more potent than their enkephalynmnide counterparts in the MVD assay and, interestingly, they are considerably less active in the GPI assay. Thus, these analogs have exceptional delta receptor selectivity, and indeed appeared to be the most delta-selective enkephalin analogs which had been obtained up to that time.

TABLE 3

Biological Activities (IC_{50}) of Cyclic Enkephalin Analogs in the GPI and MVD Assays

Compound[a]	IC_{50}(nM)[b] GPI	MVD	$\frac{IC_{50}(GPI)}{IC_{50}(MVD)}$
[D-Pen2, Cys5]EA[c]	117 ± 19	3.6 ± 0.67	32.4
[D-Pen2, Cys5]E[d]	213 ± 63	0.32 ± 0.03	666.
[D-Pen2, D-Cys5]EA[c]	116 ± 21	16.8 ± 3.1	6.9
[D-Pen2, D-Cys5]E[d]	1350 ± 340	6.27 ± 1.2	215.
[D-Cys2, Pen5]E[d]	40. ± 1.5	0.75 ± 0.05	53.
[D-Cys2, D-Pen5]E[d]	67 ± 1.3	0.13 ± 0.06	515.

[a]Ea = enkephalinamide, E = enkephalin.
[b]values are the mean (± s.e.m.) of 3 to 4 detenninations.
[c]Mosberg et al. 1982
[d]Mosberg et al. 1983a

TABLE 4

Receptor Binding Affinities for Cyclic Enkephalin Analogs in Rat Brain Preparation

Compound[a]	IC_{50} (nM)[b]		$\frac{IC_{50}(NAL)}{IC_{50}(DADLE)}$
	[^{3}H]NAL	[^{3}H]DADLB	
[D-Pen^2, Cys^5]EA	73.4 ± 14.9	3.35 ± 0.15	21.9
[D-Pen^2, Cys^5]E	178. ± 15.8	11.7 ± 1.20	15.2
[D-Pen^2, D-Cys^5]EA	16.2 ± 34.8	7.20 ± 1.80	22.6
[D-Pen^2, D-Cys^5]E	157 ± 736	26.0 ± 0.50	6.0
Morphine • HCl	23.3 ± 2.4	27.2 ± 1.2	0.9
[D-Cys^2, Pen^5]E	53. ± 2.3	5.4 ± 0.10	9.8
[D-Cys^2, D-Pen^5]E	22. ± 2.8	3.5 ± 0.8	6.3

[a]EA = enkephalinamide, E = enkephalin.
[b]Values are the mean (± s.e.m.) of 3 to 6 determinations, each done in triplicate.
[c]Mosberg et al. 1982
[d]Mosberg et al. 1983a
[e]Mosberg et al. 1983b

In order to further evaluate the receptor specificity of these cyclic enkephalin analogs, we (Mosberg et al. 1982) also have examined their receptor binding to enkephalin (opiate) receptors in rat brain membrane preparations in competition with the putative μ receptor-selective radioligand [^{3}H]naloxone and the putative δ receptor-selective radioligand [^{3}H]DADLE ([^{3}H]-D-Ala^2, D-Leu^5]E). Through neither of these ligands have particularly high μ and δ receptor selectivity, respectively, they have served as the prototypical ligands for these receptors. Thus, though the results from these binding assays must be interpreted with caution, they can provide insight into the structural and conformational trends for receptor-specific ligands.

Interestingly, in these binding assays, the very high delta selectivity for the cyclic D-Pen^2 enkephalin analogs was less striking. In fact, the enkephalinamide cyclic analogs appeared to be slightly more delta selective than the corresponding enkephalin analogs in this assay system. Thus, a lack of quantitative, and in some cases qualitative, agreement between the muscle contraction assays and the receptor binding assays is observed. This lack of agreement could be the result of one or more of the following: a) species differences; b) different assay conditions; c) lack of discrimination of receptor subtypes by competing ligands; and d) heterogeneity of receptor subtypes. Nonetheless, the high potency and significant δ selectivity of

these compounds in all assay systems prompted us to attempt to further increase this receptor selectivity.

Examination of possible conformational models for the D-Pen^2- and the D(or L)-Pen^5-analogs suggested that the ß-hydrogens on the half-cystine residues, L(or D)-Cys^5 and D-Cys^2, respectively, were not sterically hindered, and that it thus might be possible to prepare the more conformationally constrained bis-penicillamine cyclic enkephalin analogs. Placing two geminal dimethyl substituents in a 14-membered ring would generally be considered very difficult due to energetically unfavorable transannular interactions. However, we felt that if our conformational model was correct, and if the linear sulfhydryl peptides would assume a similar conformation, ring closure might be possible. Therefore, we decided to prepare [D-Pen^2, Pen^5]E (DPLPE) and [D-Pen^2, D-Pen^5]E (DPDPE) (Mosberg et al. 1983c). The synthesis proceeded well, and a good yield of the desired monomer was obtained after purification by partition chromatography and gel filtration (Hurst 1984). The biological activities in the MVD, GPI, and binding assays are shown in tables 5 and 6, and the results are compared with those obtained for the D(or L)-Cys^5 analogs and notmorphine.

Examination of the MVD and GPI bioassay data (table 5) and the binding data (table 6) provide convincing evidence that DPLPE and

TABLE 5

Biological Activities (IC_{50}) of Cyclic Enkephalin Analogs in the GPI and MVD Assays

Compound[a]	(IC_{50})[b] GPI	MVD	IC_{50}(GPI) / IC_{50}(MVD)
[D-Pen^2, Pen^5]E[c]	2720 ± 50.1	2.50 ± 0.03	1088
[D-Pen^2, Cys^5]E[d]	213 ± 63	0.32 ± 0.03	666
[D-Pen^2, D-Pen^5]E[c]	6930 ± 124	2.19 ± 0.30	3164
[D-Pen^2, D-Cys^5]E[d]	1350 ± 340	6.27 ± 1.2	215
[D-Ser^2, Leu^5, Thr^6]E[c]	234 ± 85.6	0.70 ± 0.08	334
[D-Thr^2, Leu^5, Thr^6]E[c]	100 ± 19.6	0.58 ± 0.06	172
[D-Pen^2, Cys^5, Thr^6]E[e]	228 ± 52	0.60 ± 0.06	380

[a]E = enkephalin
[b]Values are the mean (± s.e.m.) of 3 to 4 determinations, each done in triplicate.
[c]Mosberg et al. 1983c
[d]Mosberg et al. 1983b
[e]Hurst 1984

TABLE 6

Receptor Binding Affinities for Cyclic Enkephalin Analogs in Pat Brain Preparations

Compound[a]	IC_{50} (nM)[b] [^{3}H]NAL	[^{3}H]DADLB	IC_{50}(NAL) / IC_{50}(DADLE)
[D-Pen^2, Pen^5]E[c]	3710 ± 740	10.0 ± 2.2	371.
[D-Pen^2, Cys^5]E[d]	178 ± 15.8	11.7 ± 1.2	15.2
[D-Pen^2, D-Pen^5]E[c]	2840 ± 670	16.2 ± 0.9	175.
[D-Pen^2, D-Cys^5]E[d]	157 ± 73.6	26.0 ± 0.5	6.0
Morphine[c]	23.3 ± 2.4	27.2 ± 1.2	0.86
H-Tyr-D-Ala-Gly-Phe-NH[e] $(CH_2)_{12}$ H-Tyr-D-Ala-Gly-Phe-NH	96.3	1.06	91
[D-Ser^2,Leu^5,Thr^6]E[c]	88 ± 6.0	5.7 ± 0.4	15.4
[D-Thr^2,Leu^5,Thr^6]E[c]	36.3 ± 3.8	6.4 ± 0.6	5.7
[D-Pen^2, Cys^5, Thr^6]E[f]	146 ± 25	5.2 ± 0.70	28.1

[a] E = enkephalin
[b] Values are the mean (± s.e.m.) of 3 to 6 determinations, each done in triplicate.
[c] Mosberg et al. 1983c
[d] Mosberg et al. 1983b
[e] Shimohigashi et al. 1982
[f] Hurst 1984

DPDPE are exceptionally delta receptor-specific peptides. Interestingly, the DPDPE is more delta selective based on the MVD versus GPI assays, while DPLPE is more selective based on the binding assays. The reasons for this are that whereas both Pen^5 diastereimeric analogs are much less potent in the GPI assay than the corresponding D(or L)-Cys^5-containing analogs, DPDPE is more potent (almost threefold) than the corresponding D-Cys^5 analogs, and DPLPE is much less potent (about eightfold) than the corresponding L-Cys^5 analogs. Thus, the relative receptor specificity becomes much greater for DPDPE than for DPLPE in these assays. On the other hand, while DPLPE has slightly better binding potencies than DPDPE (table 6) against [^{3}H]DALE (putative δ ligand), the opposite relative binding potencies are seen versus [^{3}H]NAL (putative μ ligand). Thus, the DPLPE appears to be slightly more delta selective than DPDPE in this assay system. These results have recently been confirmed by James and Goldstein (1984). Using what different assays and assay conditions,

TABLE 7

Receptor Binding Affinities of Selected Enkephalin Analogs in Neuroblastosm X-Glioma NG 108-15 Membrane Preparations

Compound[a]	Ki (nm)[b]	Hill Coefficient
[D-Pen^2, Pen^5]E	1.95 ± 0.65	1.00 ± 0.08
[D-Pen^2, D-Pen^5]E	1.78 ± 0.58	1.01 ± 0.06
[D-Ser^2, Leu^5, Thr^6]E	1.59 ± 0.20	1.01 ± 0.06
[D-Thr^2, Leu^5, Thr^6]E	1.42 ± 0.33	1.03 ± 0.04

[a]E = enkephalin
[b]values are the mean (± s.e.m.) of 3 to 6 determinations, each done in triplicate.

they have examined the δ receptor selectivities of DPDPE, DPLPE, and several other putative δ-selective analogs, and have found that on the basis of their stringent requirements for selectivity, only DPDPE and DPLPE are truly δ receptor specific. They also found that these peptides have little or no kappa receptor activity. The bis-penicillamine cyclic enkephalin analogs also appear to have considerably higher delta receptor selectivity than the dimeric enkephalin analogs of Shimohigashi et al. (1982).

To further examine the delta selectivities and the receptor potencies of our highly δ-selective cyclic analogs, we have done several additional studies. We have examined their binding affinities for receptors in neuroblastoma X-glioma NG 108-15 cell membrane preparations (Mosberg et al. 1983d) using [^{3}H]DADLE. These opioid receptors have been suggested to be a single class of δ receptors. The results given in table 7 clearly show that cur δ-selective analogs are indeed highly potent at these receptors (nmolar range). We have also prepared the delta-selective analogs of Roques and colleagues, [D-Ser^2, Leu^5, Thr^6]enkephalin (DSTLE) (Gacel et al. 1980) and [D-Thr^2, Leu^5, Thr^6]enkephalin (DTTLE) (Zajac et al. 1983), for direct comparison in the two different binding assays. As shown in table 7, these linear compounds have similar binding properties as the cyclic bis-penicillamine analogs. However, as seen in tables 5 and 6, the Thr^6 analogs of Rogues and colleagues are not highly delta selective based on these assays, and similar results have been reported by James and Goldstein (1984) using their assays.

Finally, we have made a number of analogs to test various aspects of structure-function relationships in cur cyclic series using the [D-Pen^2, Cys^5]E as the "standard" compound. First, we examined whether other D-amino acid-containing diastereoisaner might be more potent or selective. As seen in table 8, both the D-Tyr^1 and the D-Phe^4 analogs lose considerable potency in all assay systems

TABLE 8

Biological Activities of the Cyclic Enkephalin Analogs in the GPI, MVP, and Rat Brain Binding Assays[a,b]

Compound[c]	IC_{50} GPI	MVD	[^{3}H]NAL	[^{3}H]DADLE
[D-Pen^2, Cys^5]E	213 ± 63	0.32 ± 0.03	178 ± 15.8	11.7 ± 1.2
[D-Tyr^1, D-Pen^2, Cys^5]E	>10,000	960 ± 140	>10,000	1,000
[D-Pen^2, D-Phe^4, Cys^5]E	40,000	84 ± 24	>10,000	282
[D-Pen^2, Cys^5, Thr^6]E	228 ± 52	0.60 ± 0.60	146 ± 25	5.2 ± 0.70
[D-Pen^2, Leu^5, L-Pen^6]E	30,000	1550 ± 470	>10,000	550
[des-Tyr^1][D-Pen^2, Cys^5]E	N.D.[a]	10,000	>10,000	N.D.
[D-Pen^2, ß-Ala^3, L-Cys^5]E	N.D.	8,000	>10,000	N.D.

[a]Assay results were determined as in tables 2 and 4.
[b]Data from M.S. thesis of R. Burst (1984).
[c]E = enkephalin

and, in addition, their receptor specificities are not changed. Second, Rogues et al. (1982) have suggested that hexapeptide enkephalin analogs may have higher delta receptor selectivity. We first prepared [D-Pen^2, Cys^5, Thr^6]enkephalin and found that though it did retain high potency in delta receptor assays, its δ selectivity was poorer than several other analogs.. We then prepared [D-Pen^2, Leu^5, L-Pen^6]enkephalin, a cyclic 17-membered ring analog which is pseudoisosteric to the previously reported δ-selective analog MTLE. However, this compound was quite inactive (though it did retain some 6 receptor selectivity) and, thus, it appears that the conformational restrictions impose by the disulfides and geminal dimethyl groups do not permit this analog to interact effectively with opioid receptors. Finally, we increased the ring size one CH_2 group (to 15-membered) by pre ring [D-Pen^2, ß-Ala^3, Cys^5]enkephalin, and we deleted the Tyr^1 residue in the cyclic 14-membered ring series. Both of these compounds had little or no activity.

In summary, we have prepared a series of cyclic penicillamine-containing 14-membered ring analogs of methionine enkephalin. All have delta opioid receptor specificity, and some are the most selective delta receptor ligands available to date. Based on our results and the results of James and Goldstein (1984), [D-Pen^2, Pen^5]E and [D-Pen^2, D-Pen^5]E would appear to be the ligands of choice for exploring the physiological and pharmacological roles of delta opioid receptors in the central nervouss system and in peripheral tissue. Indeed, though it is not the subject of this chapter, in collaboration with Prof. Tom Burks, Dr. Frank Porreca, Dr. James Galligan, and Mr. Lane Hirning, we have examined several aspects of this problem (Porreca et al. 1983, 1984; Galligan et al., 1984; Burks et al., in press). Suffice it to say, these studies suggest that highly delta-selective opioid peptides have unique biological properties which make them candidates for therapeutic applications.

CYCLIC MU RECEPTOR-SELECTIVE PEPTIDES

Our success in designing δ-selective peptides prompted us to examine the possibility of preparing μ-selective cyclic peptides. At the time we began this work (early 1983), the most μ-selective peptide agonist analog appeared to be Tye-D-Ala-Gly-(Me)Phe-Gly-ol (DAGO) (Handa et al. 1981), and this still appears to be the case (James and Goldstein 1984). Schiller and coworkers (DiMaio and Schiller 1980; Schiller and DiMaio 1982; DiMaio et al. 1982) have prepared a series of cyclic enkephalin analogs of which H-Tyr-cyclo-[N^{α}-D-A_2bu-Gly-Phe-Leu-] is the paradigm example. These peptides all have μ receptor selectivity with an optimum μ-selectivity value of 38.8 (as measured for H-Phe-cyclo[-N^{ε}-D-Lys-Gly-Phe-Leu]. However, by the criteria of James and Goldstein (1984), this is not a sufficiently μ-selective analog for many studies. Undoubtedly, Schiller and his coworkers will continue to further develop this cyclic series for higher μ selectivity and it is certain such will be learned in the process regarding the conformational requirements for the μ receptor (Schiller 1984).

We have taken another starting point for the development of a μ-selective peptide. Several years ago, it was reported (Terenius 1976; Razek et al. 1978) that sanatostatin can weakly inhibit the binding of [^{3}H]NAL and [3H]DADLE to rat brain homogenates and also can give an analgesic response in mice. More recently, it was reported that the much modified somatostatin analog **D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)** had an IC_{50} of 38 nM at a putative μ receptor, and was capable of antagonizing the excitory effects of a stable enkephalin analog in electrophysiological experiments (Maurer et al. 1982). The compound also was a potent antagonist of insulin release. We became interested in utilizing this lead compound to develop conformationally constrained somatostatin analogs with high potency and high receptor selectivity with regard to glucagon release, growth hormone release, insulin release, or μ opioid receptor interactions. We report here our progress in developing a potent μ receptor-selective conformationally constrained sunatostatin analog (Pelton et al. 1985).

We began our studies by examining two parameters in the structure of Maurer et al. We replaced the carbinol terminal group with a carboxylate, and placed a geminal dimethyl group in the 20-membered ring by substituting either the Cyst or Cys^{11} residue (the numbering schema used in sanatostatin analogs) for a penicillamine residue. The biological activity at mu and delta receptors was assessed by examinating the binding of the analogs to opioid receptors in rat brain membranes in competition with [^{3}H]naloxone ([^{3}H]NAL) and [^{3}H] [D-Ala^2, D-Leu^5]enkephalin ([^{3}H]DADLE), respectively. The results are shown in table 9 along with the data for sanatostatin (control), morphine-HCl (control), and the [Cys^6, Cys^{11}]-analogue **H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-OH,** in which only the carboxylate residue change is made (control). The Pen^6 substitution greatly reduces potency relative to the cyclic somatostatin$_{5-12}$ analog and even relative to sanatostatin at both

TABLE 9

Receptor binding Affinities of Samatostatin Analogs
in Rat Brain Membrane Preparations

Compound[a,c]	IC_{50}(nM)[b]		IC_{50}DADLE / IC_{50}NAL
	[^{3}H]NAL	[^{3}H]DADLE	
Somatostatin	27,400 ± 4,200	16,400 ± 8,500	0.60
Morphine • HCl	23 ± 2.4	27 ± 0.9	1.17
Ac-Phe-D-Trp-Lys-Thr-OH	51,500 ± 4,100	5,800 ± 1,000	0.11
[D-Phe^5, Cys^6, D-Trp^8, Cys^{11}]S_{5-12}	2,600 ± 260	3,100 ± 720	1.21
[D-Phe^5, Pen^6, D-Trp^8, Cys^{11}]S_{5-12}	61,000 ± 17,500	38,100 ± 3,000	0.62
[D-Phe^5, Cys^6, D-Trp^8, Pen^{11}]S_{5-12}	930 ± 72	5,400 ± 800	5.8

[a] S = somatostatin
[b] Assay values are the mean (± s.e.m.) of 5 to 6 determinations, each done in
[c] Pelton et al. 1985

the mu and delta receptor sites. On the other hand, the Pen^{11} cyclic analog [D-Phe^{5}, Cys^{6}, D-Trp^{8}, Pen^{11}]somatostatin$_{5-12}$ shows increased binding potency at the μ receptor and slightly decreased binding potency at the δ receptor and, hence, is slightly μ selective. It is interesting to note that the putative "active site" peptide for sanatostatin, Ac-Phe-D-Trp-Lys-Thr-OH (Ac-D-Trp^{8}-somatostatin$_{7-10}$-OH), is somewhat less potent than sanatostatin at the μ binding site, but somewhat more potent than somatostatin at the delta binding site. Thus, it is quite delta selective, while sanatostatin has very little selectivity for either the delta or the mu binding site.

In an effort to further increase the selectivity and potency of these somatostatin-based cyclic analogs, three different strategies were followed. First, we further conformationally constrained the cyclic 20-membered ring by preparation of the bis-penicillamine analog [D-Phe^{5}, Pen^{6}, D-Trp^{8}, Pen^{11}]-somitostatin$_{5-12}$. Second, we substituted the Phe^{7} residue for a tyrosine residue to give **H-D-Phe-Pen-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH** and **H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-OH.** Third, we replaced the carboxylate terminal group with a carboxamide group. The bis-penicillamine analog was intermediate in binding potency between the Pen^{6} and Pen^{11} analogs at both the μ and δ receptor (table 10), but had little discrimination for the μ or δ receptor. Replacement of the Phe^{7} for a Tyr^{7} residue greatly increased the binding potency of the compound at the μ receptor in both the Pen^{6} and the Pen^{11} series and slightly increased potency at the δ receptor as well. The differential effect was greatest in the Pen^{11} series and **H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-OH** is now quite μ receptor selective.

However, the most dramatic effect came by converting the carboxylate terminal to a carboxamide terminal group. This simple change in the Pen^{11} series led to an analog H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 which was over 90 times more potent in binding at the μ receptor, but only about 5 times more potent in binding at the δ receptor. As a result, this compound now has an EC_{50} of 10 nM at the μ receptor and is 110-fold selective for this receptor versus the δ receptor. Addition of the Tyr^{7} residue in place of the Phe^{7} residue led to an even more selective analog **H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2,** which is our most potent (EC_{50} 3.5 nM) and most selective (270-fold) analogue for the μ receptor system as measured in this assay system. Preliminary studies show that these analogs act as μ receptor antagonists in *in vitro* and *in vivo* experiments.

Careful analysis of the competitive binding experiments against [3H]NAL gave Hill numbers near unity, suggesting that these analogues were interacting with the receptor in a manner similar to that of the labeled antagonist. However, the Hill number obtained from binding experiments with [^{3}H]DADLE were substantially less than one, indicating that the somatostatin octapeptide analogs were interacting with either multiple receptor sites or multiple

TABLE 10

Receptor Binding Affinities of Somatostatin Analogs in Rat Brain Membrane Preparations

Compound[a]	IC_{50} (nM)[b]		$\frac{IC_{50}DADLE}{IC_{50}NAL}$
	[^{3}H]NAL	[^{3}H]DADLE	
Somatostatin[c]	27,400 ± 4,200	$6,400 ± 8,500	0.60
[D-Phe^5,Pen^6,D-Trp^8,Cys^{11}]S$_{5-12}$[c]	61,000 ± 17,500	38,100 ± 3,000	0.62
[D-Phe^5,Pen^6,D-Trp^8,Pen^{11}]S$_{5-12}$[d]	8,300 ± 1,000	11,000 ± 2,000	1.3
[D-Phe^5,Pen^6,Tyr^7,D-Trp^8,Cys^{11}]S$_{5-12}$[c]	470 ± 10	2,600 ± 410	5.5
[D-Phe^5,Cys^6,D-Trp^8,Pen^{11}]S$_{5-12}$[c]	930 ± 72	5,400 ± 800	5.8
[D-Phe^5,Cys^6,Tyr^7,D-Trp^8,Pen^{11}]S$_{5-12}$[c]	290 ± 58	3,800 ± 610	13.
[D-Phe^5,Cys^6,D-Trp^8,Pen^{11}]S$_{5-12}$-NH$_2$[c]	9.9 ± 1.6	1,100 ± 120	110.
[D-Phe^5,Cys^6,Tyr^7,D-Trp^8,Pen^{11}]S$_{5-12}$-NH$_2$[c]	3.5)± 0.20	950 ± 210	270.
[D-ΦGly^5,Pen^6,D-Trp^8,Cys^{11}]S$_{5-12}$[d]	2,500 ± 400	13,500 ± 3,000	5.4

[a]S = somatostatin
[b]Assay values are the mean (± s.e.m.) of 5 to 6 determinations, each done in duplicate.
[c]Pelton et al. 1985
[d]Pelton et al., unpublished results

TABLE 11

Inhibition of [^{3}H]DADLE Binding to Bat Brain Homogenates by Various Analogs Fitted to a Two-Receptor Site Model

Compound[a]	[^{3}H] DADLE			
	$IC^{50}(1)$	$IC_{50}(2)$	$BMax_1$	$BMAX_2$
[D-PHE^5,CYS^6,TYR^7,D-TRP^8,PEN^{11}]S$_{5-12}$-NH$_2$	19	24,000	43	56
[D-PHE^5,CYS^6,D-TRP^8,PEN^{11}]S$_{5-12}$-NH$_2$	7	22,000	43	60
[D-PHE^5,CYS^6,TYR^7,D-TRP^8,PEN^{11}]S$_{5-12}$	260	8,100	29	72
[D-PHE^5,PEN^6,TYR^7,D-TRP^8,CYS^{11}]S$_{5-12}$	640	51,000	51	46

[a]S = Somtostatin

conformational states. The results of a computer analysis of the latter data are given in table 11. The data can be fitted to a two-site model with high- and low-affinity sites and approximately equal nunbers of receptors (Pelton et al. 1985).

In an effort to establish the differential binding characteristics of our analogs to somtostatin as compared to opiate receptors, we have examined the ability of these analogs to inhibit the binding of the biologically stable ^{125}I,labeled somtostatin analogue GCP 23,996, [des-Ala^1, Gly^2] [desamincCys^3, [Tyr^{11}], dicarba^{3,14}]somtostatin. The results (table 12) show that the

TABLE 12

Binding Affinities of Somtostatin Analogs to Somtostatin Receptors in Rat Brain Membrane Preparations

Compound[a]	IC_{50}(nM)[b,c]
Somatostatin	3.3 ± 0.30
[D-Phe^5,Cys^6,D-Trp^8,Pen^{11}]S$_{5-12}$	170. ± 99
[D-Phe^5,Pen^6,D-Trp^8,Cys^{11}]S$_{5-12}$	400. ± 200
[D-Phe^5,Cys^6,Tyr^7,D-Trp^8,Pen^{11}]S$_{5-12}$-NH$_2$	690. ± 220
[D-Phe^5,Pen^6,D-Trp^8,Pen^{11}]S$_{5-12}$	800. ±300
[D-Phe^5,Cys^6,D-Trp^8,Cys^{11}]S$_{5-12}$	980. ± 480
[D-Phe^5,Cys^6,D-Trp^8,Pen^{11}]S$_{5-12}$-NH$_2$	1500. ± 470
[D-Phe^5,Cys^6,Tyr^7,D-Trp^8,Pen^{11}]S$_{5-12}$	1600. ± 460
[D-Phe^5,Pen,Tyr^7,D-Trp^8,Cys^{11}]S$_{5-12}$	4000. ± 780

[a]S = somatostatin
[b]Determined by inhibitor ^{125}I-labeled OGP 23,996.
[c]Pelton et al. 1985

selectivity of the cyclic octapeptide analogs extend to their interaction with the sanatostatin receptor system in the brain. However, the selectivities are much different than seen in the opioid receptor systems. Indeed, our most potent μ receptor-selective analogs, [D-Phe^{5}, Cys^{6}, Tyr^{7}, D-Trp^{8}, Pen^{11}]-somatosting$_{5-12}$-NH_2 and [D-phe^{5}, Cys^{6}, D-Trp^{8}, Pen^{11}]-somatostatin$_{5-12}$-NH_2, are respectively 210 and 450 times less potent than sanatostatin in this somatostatin receptor binding assay, but about 8,000 and 2,700 times more potent, respectively, than sanatostatin in the μ receptor binidng assay. A discussion of the data in the sanatostatin binding assay is outside the scope of this chapter. Suffice it to say, these studies show that the opiate and somatostatin receptor systems, at least in the rat brain, have substantial differences in the structural and conformational requirements of ligands for strong receptor interactions.

The structural and conformational features which lead to the high and specific μ opioid receptor binding for the much modified somatostatin analogs reported here are not clear as yet. The relatively small effect of replacing Phe^{7} with Tyr^{7} in cur analogs suggests that the increases seen may not be directly related to the well-known requirement for a phenolic hydroxyl moiety in the opiates and enkephalins. The much larger effect of substituting the carboxylate terminal for a carboxamide terminal might suggest that the increased potencies and selectivities are a result of a general increase in lipophilicity. However, octanol-water partition coefficients of the sanatostatin analogs do not correlate well with receptor affinity or selectivity. Hence, other features such as conformational changes or specific peptide-receptor inter actions may be involved. Work is in progress in cur laboratory to examine these questions.

C0NCLUSIONS

We have utilized principles of conformational restriction in conjunction with careful examination of structure-biological activity relationships to develop a series of highly receptor-selective, potent cyclic peptide analogs of enkephalin and somatostatin which have exceptionally high delta and mu receptor selective respectively. Two of these compounds [D-Pen^{2}, D-Pen^{5}]enkephalin and [D-Phe^{5}, Cys^{6}, Tyr^{7}, D-Trp^{8}, Pen^{11}]somatostatin$_{5-12}$-NH_2 appear to be, respectively, the most receptor-specific delta receptor agonist and mu receptor antagonist reported thus far. These compounds appear to have sufficient receptor selectivity, so that they should be excellent tools for examining the physiological roles of δ and μ receptors in peripheral and central nervous system tissue. Indeed, considerable progress in this direction has already appeared in the case of the delta-selective agonist. In addition, since these compounds are cyclic, conformationally constrained peptides, it may be possible to establish their preferred solution conformations and from this develop a model for conformation-biological activity relationship of enkephalins and sanatostatin at δ and μ opioid receptors, respectively. Conformationally constrained peptides provide a more valid approach to

utilizing solution conformations for assessing the conformational and topological properties of a peptide ligand which may be important to biological activity, since in such constrained molecules the overall conformational properties found in solution are more likely to have relevance to the peptide-receptor complex. Thus, insight into conformation-biological activity relationships are more readily obtained using such analogs. Such insight should lead to a rational design of more potent, more receptor-selective, more biologically stable, etc., peptide analogs. There is still much to be done utilizing this approach, and it is expected that many important new insights regarding the physical-chemical basis for the biological activity of peptide hormones and neurotransmitters will follow fran these studies.

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Dehydro and Cyclopropyl Amino Acids and Peptides

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In the recent past, there have been several excellent reviews covering the synthesis of dehydro amino acids and peptides (Stammer 1982; Schmidt et al. 1979; Noda et al. 1983). This review will cover only the most recent literature on this subject and is not intended to be exhaustive. The work on cyclopropyl amino acids is quite new, coming mostly from the author's laboratory, and the literature can be covered completely.

DEHYDRO AMINO ACIDS

Some of the latest work on dehydro amino acid synthesis will be summarized here. α-Phosphoryl glycine esters have recently been synthesized in two different laboratories (Schmidt et al. 1982; Kober and Steglich 1983). One procedure used an α-methoxy glycine derivative, previously prepared in Israel (Zoller and Ben-Ishai 1975), in which the α-methoxyl function is replaced with the diethoxyphosphoryl group. The other process replaces an α-bromine atom with the diethoxyphosphoryl group to give the same intermediate. The N-acyl α-phosphoryl glycine exter was then used in a Wittig condensation with aryl or aliphatic aldehydes to give, in one step, the dehydro amino acid derivative. Both of these methods appear to be imminently useful. A new kind of specific synthesis of dehydro tryptophan has also appeared (Moriya et al. 1982). A readily prepared pyrrolidino enamine of 3-indole aldehyde was treated with N-fornyl glycine ester to give the dehydro tryptophan derivative which is easily N-deblocked with ethanolic HCl. In 1983, a stereospecific synthesis of dehydro amino acids was described (Somekh and Shanzer 1983) which proceeded through the dehydration of ß-hydroxy amino acids synthesized by a previously reported method

(Shanzer et al. 1979). The dehydration was accomplished using (diethylamino)sulfur trifluoride (DAST) in pyridine.

DEHYDRO PEPTIDES

Only a few new ways to prepare dehydro peptides have been reported recently. A series of communications describing the use of dehydro N-carboxy anhydrides (NCA'S) has appeared (Shin et al. 1981). The synthesis of the NCA directly from an N-Cbz dehydro amino acid using thionyl chloride proceeds normally to give the desired product. This compound undergoes alcoholysis to give N-free dehydro esters or can be condensed with amino acid esters or peptide esters to give N-terminal dehydro peptides.

Another novel approach to the synthesis of dehydro peptides incorporates 2-aziridine carboxylic acids (Azy) into a peptide chain followed by base-catalyzed rearrangement of the aziridine ring to give a dehydro amino acid moiety (Nakajima et al. 1982). The method has been used to make several actinomycin analogs.

Some years ago in the author's laborator, the first dehydro peptide hormone, D-Ala^{2}Δ^{Z}Phe^{4},Met^{5}-enkephalin amide, was synthesized and shown to be bioactive and stable to enzymatic attack (English and Stammer 1978). The *in vivo* activity of this dehydro Phe^{4}-enkephalin was confirmed later (Chipkin et al. 1979), and various other dehydro Phe^{4}-enkephaline were prepared and their binding affinity to the various opiate receptors were compared (Shimohigashi et al. 1982b). The dehydro Phe moiety in these compounds was prepared by two methods, i.e., dehydrogenation of an azlactone with dicyanodichlorobenoquinone (DDQ) (Konno and Stammer 1978; Shimohigashi et al. 1983b) and by the more convenient dehydration of an azlactone formed from β-phenylserine. In all cases, the presence of a dehydro amino acid was confirmed by double beam ultraviolet measurements in which the peak(s) characteristic of the dehydro moiety was isolated (Shimohigashi et al. 1981b). The dehydro Ala^{2}-, Ala^{3}-, and Leu^{5}-enkephalins were all synthesized and tested for binding and *in vivo* activity (Shimohigashiet al. 1981a; Shimohigashi Stammer 1982a). In general, these compounds were of somewhat reduced biological activity, but showed a preference for the delta receptor over the mu. The dehydro Ala^{3}, Leu^{5}-enkephalin was particularly skewed in this direction even though its activity was below that of the naturalcompound (Shimohigashi and Stammer 1982d).

Most impressive was the dehydro Leu^5-enkephalin in this respect, since it was almost four times more active than its D-Leu^5 analog in the radioligand binding assays and showed good delta selectivity. More interestingly, it was very resistant to hydrolysis by carboxypeptidasey, showing that the presence of the dehydro moiety again caused the peptide to be incompatible with the enzyme active site (Shimohigashi and Stammer 1983). An attempt to prepare Tyr^1-enkephalin was successful up to the last step when the N-Boc group was removed and gel chromatography in aqueous acetic acid was attempted (Shimohigashi et al. 1983a). Hydrolysis of the N-terminal enamine function was apparently responsible for the loss of ninhydrin positive material. Difference spectroscopy showed the presence of the dehydro tyrosine residue in the N-blocked peptide with a strong peak just above 300 nM. Later, quantitative amino acid analysis was used to measure the stability, relative to the saturated compound, of all the dehydro enkephalins toward carboxypeptidase. They showed very high stability to this enzyme (Shimohigashi et al. 1982a).

The configurations of dehydro peptide moieties have been determined in various ways (Stammer 1982; Schmidt et al. 1979 ; Noda et al. 1983). X-ray crystallography has been used to prove the configuration of dehydro Phe and dehydro Leu (Chauhan et al. 1979), but nuclear magnetic resolution (NMR) methods have been used to infer the configurations of the other unsaturated amino acids. When a derivative of dehydro E-Phe became available (Nitz et al. 1981), nuclear Overhauser effect measurements were carried out in which the N-H and the vinyl protons were irradiated using a Nicolet NMC-500 NMR spectrometer (Shimohigashi et al. 1982c). Only the E-form showed any effect at all.

Some recent work has been done to determine the effect of a dehydro amino acid moiety on peptide conformation. An NMR study of a di- and tri-peptide containing a dehydro Phe residue showed that this residue can occur in the i+2 position of a ß- or γ-turn without greatly affecting the stability of the turn (Bach et al. 1983).

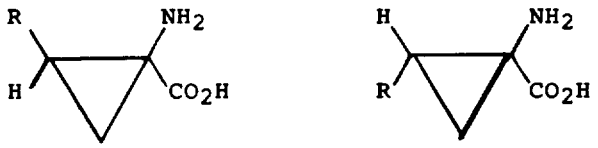
More recently, dehydro E-Phe^4-enkephalin has been synthesized in the author's laboratory. Compared with the Z-isomer, the E-compound showed a drastically reduced potency in the radioligand binding assays, i.e., 260- and 150-fold loss of affinity toward the γ and µ receptors, respectively (Shimohigashi

et al. 1984). It is interesting to note that the recently reported crystal structure of Leu^5-enkephalin (Camerman et al. 1983) showed the phenylalanine side chain oriented toward the amino end of the sequence, just as it appears in the Z-isomer of the dehydro Phe residue.

A dehydro cyclic hexapeptide analog of somatostatin containing a dehydro Z-Phe residue in the 2-position has recently been prepared. The NMR spectra of the saturated and unsaturated cyclic compounds were very similar, even though the dehydro peptide had only one-tenth the biological activity of the saturated material (Brady et al. 19841. The dehydro E-Phe isomer may have the preferred configuration in this case.

CYCLOPROPYL AMINO ACIDS

The synthesis and incorporation of cyclopropyl amino acids into various peptides is a new area. These amino acids have a cyclopropane ring superimposed upon the α,β bond of the amino acid moiety just as the dehydro amino acids have a double bond in this position. Similarly, the information carrying ß-group of the cyclopropyl amino acid is fixed in space, just as it is in the dehydro compounds (figure 1). These two kinds of residues differ,



∇'Z Amino Acid ∇E Amino Acid

FIGURE 1

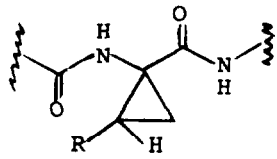
however, in that dehydro amino acid residues are achiral while cyclopropyl moiety has two chiral centers at the α - and ß-carbon atoms. This, of course, means that all cyclopropyl amino acids (except Ala, Val, and Pro) exist as four stereoisomers, i.e., two diastereoisomeric pairs of racemates. Thus, in order to determine the effect of such a residue on the bioactivity of a peptide, four peptides must be prepared and tested. It is clear, also, that these rigid amino acids will have profound effects on the overall conformation of a peptide, especially in the space proximal to the rigid moiety. It seems quite probable that one of the four stereo-

isomers may cause the peptide to approximate a shape which is most compatible with the enzyme or receptor site normally occupied by the natural peptide in question. Thus, at least one of the four possible cyclopropyl peptides should be bioactive and, most important, stable to indiscriminate enzymatic cleavage. Very stable peptide hormones and/or enzyme inhibitors should then result.

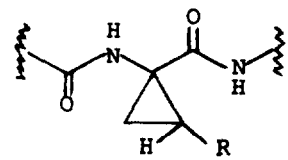
We have recently published the synthesis of both racemic cyclopropyl Phe isomers and have now resolved these racemates and incorporated them into D-Ala^2, Leu^5-enkephalin (King et al. 1982; Ximura et al. 1983). The amino acid synthesis requires the preparation of known azlactones followed by cyclopropanation with diazomethane. The deblocking of this material to give the free amino acids follows relatively easily depending upon which amino acid is being prepared. Cyclopropyl Phe requires relatively mild deblocking conditions due to the ease with which the cyclopropane ring is opened in hot acid. To overcome this problem, another N-blocking group has been developed (Suzuki et al. 1983b) in which an incipient phthaloyl group is incorporated into the azlactone molecule. A completely different and more general approach was later used in the synthesis of coronamic acid (Suzuki et al. 1983a). In this case, a substituted diasomethane is added to a dehydro alanine derivative followed by pyrolysis or photolysis of the resulting pyrazoline. A halo substituted cyclopropyl amino acid has also been prepared by the azlactone method, but this is not an analog of a protein amino acid (Bland and Stammer 1984).

CYCLOPROPYL PEPTIDES

Incorporation of a cyclopropyl amino acid residue into a peptide chain will strongly affect the conformation of the peptide. Examination of extended conformations (figure 2) shows that the cyclopropyl Z-residues will be most restrictive ($+90^\circ > \phi < -90^\circ$)



Z-peptide



E-peptide

FIGURE 2

of the ϕ (N-C_α) angle while the E-residues restrict the ψ (C_α-C') angle to small values. These effects will most probably promote the formation ß- and γ-turns in that area.

A preliminary account of the synthesis of the first cyclopropyl peptide hormone has been published by Kimura et al. (1983). This cyclopropyl enkephalin incorporated cyclopropyl E-Phe in the 4-position. As in the case of the dehydro Phe^4 enkephalin, the E-configuration of this rigid phenylalanine residue was extremely deleterious to the binding affinity of the peptide, with the more active isomer being less active than its counterpart by a factor of 3000. Most recently, both cyclopropyl Z-Phe^4-enkephalins have been prepared. These compounds are more active by a factor of about 8000 than the E-isomers, and they bind to rat brain receptors with about the same affinity as the natural compounds do. All of these compounds are stable in vitro to enzymic attack and have not yet been assayed in the muscle assays or in vitro. The synthesis of cyclopropyl Tyr is under way and we hope soon to see the effect of a rigid tyrosine residue on the bioactivity of enkephalins.

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Affinity Labels as Probes for Opioid Receptor Types and Subtypes

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Progress in most areas of pharmacology and medicinal chemistry depends upon the availability of highly selective molecular probes. The opioid field is a case in point. With the burgeoning evidence that the actions of opioids are mediated through multiple receptor types and subtypes (Martin 1983). it has become apparent that more selective ligands are required as tools. Thus, the narcotic antagonist naloxone, which proved to be extremely useful in determining opioid receptor involvement, is now considered to be inadequate for sorting out actions mediated by different receptor types. This presentation reviews our approach to addressing this problem through the design of highly selective, nonequilibrium molecular probes for opioid receptors. Because such ligands become bound covalently to opioid receptors, they are preferred where firm attachment of a ligand is desirable. In this connection, they are finding utility in receptor isolation, in mapping receptor distribution, in receptor binding paradigms, and in determining the relative involvement of different opioid receptor types in pharmacologic studies.

DESIGN RATIONALE

The original impetus for pursuing affinity labels for opioid receptors stemmed from conclusions (Portoghese 1965, 1966) drawn from our stereochemical studies on opioid ligands. In this connection, the divergent stereochemical requirements. Convergent pharmacologica studies (Martin et al. 1976; Ward and Takemori 1976; Lord et al. 1977) subsequently complemented and provided a detailed framework for this concept. During that period, we had begun to investigate electrophilic affinity labels as an alternative to stereochemical probes to explore opioid receptors (Portoghese et al. 1971, 1977; Takemori et al. 1974).

The rationale for our approach was based upon studies of Baker (1967) who pioneered the area of active-site, irreversible enzyme inhibitors. In theory, when the affinity label contains an electrophilic center, high selectivity for opioid receptors should be dependent on four parameters. These are: a) affinity of the receptor for the ligand; b) equilibrium-controlled receptor selectivity; c) location of electrophilic center in the ligand, and d) reactivity and chemical selectivity of the electrophile.

In considering these parameters, it is evident that an electrophilic affinity label may be involved in two consecutive recognition processes which lead to covalent attachment to the receptor (figure I). The first recognition step is manifested by

receptor affinity and the second recognition step involves proper alignment of the electrophilic center of the ligand with a compatible receptor-based nucleophile.

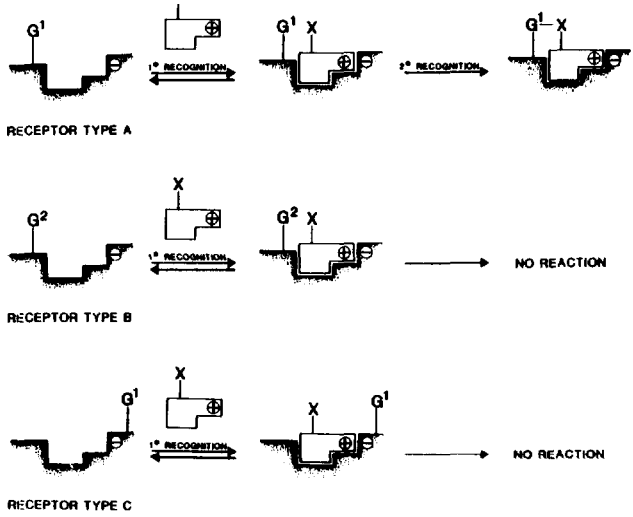


FIGURE 1

A schematic illustration of the principle of recognition amplification in the covalent binding of receptor type A by affinity label containing a group-selective electrophile X. Note that receptor types A-C have similar topographic features which lead to reversible binding (1° recognition). However, the receptor types differ with respect to the reactivity (G_1 vs G_1 in A and B) and location (G_1 in A and C) of nucleophiles. Only in A is the nucleophile G_1, reactive with respect to X and within covalent binding distance (2' recognition).

Because two recognition steps rather than one lead to covalent binding, enhanced receptor selectivity (recognition amplification) is possible, particularly with chemically selective electrophiles. On the other hand, if an affinity label contains a highly reactive electrophilic group, such recognition amplification is minimal and covalent binding selectivity is determined primarily by the first recognition step. For example, since the promiscuity of the aziridinium ion enables it to alkylate almost any nucleophile within covalent bonding distance. the selectivity of a ligand which contains the active electrophilic group will be determined primarily by its relative affinity for different receptor populations.

The concept of recognition amplification may be utilized for the design of affinity labels that are highly selective for a single opioid receptor type among multiple types if each receptor type contains a unique array of nucleophiles that differs with respect to nucleophilicity and accessibility. This is illustrated schematically in figure 1 where three receptor types with similar topographic features (A, B, C) all are capable of associating reversibly with the affinity label. However, the second recognition step takes place only with receptor type A because the other receptor types contain nucleophiles that either are insufficiently reactive (type B) or are not within covalent bonding distance (type C) of the electrophile (X).

EXPLORING THE FIRST RECOGNITION STEP

An important consideration in the design of opioid receptor affinity labels is the nature of a ligand which will be modified with an electrophilic moiety. Since our prior efforts and those of others with the modification of agonist ligands did not yield compounds that have had wide utility, we turned our attention to the antagonist pharmacophore as the recognition unit. A consideration in this choice was based on the fact that antagonists, especially those without agonist activity, have been extremely useful in pharmacologic investigations and in characterizing opioid receptor involvement.

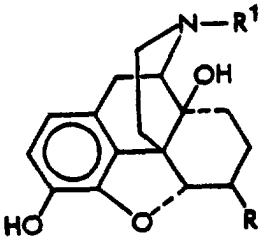
We therefore selected naltrexone 1 (table 1) for modification, as this pure antagonist possesses high affinity for opioid receptors and contains convenient functionality at the C-6 position for molecular modification. Moreover, C-6 was chosen as a point of attachment for the electophilic group because it was reported previously (Hahn et al. 1975) that a variety of C-6 substituents did not destroy receptor affinity (first recognition step). In this connection, the 6-amino derivative of naltrexone, beta-naltrexamine 2, possessed an affinity equal to that of naltrexone (Jiang et al. 1977). This was relevant to our approach in that 6 beta-amino group was employed as a handle to attach various electrophilic moieties. Our preference for the 6 beta-isomer rather than the 6 alpha-isomer was based on the greater affinity of the former in the binding assay.

THE SECOND RECOGNITION STEP: RANGE-FINDING STUDIES

After it was established that the first recognition step was unimpaired by the presence of a 6 beta-amino group, our design strategy involved the introduction of a highly reactive electrophilic moiety (i.e., aziridinium ion) to assess the availability of a proximal receptor-based nucleophile. In this case, it was expected that the selectivity for different receptor types would be conferred largely by the relative affinity (first recognition step) of the ligand at these sites. Once nonequilibrium blockage was demonstrated, then less reactive and more selective electrophilic groups were introduced in an effort to "fine-tune" the ligand to allow a second recognition step to occur at one receptor type in a population of multiple opioid receptors.

This rationale led first to the development of a nitrogen mustard derivative of naltrexone, beta-chlornaltrexamine 3 (beta-CNA) (table 1) (Portoghese et al. 1978). Beta-CNA was found to be highly selective for opioid receptors and it inhibited irreversibly the specific binding of opioids to brain membranes (Portoghese et al. 1979). In both the guinea pig ileal (GPI) longitudinal muscle and mouse vas deferens (MVD) preparations, beta-CNA produced irreversible antagonism to opioids (Ward et al. 1982a; Caruso et al. 1979). This antagonism, which was characterized by a shift of the concentration-response curve to the right, followed by a decline in the maximum effect, is typical of classical nonequilibrium antagonism involving spare receptors. The antagonism could be prevented but not reversed by naloxone. Since nonopioid nitrogen mustards had no selectivity for opioid sites, the data suggested that beta-CNA exerts its sustained antagonism by alkylating opioid receptor nucleophiles. The selectivity of its action was demonstrated by the fact that beta-CNA failed to block the effect of norepinephrine in the GPI or block other binding sites (cholinergic, prostaglandin, benzodiazepine) (Fantozzi et al. 1981). As beta-CNA was found to be highly reactive toward nucleophilic reagents, it presumably reacts with receptor nucleophiles via its aziridinium ion 4 (table 1).

Table 1
Naltrexone and Related Compounds



R^1 = $CH_2CH(CH_2)_2$ unless otherwise indicated

R

1 = 0 (naltrexone)

2 NH_2

3 $N(CH_2CH_2Cl)_2$(ß-CNA)

4 $N(CH_2CH_2)CH_2CH_2Cl$

5 $N(CH_2CH_2Cl)_2$,R^1= CH_3 (ß-COA)

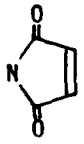
6 $NHCOCH{=}CHCOOMe$ (ß-FNA)

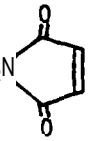
7 $NHCOCH{=}CHCOOMe$, R^1-CH_3 (ß-FOA)

8 $NHCOCH{=}CHCOMe$

9 $NHCOCCl{=}CH_2$

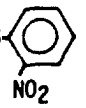
10 $NHCOCH{-}CHCOOMe$

11 

12 $NHCOCH_2N$ 

13 N-C-S

14 $NHCOCH_2I$

15 $NHCOCH_2SS$ 

16 $NHCOCH_2HgCl$

17 $NHCOCOC_6H_5$

18 $NHCOCH_2CH_2COOMe$

The ease with which beta-CNA blocked different opioid receptor types was mu > kappa > delta, which paralleled its reversible binding affinity for these sites (Sayre et al. 1983c; Goldstein and James 1984). This is in harmony with the idea that little or no distinction between receptor-based nucleophiles occurs with a highly reactive and promiscuous electrophile such as aziridinium ion.

The pharmacologic profile of beta-CNA in mice also was consistent with the alkylation of opioid receptors, in that it produced ultralong antagonism (> 3 days) of morphine-induced analgesia after a single (i.c.v.) injection (Caruso et al. 1980b). By comparison, naltrexone antagonism lasted less than 2 hours. It was determined that the opioid binding capacity of brain membranes of beta-CNA-treated mice was significantly decreased during this 3-day period, which suggested covalent binding had occurred in vivo. When administered intrathecally (i.t.), beta-CNA produced even longer antagonism (> than 13 days) against it. morphine antinociception (Larson and Armstrong 1980).

In view of the potent nonequilibrium opioid antagonism displayed by beta-CNA, we investigated the consequence of attaching a nitrogen mustard group to the same position in oxymorphone. a potent agonist. This derivative, designated as beta-chloroxymorphamine 5 (beta-COA) (table 1) (Caruso et al. 1979). was of interest as a tool to test the generality of the rate theory of drug action proposed by Paton (1961). According to this hypothesis, a characteristic feature of antagonists is their slow dissociation from receptors when compared to agonists. Beta-COA behaved as an irreversible agonist in the GPI. The fact that naloxone protected against irreversible agonism, but could not reverse it, implicated the involvement of opioid receptors. These results suggested that, at least in the GPI, ligand receptor occupation rather than rate of dissociation is important for agonist activity. Beta-COA administered i.c.v. in mice produced analgesia that lasted 4 times longer than oxymorphone (Caruso et al. 1980a). The brains of beta-COA-treated animals exhibited a decreased capacity to bind opiates, suggesting covalent binding had occurred. Interestingly, beta-COA given i.c.v. displayed a long-lasting antagonism (6 days) after the agonism had disappeared. The antagonism was more pronounced (> 21 days) when beta-COA was administered i.t. (Larson and Armstrong 1980).

BETA-CNA AS A TOOL

The irreversible nature of beta-CNA has made it useful as a research tool. [$_3$H]-beta-CNA has been employed to isolate opioid receptor components from brain membranes (Caruso et al. 1980b). The elution profile suggested four covalently bound fraction, two of which migrated as single-peak calibrated to be 590,000 daltons. These complexes may represent multiple forms of opioid receptors.

Since beta-CNA alkylates all opioid receptor types, it has been used in protection studies. For example, in the MVD it has been used in the presence of a protecting concentration of a delta agonist to irreversible block mu and kappa receptors (Ward et al. 1982b). Such a preparation contains the highly enriched population of delta receptors. Other protecting ligands have been employed with beta-CNA in the smooth muscle preparations (Chavkin et al. 1982; Chavkin and Goldstein 1981). Using the protection procedure, beta-CNA has been used to enrich specific binding sites in brain membranes (Goldstein and James 1984; James and Goldstein 1984). Beta-CNA has been employed to progressively block dynorphin receptors in the GPI and MVD. It was concluded that the GPI has more spare kappa receptors than the MVD (Cox and Chavkin 1983). An effort

to estimate the dissociation constant of the normorphine-receptor complex in the GPI using beta-CNA has been reported (Porreca and Burks 1983).

Beta-CNA also has been employed as a pharmacologic tool in vivo. These include studies on the opioid nature of delta-tetrahydrocannabinol (Tulunay et al. 1981) in rats and behavioral studies in mice (Quack and Lucas 1981), rats (Messing et al. 1982), and chicks (Panksepp et al. 1982). The inhibition of tolerance development to, and withdrawal from, morphine after i.t. administration of beta-CNA also was investigated (DeLander and Takemori 1983).

THE SECOND RECOGNITION STEP: FINE TUNING

Since the pharmacologic characterization and binding studies of beta-CNA and beta-COA suggested that these ligands selectively and irreversibly bind to opioid receptors, it seemed likely that neighboring receptor-based nucleophiles were involved in covalent bond formation with the aziridinium ion that was generated. The reactivity and promiscuity of this electrophile minimize the importance of the second recognition step. Consequently, the facility with which beta-CNA alkylates different opioid receptor types is determined by the first recognition step (affinity of reversibly bound ligand for the receptor type). Thus, based on the assumption that different opioid receptor types contain different arrays of nucleophiles, our strategy for enhancing selectivity of the second recognition step was to employ electrophilic groups with a reactivity that is limited to only one or two different nucleophiles. In this regard, Michael acceptors appeared to fulfill this requirement because they react readily with sulfhydryl and sluggishly or not at all with other nucleophiles. Another reason for selecting a Michael acceptor groups was that studies with N-ethylmaleimide implicated the presence of a receptor-based sulfhydryl (Simon et al. 1973).

BETA-FUNALTREXAMINE (BETA-FNA)

The fumarate methyl ester group was one of several Michael acceptor moieties that were attached to the C-6 position of naltrexone (Portoghese et al. 1980). This compound, which has been named beta-funaltrexamine [6] (beta-FNA), was the most selective ligand in the series (table 1).

In the GPI, beta-FNA is a reversible kappa agonist and irreversible blocks mu receptors in a concentration- and time-dependent manner (Takemori et al. 1981). The lack of inhibition of kappa agonists (e.g., ethylketazocine) revealed that this antagonism was selective for mu receptors. It is noteworthy that beta-FNA did not antagonize its own agonist effect (mediated through kappa site). The inhibition of morphine antagonism was manifested as a parallel shift of the morphine concentration-response curve with no diminution in the maximal response. Using naloxone as an antagonist, PA^2 analysis revealed that beta-FNA depleted the GPI of functional mu receptors and that the residual opioid receptor population was of the kappa type (Ward et al. 1982b). Thus, the presence of a maximal response to morphine after mu receptor blockage was due to the fact that morphine can act as a full agonist at kappa receptors. This pharmacologic profile was in contrast to beta-CNA, which produced both a rightward shift and a decline in the concentration-response relationship of morphine as a consequence of blockage of both mu and kappa receptors (Caruso et al. 1979). In the MVD, beta-FNA displayed all of the reversible kappa agonist and irreversible mu antagonist features it exhibited in the GPI, and it did not significantly inhibit delta agonists at concentrations employed for the blockage of

mu receptors (Ward et al. 1982a). The binding characteristics of beta-FNA binding appeared to be consistent with its profile in the GPI and MVD in that the irreversible portion of beta-FNA binding demonstrated selectivity for mu over delta binding sites, while the reversible component of beta-FNA binding exhibited selectivity for kappa over mu or delta binding sites (Ward et al., in press).

In mice, beta-FNA exhibited a pharmacologic profile similar to that observed on smooth muscle preparations. It produced an antinociceptive effect of short duration which appeared to be mediated by kappa receptors. In contrast. the antagonist action was of remarkably long duration (> 4 days) (Ward et al. 1982c).

BETA-FNA AS A TOOL

Because of the high mu selectivity of beta-FNA, it has many applications as a pharmacologic tool and opioid receptor probe. One of the initial uses was in depleting the GPI of functional mu receptors, thereby affording a more homogeneous population of kappa receptors (Ward et al. 1982b). This preparation was useful in evaluating the kappa activity of so-called mu agonists. It has been found that even mu agonists that were presumed to be highly selective exhibited kappa activity at high concentrations (Takemori and Portoghese, unpublished data). Since the high selectivity of beta-FNA obviates the necessity of protection experiments involving mu receptors, the beta-FNA-treated GPI has been employed to characterize dynorphin as a kappa agonist (Huidobro-Toro et al. 1982) and to demonstrate the presence of cross-tolerance to opioids in the GPI (Seidl and Schulz 1983).

A number of studies in vivo have made use of beta-FNA to study the relative involvement of mu receptors in various opioid actions. These include opioid-induced respiratory depression (Ward and Takemori 1983a). decrease in gastrointestinal transit (Ward and Takemori 1983b). cardiovascular effects (Holaday and Ward 1982; Pfieffer et al., in press), spinal analgesia (Hylden and Wilcox 1983a. 1983b), antidiuresis (Zimmerman et al. 1984) and prolactin release (Holaday et al. 1983). Beta-FNA also has been used to demonstrate the non-involvement of mu receptors in certain opioid actions such as endotoxic shock (Holaday et al. 1983) and post-ictal analgesia (Belenky et al. 1983). The mu receptor involvement in analgesia (Hynes et al. 1384; Dystra 1984; Frederickson et al. 1984; Leander et al. 1984; Schafer et al. 1984) produced by kappa and delta agonists and the phenomena of tolerance and physical dependence (Aceto et al. 1984; DeLander et al. 1984; Gmerek and Woods, in press) also have been investigated using beta-FNA.

An interesting application of the high mu selectivity of beta-FNA stemmed from the finding that its N-methyl analogue 7 (table 1) derived from oxymorphone, beta-FOA (Portoghese et al. 1980; Takemori et al. 1981). is reversibly acting mu agonist in the GPI. No irreversible blockage of opioid receptors was observed. The possibility that mu agonists and antagonists interact with separate recognition sites was studied by evaluating a series of opioid agonists and antagonists for their ability to protect against the irreversible mu antagonism by beta-FNA (Portoghese and Takemori 1983). Antagonists afforded excellent protection against irreversible blockage by beta-FNA, whereas most agonists were relatively poor protectors. Moreover, the ability of the compounds to protect against beta-FNA appeared to correlate with their antagonist potencies (K_e) but not their agonist potencies (IC_{50}). These results suggested that mu agonists and antagonists interact at separate sites on the mu receptor system. It was proposed that the

antagonist recognition site is a regulatory site which controls the agonist effect mediated by mu receptors.

STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

A number of Michael acceptor analogues of beta-FNA were synthesized in order to delineate the steric requirements of covalent bond formation with opioid receptors (table I) (Sayre et al. 1983a. 1984). In the beta-series, it was found that the ketone 8 and acrylamide 9 exhibited irreversible blocking activity against morphine but not ethylketazocine. This antagonism was in the range of that displayed by beta-FNA. It is noteworthy that dihydro-beta-FNA 18 is a reversibly acting compound, as would be expected for an unreactive ligand. The fact that the Michael acceptors with cis double bonds (10, 11) were not active as irreversible antagonists suggests that steric factors do play an important role in the second recognition step. This is supported by kinetic studies which show that the reactivities of the Michael acceptors toward cysteine do not parallel the rank order of irreversible activities. Thus, it is plausible that proper alignment of the electrophile with a receptor-based nucleophile is critical for covalent binding with mu receptors in the GPI preparation. In support of this idea it was reported that, even at high concentrations, alpha-FNA was incapable of irreversible blockage of opioid receptors (Sayre et al. 1983b). The fact that irreversible antagonism of beta-FNA could be blocked by protection with alpha-FNA established that the electrophilic moiety is not properly oriented in the alpha-series. Significantly, the C-6 stereospecificity observed for beta-FNA was not found among the CNA C-6 epimers. as both beta-CNA and alpha-CNA (Sayre et al. 1983c) are irreversibly acting ligands. This indicates that the second recognition step is important only with relatively selective electrophilic groups.

It was noted that the placement of a spacer between the opiate and the Michael acceptor group dramatically changed the pharmacologic profile. While the maleimide 11 was devoid of irreversible antagonist activity, its N-acetyl analogue 12 selectively blocked my receptors in the MVD without a significant effect on the GPI preparation (Sayre et al. 1984). The rationale for the synthesis of 12 was based on the assumption that different receptor subpopulations contain a different array of nucleophiles. Thus, by altering the distance of the electrophile from the pharmacophore, opioid receptor selectivity can be altered to distinguish opioid receptor subtypes.

Electrophiles other than Michael acceptors exhibited varying degrees of selectivity in irreversibly blocking the effects of different agonists (Sayre et al. 1983c, 1984). Most notable was the isothiocyanate 13 which appeared to block mu receptors in the GPI and mu and delta receptors in the MVD. Interestingly, the alpha-isomer of 13 did not irreversibly block mu receptors in the GPI. Thus, the stereoselectivity is similar to that of beta-FNA and other active Michael acceptors in this preparation. The iodoacetamide 14 showed considerably less cross-reactivity with different receptor types by selectively blocking the mu receptors in the MVD. The pharmacologic profiles of 12 and 14 indicate that the mu receptors in the MVD are different from those in the GPI.

Other compounds in this series (15 to 17) are electrophilic but produced no significant blockage of morphine, ethylketazocine. or [D-Ala^2-D-Leu^6]enkephelin (DADLE) in the smooth muscle preparations. The reason for the inability of these compounds to act as irreversible antagonists is not well understood at this time. However, it may be related to a deficient secondary recognition step.

CONCLUSIONS

The extraordinary selectivity of beta-FNA for mu opioid receptors can be viewed as arising from two sequential recognition steps leading to covalent bond formation. While reversible binding to the mu receptor system is important, the proper alignment of the electrophiles with a compatible receptor-based nucleophile is critical for the selectivity of irreversible blockage. In this connection, the fumarate moiety appears to possess a narrow spectrum of reactivity and a geometry which permits facile access to the receptor-based nucleophile. Refinement of this approach has led to development of subtype-selective blockers of mu receptors. The enhanced selectivity of these probes is proving to be very useful in the pharmacologic and biochemical characterization of opioid receptors.

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Peptide Conformation and Biological Activity: A General Review With Examples of Approaches Relevant to Opioid Peptide Function

Dan W. Urry, Ph.D.

INTRODUCTION

In this International Conference on the Medicinal Chemistry and Molecular Pharmacology of the Opioid Peptides and the Opiates, it is a challenge to respond to the invitation to give a review of a general nature with examples of both methodological approaches and conformational analyses of peptides, and to do so in a manner that could have some relevance to the theme of the conference. This is particularly so as it has been a number of years since, working with my colleague M.A. Khaled, we were directly concerned with the solution conformation of Met- and Leu-enkephalins (Khaled et al. 1977, 1979). Nonetheless, it is satisfying that the fundamental perspective of enkephalin conformational flexibility is retained and that the two conformations considered in that work, a β-chain conformation capable of antiparallel association and a monomeric β-turn conformation, continue to be relevant to current considerations in solution and in the crystalline states (Schiller 1984; Karle et al. 1983a). It is the conformational flexibility of the enkephalins and the opioid peptides in general, coupled to the objective of determining a receptor bound state, that makes the conformation-function studies of opioid peptides so challenging.

An attractive approach is to impose structural restrictions on conformational freedom which would allow probing of receptors and which could lead to more selective binding at one of the several receptors now recognized for the opioid peptides (Atweh and Kuhar 1983; Paterson et al. 1983; Barnard and Demoliou-Mason 1983). Accordingly, amino isobutyric acid (Aib) and D•Ala analogs have been synthesized with interesting results (Gorin et al. 1978; Sudha and Balaram 1983; Beddell et al. 1977). Enkephalins with restricted side chains have been prepared and studied with delineation of different receptor activities and with increased potency at particular receptors (English and

Stammer 1978; Shimohigashi and Stammer 1982a, 1982b; Shimohigashi et al. 1982; Deeks et al. 1983). Additionally, cyclic enkephalin analogs have been synthesized and their activities determined. sometimes with strikina increase in activity in one assay and decrease in activity in another (DiMaio and Schiller 1980; Schiller et al. 1981). It has been concluded that "Activity profiles obtained with a cyclic enkephalin analog and its linear correlate revealed that μ and δ-receptors differ in their conformational requirements" (Schiller 1984). Such approaches, coupled with graphic volume analyses such as those of Marshall and colleagues (Gorin and Marshall 1977; Gorin et al. 1980), can be expected to provide significant progress on the conformation-activity problem of the opioid peptides and receptors.

Much progress has been made in the characterization of the opioid receptors and of the actions of opioid peptides. It has been found that opioid receptors reside in the plasma membrane of nerve cells and that they are protein in nature with a significant phospholipid component (Barnard and Demoliou-Mason 1983). The opioid peptides alter membrane conductance (West and Miller 1983) with both pre- and post-synaptic actions in mammalian parasympathetic ganglia (Katayama and Nishi 1984). It has been reported that they hyperpolarize cells by means of increasing potassium ion membrane conductance in the central nervous system (Henderson 1983) and specifically by opening potassium ion channels in peripheral nerve cells (North and Egan 1983; Morita and North 1981). A significant action in dorsal root ganglion cells and peripheral nerve cells is the inhibition of neurotransmitter release (Hughes and Kosterlitz 1983; North and Egan 1983; Wouters and van den Bercken 1980; Werz and Macdonald 1982). Additionally, opioid peptides stimulate hormone release (Grossman and Rees 1983) and there are yet other actions reported, including some which relate to the development of tolerance and addiction (West and Miller 1983). What clearly emerges, of course, is action at a membranous site with dominant effects being on channel conductance and neurotransmitter release.

This conformation-activity review with concern for methodological approaches will attempt to address peptide channel activity at a membranous site with some effort to note the wide-ranging set of methodologies that are required to attack such a problem effectively. Disciplines involved in a comprehensive approach to the understanding of opioid peptide function include: 1) synthesis of peptide analogs (Peptide Chemistry); 2) characterization of molecular conformation by physical methods and by molecular mechanics calculations (Molecular Biophysics); 3) isolation and reconstitution of receptors and associated transport components (Membrane Biochemistry); and 4) characterization of opioid peptide and analog activities (Molecular Pharamacology, Membrane Biophsyics. and Electrophysiology). These components

for a comprehensive attack on the mechanism of action of opioid peptides all have analogous components in determination of the molecular structure and ionic mechanism of Gramicidin A channel transport.

Accordingly, this chapter reviews a general conceptual and experimental approach of considering cyclic conformations and linear conformational correlates with use of the physical methods of nuclear magnetic resonance (NMR), circular dichroism, and dielectric relaxation. This will allow a conceptual derivation of the Gramicidin A channel structure, an experimental development of a dynamic molecular structure wherein molecular flexibility is an integral part of function, and the conceptual derivation of a voltage-dependent channel structure. There will be a brief consideration of channel transport phenomenology combined with the utilization of an array of disciplines, with similarity to those noted above for approaching the opioid peptide structure-activity problem, to derive the molecular structure and mechanism of Gramicidin A channel transport. Finally, there will be a few comments on the patch clamp methodology of Neher and Sakmann and their colleagues (for an excellent review of the subject, see Sakmann and Neher 1983) which is being used in the Gramicidin A channel tranport study and which appears to be particularly well-suited for the detailed study of opioid peptide activity. This approach provides a means of studying single whole cells or small membrane patches from cells, or of reconstituted protein-membrane systems. It can be expected to give rise to more detailed and less ambiguous demonstration of activities and mechanisms related to ion transport across cell membranes. It could also be used to follow, by means of cell membrane capacitance changes, the fusion of neurotransmitter loaded vesicles with plasma membrane in this mechanism of neurotransmitter release (Neher and Marty 1982).

CYCLIC CONFORMATIONS WITH LINEAR CONFORMATIONAL CORRELATES (CCLCC): AN ANALYTICAL CONCEPT AND AN EXPERIMENTAL APPROACH

The analytical concept of a cyclic conformation with a linear conformational correlate (Urry 1972) derives from the realization that, if a cyclic peptide with an adequate number of residues forms a definable conformation with reasonable stability, then one can anticipate that a linear polymer comprised of the repeating sequence of the cyclic structure could exist in a related conformation. This is because, with two torsion angles per peptide and with a substantial number of residues in the cycle, only small changes in the torsion angles could result in a left- or right-handed helical structure comprised of the repeating unit. The resulting helical structure is particularly favorable when the conformation gives rise to favorable interturn interactions. This concept is relevant to the channel structure of Gramicidin A (see below): it was originally described in the first enunciation of voltage-dependent channel formation using only dipole moments, in this case the dipole

moment of the peptide moiety (Urry 1972); it will be used as an analytical concept below to derive two different channel structures; and it will be used as an experimental approach to derive a particularly interesting helical conformation referred to as a **β**-spiral.

The Channel Conformation of Gramicidin A

If one considers the L•amino acid-Doamino acid (L-D) repeating dipeptide unit contained in a cyclic hexapeptide, the relative orientation of the peptide moieties would be as depicted in figure 1A, pointing alternately up and down with respect to the mean plane of the cyclic hexapeptide.

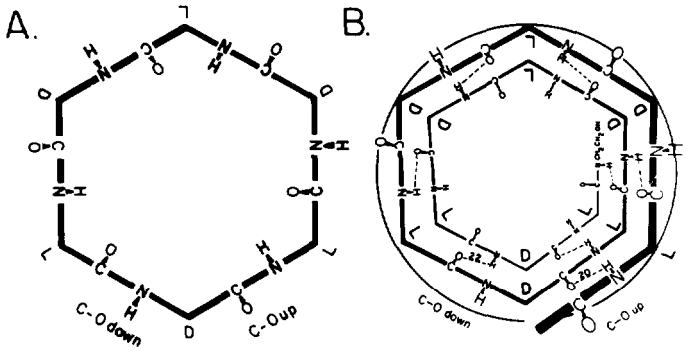

FIGURE 1

Representation of a cyclic conformation A, and a linear conformational correlate B, comprised of repeating L-D dipeptides. Part B is from Urry et al. 1971. Copyright 1971, National Academy of Science.

The side chains (R groups) would be oriented in a radial manner. This is closely the structure of the ionophore enniatin B (Plattner et al. 1963), wherein the cation is held centrally by peptide carbonyl oxygens that are librated inward with three carbonyl oxygens above and three below the plane to achieve coordi-nation of the cation. To convert this to a linear conformational correlate, the cyclic structure is ruptured; small torsion angle changes are made to give a helical pitch of about 5A; the hexapeptide is repeated; and it is found that hydrogen bond formation readily occurs between turns of the helix, as schematically shown in figure 1B. The hydrogen bonding pattern between turns exhibits the same pattern as between chains in the parallel **β**-pleated sheet. For this reason, the structure is referred to as a single stranded **β**-helix. In fact, the structure is also readily derived from a single **β**-chain by

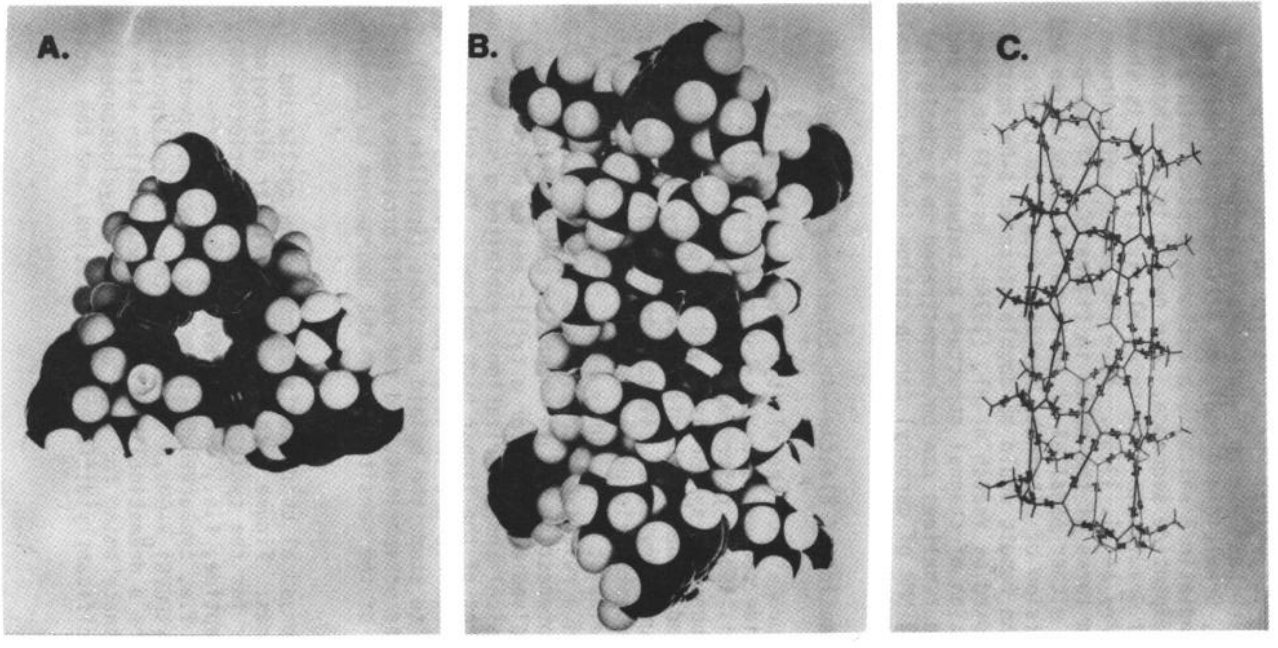

FIGURE 2

A. and B. Spacefilling model of the Gramicidin A transmembrane channel in channel view (A) and in side view (B). C. Wire model of the backbone and β-carbons of the channel seen in side view as in B. From Urry et al. 1975. Copyright 1975, New York Academy of Science.

interchanging backbone and side chain (i.e., converting from an L-residue to a D-residue) at every other residue in the **β**-chain. The primary Structure of Gramiciden A is HCO-L•Val^{1}-Gly^{2}-L•Ala^{3}-D•Leu^{4}-L-Ala^{5}-D•Val^{6}-L•Val-D-Val^{8}-L•Trp^{9}-D•Leu^{10}-L•Trp^{11}-D•Leu^{12}-L•Trp^{13}-D•Leu^{14}-L•Trp^{15}-$NHCH_2CH_2OH$ (Sarges and Witkop 1964); that is, Gramicidin A could be a linear conformational correlate of enniatin B which is a cyclohexapeptide with a repeating L-D dipeptide sequence. The result is the channel conformation of Gramicidin A (Urry et al. 1971) which is shown in figure 2 (Urry et al. 1975). Note the pattern of hydrogen bonding between turns of the helix in figure 2C. If two **β**-chains were in either parallel or antiparallel association and the same conversion of alternate residues from L to D were carried out, the result would be double stranded **β**-helices--a structure proposed by Veatch et al. (1974). As will be noted below, delineation of channel structure as between single and double stranded **β**-helices can be demonstrated in a simple way using carbon-13 NMR of carbonyl carbon cation induced chemical shifts.

The Polypentapeptide of Elastin --An Experimental Use of CCLCC

The precursor protein of the elastic fiber contains as a repeating peptide sequence, L•Val$_1$-L•Pro$_2$-Gly$_3$-L•Val$_4$-Gly$_5$ (Sandberg et al. 1981), referred to simply as VPGVG. When this pentameric sequence is synthesized and the pentamer polymerized and cross-linked either by chemical means (Urry et al. 1976) or by y-irradiation (Urry et al. 1984c), the resulting matrix is found to have elastomeric properties strikingly similar to those of the native elastic fiber (Urry et al. 1985). The polypentapeptide forms a relatively complex and dynamic structure. One particularly interesting way in which its conformation was approached involved a search for a cyclic analog which, by means of various physical characterizations, exhibited a similar conformation to that of the linear high polymer. The solution conformational approaches are in large part the same as those that have been used to study the opioid peptides (Schiller 1984), particularly the enkephalins, and the result provides a clear demonstrations of the sensitivity of the approaches to conformation.

Comparison of physical characterizations of the cyclic and linear structures: Figure 3 contains the circular dichroism curves in water for the cyclopentapeptide, i.e., cyclo(VPGVG); the cyclodecapeptide, i.e., cyclo(VPGVG)$_2$; the cyclopentadecapeptide, i.e, cyclo(VPGVG)$_3$; and the linear polypentapeptide, i.e., H-(VPGVG) V-OMe. It is seen that the cyclopentapeptide exhibits a dramatically different conformation with the right-hand ordinate being for this curve. The cyclodecapeptide exhibits a significantly different conformation, and the cyclo-

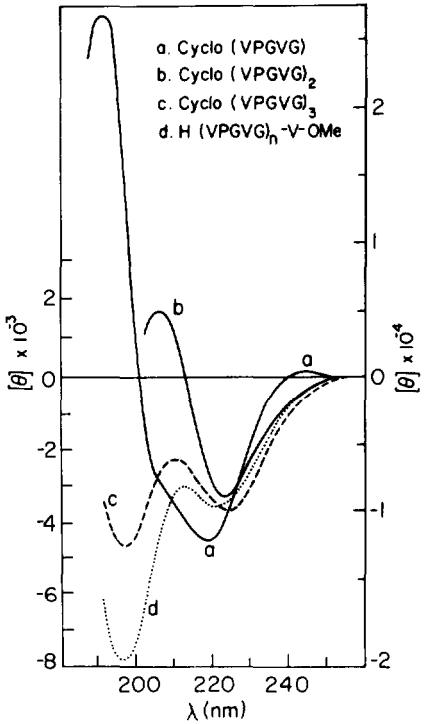

FIGURE 3

Circular dichroism spectra of cyclic analogs and the linear high polymer. From Urry 1985a. Copyright 1985, Elsevier Science.

pentadecapeptide exhibits a conformation which is more similar to that of the linear polymer. Thus, the cyclopentadecapeptide is a candidate for the cyclic conformational correlate of the linear polymer.

Using proton NMR, the temperature dependence of peptide NH proton chemical shift in dimethylsulfoxide was determined and is shown in parts a, b, and c of figure 4 (Urry et al. 1981) for the cyclic analogs. The dotted curves give the data for the linear polymer. The cyclopenta- and cyclodecapeptides exhibit very different patterns of temperature dependence of chemical shift, whereas the patterns are very similar for the cyclopentadecapeptide and the linear polypentapeptide. Since in each case the sequence is the same, i.e., each residue has the same nearest neighbor and next nearest neighbor etc., the differences that appear must be solely due to differences in conformation. This demonstrates that the approach is very sensitive to conformation and that the cyclopentadecapeptide is a cyclic conformational correlate of the linear polypentapeptide. The small

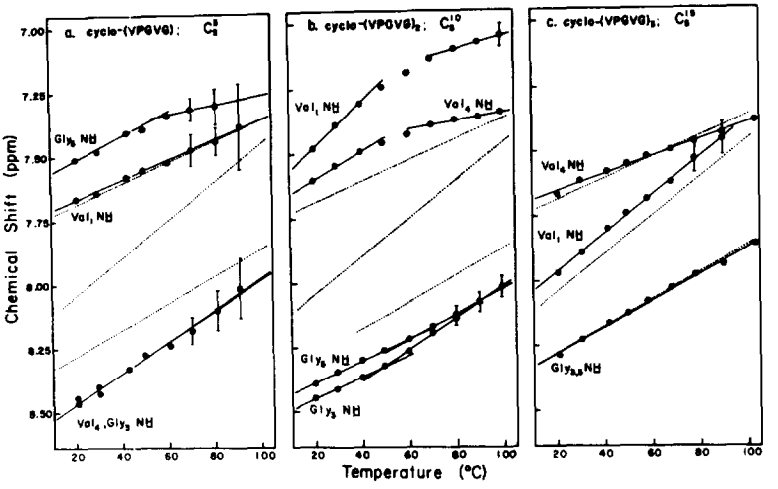

FIGURE 4

Temperature dependence of amide proton in dimethylsulfoxide of cyclic analogs of the elastin pentamer. Adapted with permission from Urry et al. 1981. Copyright 1981, American Chemical Society.

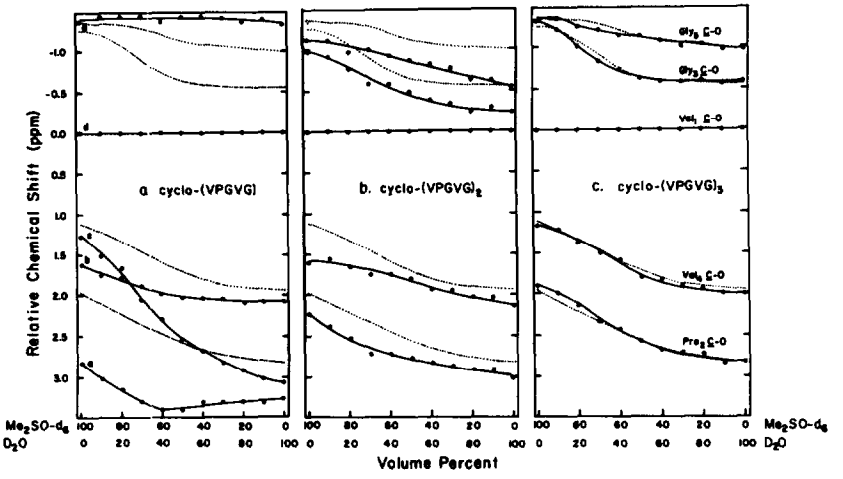

FIGURE 5

Solvent dependence of peptide carbonyls in dimethylsulfoxide and water of cyclic analogs of the elastin pentamer. Adapted with permission from Urry et al. 1981. Copyright 1981, American Chemical Society.

chemical shift in the $Val_1N\underline{H}$ proton, which is essentially constant with temperature, correlates with a small difference in the Val_1 $\alpha\underline{CH}-\underline{NH}$ coupling constant. Rotation of the ϕ_{1} torsion angle by a few degrees in a direction that would make the coupling constants similar would convert the cyclic structure to a right-handed helical structure. This would also move the $Val_1N\underline{H}$ peptide proton toward the edge of a peptide plane in a manner that would lead to a downfield chemical shift due to the magnetic anisotropy of the peptide moiety. Thus, the data not only argues that the conformations of the cyclopentadecapeptide and linear polymer are similar, but it also suggests that the linear polymer forms a right-handed helical structure.

In figure 5, carbon-13 NMR is used to compare the solvent dependence of peptide carbonyl carbon chemical shifts of the cyclic and linear structures (Urry et al. 1981). The solvent pair is dimethylsulfoxide and deuterated water and, again, dotted curves are used for the linear polymer data to facilitate comparison with each of the cyclic structures. The peptide $\underline{C}$-O chemical shift dependence on solvent also shows the cyclopenta- and cyclodecapeptides to be in conformations very different from each other and very different from the linear polymer, whereas again the cyclopentadecapeptide conformation is very close to that of the linear polypentapeptide.

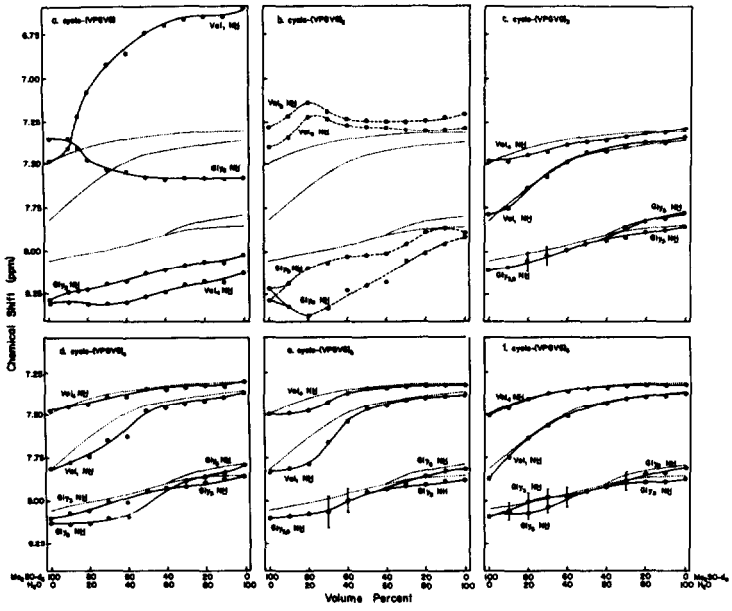

FIGURE 6

Solvent dependence of amide proton in dimethylsulfoxide and water of cyclic analogs of the elastin pentamer. From Urry et al. 1981. Copyright 1981, American Chemical Society.

It is also interesting to look at the solvent dependence of the peptide NH chemical shifts. This is shown in figure 6, using the same Solvent pair as in figure 5 but for the six cyclic analogs with n=1, 2, 3, 4, 5, and 6. As with the data of figures 4 and 5, the data for the linear polypentapeptide is the dotted set of curves superimposed on each of the sets of curves for the cyclic analogs. Again, the patterns for the cyclopenta- and cyclodecapeptides are dramatically different from each other and from that of the linear polymer. The data for the cvclopentadecapeptide, as before, closely follow that of the linear polypentapeptide. The data for the cycloicosapeptide (n=4) and the cyclopentaicosapeptide (n=5) do not follow so closely. But the data for the cyclotricosapeptide (n=6) follow extraordinarily closely, even more so than for the cyclopentadecapeptide. This is thought to be due to a figure 8 type of structure comprised of two pentadecapeptide moieties related by twofold symmetry and differing from the cyclopentadecapeptide primarily by the introduction of a helical pitch resulting from chain crossing.

These results demonstrate the validity of such approaches as sensitive monitors of conformation. The use of these methods is particularly favorable for a sequence such as this pentamer, where all side chains are aliphatic--having neither hydrogen bonding capability nor magnetic anisotropies that can complicate interpretation of temperature and solvent dependence data. The interpretation of such data for the enkephalins is complicated by the ring current effects of the Phe and Tyr side chains and the hydrogen bonding capabilities of the Tyr OH and the amino and carboxyl end groups.

Conformations of the Cyclic and Linear Structures of the Polypentapeptides of Elastin

The solution derived conformations are shown in figure 7A, B, and C for the cyclopentapeptide, the cyclodecapeptide, and the cyclopentadecapeptide, respectively (Khaled et al. 1981, 1982; Venkatachalam et al. 1981). These were derived using the above type of NMR data to identify secondary structure, with coupling constants to limit αCH_i-NH_i torsion angles, and with the nuclear Overhauser effects to limit the αCH_i-NH_{i+1} distances, all in combination with conformational energy calculations to limit further the allowed torsion angle ranges. Essentially the same conformation for the more rigid cyclopentapeptide has been determined in the crystal by X-ray diffraction (Einspahr et al. 1980), as has that of the more flexible cyclopentadecapeptide which is shown in figure 8 (Cook et al. 1980). The molecular structure of the cyclopentadecapeptide is analogous to a three-footed stool with the Pro_2-Gly_3 β-turns functioning as the feet of the stool. The crystal structure of the cyclopentadecapeptide is particularly interesting since the threefold symmetry is retained (see figure 8A); the intermolecular interactions are

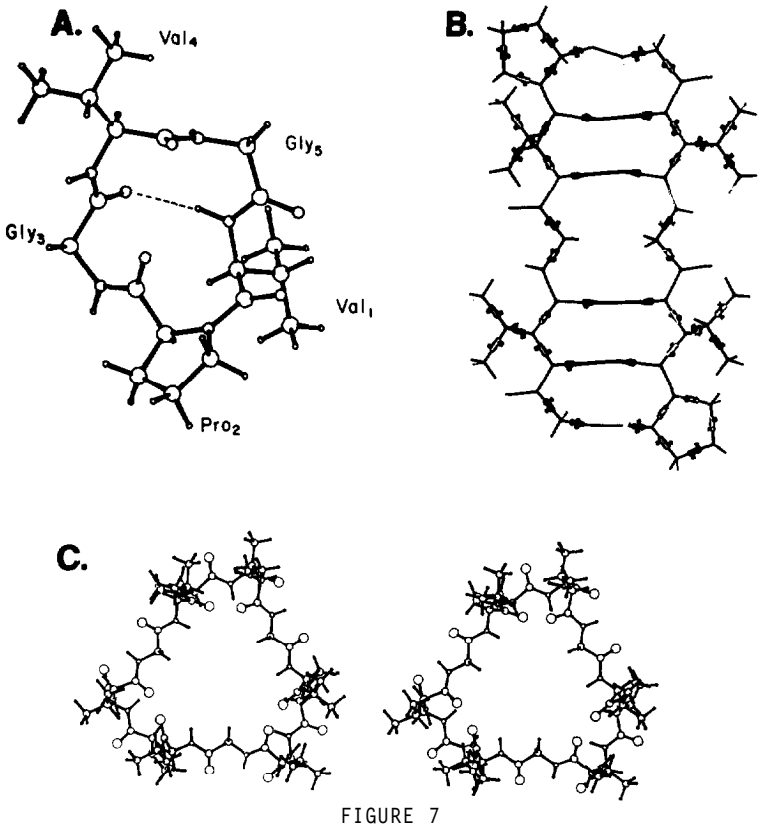

FIGURE 7

Crystal structures of cyclic analogues of the elastin pentamer. A. The cyclopentapeptide; B. the cycle-decapeptide; C. the cyclopentadecapeptide (stereo view). Reproduced with permission from Khaled et al. 1981(A). Copyright 1981, Munksgaard, Copenhagen. Khaled et al. 1982(B). Copyright 1982, Elsevier Biomedical Press. Venkatachalam et al. 1981(C). Copyright 1981, American Chemical Society.

hydrophobic, and the substantial water in the crystal is within the stack of cyclic molecules that are stacked exactly on top of each other (see figure 8B).

It is a relatively simple conceptual process to take a stack of cyclopentadecapeptide molecules and to convert them to a linear left-handed or right-handed helix as described above for forming the Gramicidin A channel from an enniatin E-like cyclic structure. When this is done by means of molecular mechanics, using the potential functions of Scheraga and colleagues (Momany et

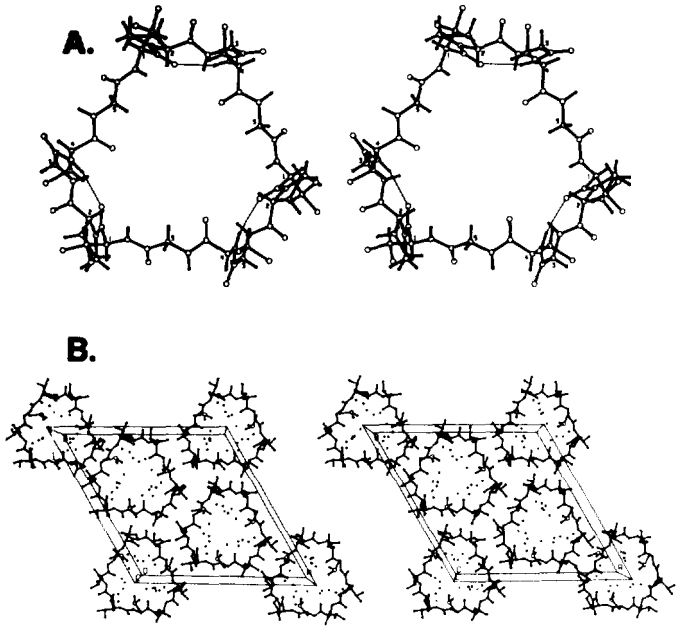

FIGURE 8

Crystal structure of the cyclopentadecapeptide. A. molecular structure; B. crystal packing. From Cook et al. 1980. Copyright 1980, American Chemical Society.

al. 1974, 1975), a right-handed helical structure is found to be preferred (see figure 9), whereas going left-handed disrupts the conformational features of the cyclic structure (Venkatachalam and Urry 1981). This is consistent with the analysis of the small $^{3}J(\alpha\underline{CH}\text{-}\underline{NH})$ coupling constant difference and the small difference in chemical shift of the $Val_1N\underline{H}$ as discussed relative to figure 4 above. [It may be noted that the same potential functions have been utilized in conformational energy calculations of Met- and Leu-enkephalins and their analogs (Manavalan and Momany 1979, 1980, 1981)]. The resulting linear structure depicted in figure 9 is termed a β-spiral, as the β-turn is the dominant repeating secondary structural feature. The term spiral is used instead of helix to emphasize in the structure that there is not the obligatory hydrogen bonding between repeating units as in the classical helical structures of polypeptides. The interturn interactions are hydrophobic; there is water within the β-spiral; and the β-turns function as spacers between the turns of the β-spiral. This is shown as stereopairs for an all atom depiction in figure 9A and B and for an α-carbon only representation in figure 9C and D. The upper structures

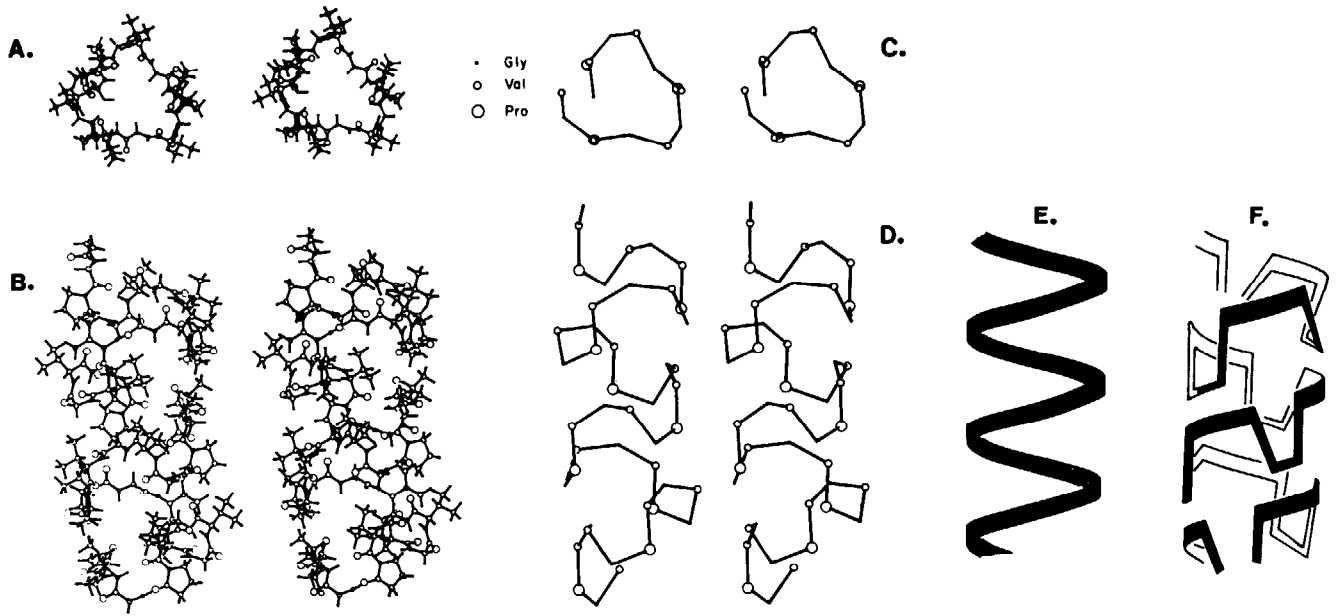

FIGURE 9

Representations of the proposed β-spiral for the polypentapeptide of elastin. A, B, C, and D from Venkatachalam and Urry 1981. Copyright 1981, American Chemical Society. E and F from Urry 1983. Copyright 1983, McGraw-Hill.

give the helix axis view. Figure 9E schematically shows the helical nature and figure 9F shows the β-turns functioning as spacers between turns.

β-Spiral Temperature Dependence and the Source of Elasticity

While the β-turns are present at temperatures below 20 °C, they are less stable at that temperature and become more stabilized as the temperature is raised. Also, the hydrophobic Val-Pro side chains come into closer association and the interturn hydrophobic interactions develop. These hydrophobic interactions are responsible for the β-spiral formation. Particularly on slight extension of the structure, as occurs on stretching, the β-turns are joined by a suspended segment, Val_4-Gly_5-Val_1, which is allowed a great deal of motional freedom as it is largely surrounded by water and as the central residue is a glycine without a sterically restricting side chain. The peptide moieties of this suspended segment are able to librate, or to rock, thereby providing motion and entropy. It is thought that the damping of this motion is the decrease in entropy that occurs on stretching and this is responsible for the entropic elastomeric retracting force. This has been termed a peptide librational entropy mechanism of elasticity (Urry et al. 1982b, 1983a). This proposed mechanism has been substantiated by

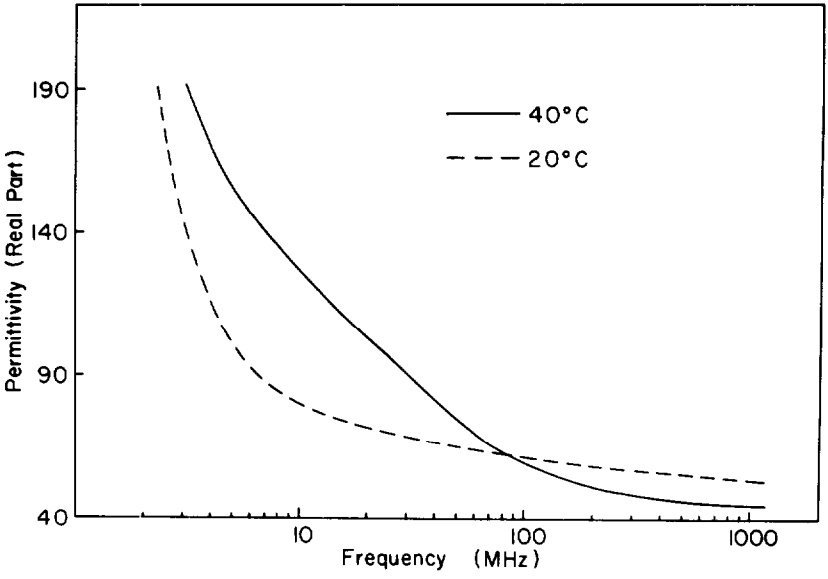

FIGURE 10

Temperature dependence dielectric relaxation spectrum of polypentaprptide of elastin. Adapted from Henze and Urry 1985. Copyright 1985, American Chemical Society.

selected synthetic analogs (Urry et al. 1983a, 1983c), and by the development of a motional mode on raising the temperature that can be detected by means of dielectric relaxation studies. As shown in figure 10, at 20 °C no resolvable relaxation is observed in the real part of the dielectric permittivity in the 3 MHz to 1000 MHz range; but on raising the temperature, an intense relaxation develops at 25 MHz as the **β**-spiral forms (Henze and Urry, unpublished data). This motional mode is due to the motions of a dipole moment with the indicated frequency. As the peptide is the only source of a dipole moment, this is due to peptide motion. As the material is very viscoelastic, the change is not whole molecule reorientation but rather a peptide rocking motion. This experimental result provides direct evidence for a structure-dependent peptide librational entropy mechanism of elasticity. Whereas structural flexibility is a problem in characterizing opioid peptide conformations, this does not mean the absence of conformation and, as in the case of the polypentapeptide of elastin, flexibility could be an essential aspect of function.

The Helical Rack Model and a Proposed Voltage-Dependent Channel

The concept of CCLCC described above was used to derive the Gramicidin A channel conformation and, in the elastin study, it was used experimentally to determine which of a series of cyclic analogs had a conformation resembling the linear structure. In the process of comparing cyclic analogs, the experimental methods used--temperature and solvent dependence of peptide NH and C-O nuclear magnetic resonance chemical shifts--were showii to be very sensitive to conformation. In this section, the concept of CCLCC is used to develop a structural motif for a membranous protein which would function as a voltage-dependent channel.

The classical helices, e.g., the **α**-helix and 3_{10}-helix, have large dipole moments which could be used to orient in an electric field or to induce helix formation on the application of such a field. In 1975 (Urry 1975; Urry et al. 1975), extending the use of peptide dipole moments to form voltage-dependent channels (Urry 1972). a helical rack model was proposed utilizing a classical helix cyclized head to tail by a **β**-chain segment continuing in sequence as shown in figure 11. The dipole moments of the individual peptide moieties of the helix sum to make a large dipole moment which, since the dipole moment of a **β**-chain is small, cause the cyclic molecules to align across a membrane on application of a potential. A schematic representation of the structure from a view perpendicular to the membrane surface is given in figure 12 (Urry 1982). Since all the **α**-helices cross the membrane in the same direction and the **β**-chains do so in the opposite direction, all the **α**-helices and **β**-chains run in parallel and it is a simple matter to convert from a series of cyclic subunits to the linear conformational correlate as shown in figure 13. This results in a

protein structural motif of a β-barrel surrounded by α-helices. This has been recognized by Richardson (1981) as the structural motif for triosephosphate isomerase, the pyruvate kinase domain I, and aldolase. A related structured motif has been derived by Marshall and colleagues (Hall et al. 1984) from studies on alamethicin voltage-dependent channel formation. Obviously, such a structural motif and the insiahts it provides aive rise to numerous experimental approaches. Thus; we have a proposed molecular structure for a voltage-dependent channel which could possibly provide a point of reference for the consideration of the effect of opioid peptides on increasing potassium ion conductance.

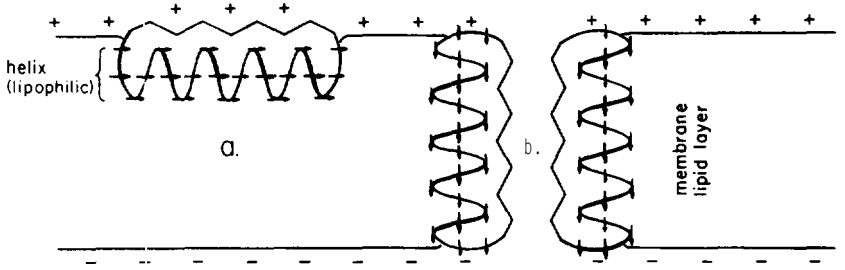

FIGURE 11

Helical rack model for voltage dependent channel formation. From Urry 1975. Copyright 1975, John Wiley & Sons, Inc.

MOLECULAR STRUCTURE AND MECHANISM OF MEMBRANE CHANNELS: THE GRAMICIOIN A CASE

As noted in the Introduction, the set of disciplines required in an approach to the mechanism of action of opioid peptides is closely similar to that required for a comprehensive approach to the molecular structure and mechanism of Gramicidin channel transport. Peptide synthesis is required to make analogs for testing structural concepts and to prepare selectively isotope-labeled molecules for determining elements of mechanism. Characterization of conformations, interactions, and rate processes by physical methods--such as circular dichroism, NMR, ultraviolet, infrared, and fluorescence spectroscopies; dielectric relaxation; etc.--provide an essential second component. Molecular mechanics calculations are required to test and refine proposed elements of mechanism and to add information and insight not directly available from experiment. In addition, characterization of peptide or protein effected ion transport across lipid bilayer membranes is by methods that provide the greatest sensitivity and time resolution, such as the planar bilayer approach of Mueller and Rudin (1967) and the

single channel recording techniques of Sakmann and Neher (1983). The interplay and essential information exchange between each of these components is extensive and the loss of any component would mean a less well-characterized and less certain mechanism. As an action of opioid peptides is to increase the opening of potassium ion channels, a complete understanding of this mechanism of action would require analogous approaches.

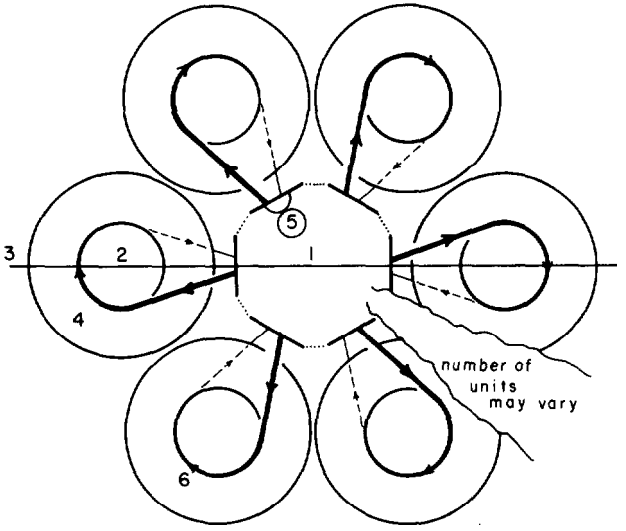

FIGURE 12

Voltage dependent channel formed from cyclic subunits with each subunit containing an α-helical and a β-chain component. This is the same structure as given in figure 11 from a different perspective. The perspective of figure 11 is the cross section indicated by the line labeled 3.

1. β-barrel at center comprised of parallel β-chains.
2. Right-handed α-helices with positive end of dipole at top.
3. Horizontal line indicates cross section for figure 11.
4. Side chains on α-helices are hydrophobic.
5. Alternate side chains on β-chain determine character of channel. Others may be hydrophobic.
6. Arrow indicates direction of chain

From Urry 1982. Copyright 1982, Alan Liss, Inc.

Phenomenology of Channel Transport

In its simplest terms, characterization of channel transport involves a pair of electrodes separated by a small lipid bilayer membrane of high resistance. Commonly, the salts in the aqueous solutions on each side are equivalent and a potential is applied. The presence of peptides or proteins that can form channels across the lipid bilayer result in conductance events as a chan-

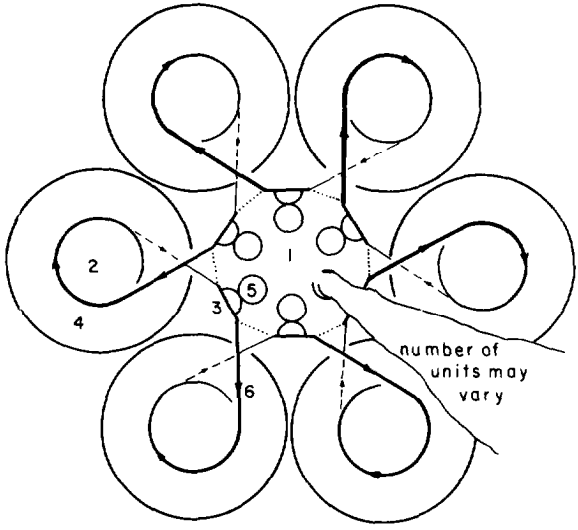

FIGURE 13

Cyclic conformational correlate of the structures shown in figures 11 and 12. Protein model for a voltage dependent channel.
1 **β**-barrel at center.
2 Right-handed **α**-helix with positive end of dipole at top.
3. Parallel aligned **β**-chains.
4. Hydrophobic side chains of **α**-helix.
5. Alternating side chains on **β**-chains determine character of the channel.
6. Arrow indicates direction of chain.
From Urry 1982. Copyright 1982, Alan Liss, Inc.

nel forms and disappears. This was first observed by Hladky and Haydon (1970, 1971, 1972) with the Gramicidin peptides isolated from bacillus brevis. Such single channel events have now been observed for many different channels from higher organisms in their natural cell membranes and reconstituted in model membranes (see, for example, Sakmann and Neher 1983). The two basic channel characteristics are their single channel conductance given in Siemens (channel current in coulombs/sec divided by applied potential in volts) and channel mean lifetime, where a single conductance event can last from milliseconds or less to seconds or even minutes. Analgesics and anesthetics can be expected to act indirectly or directly on a channel to alter one or both of these two characteristics.

Figure 14 shows single channel current traces for Gramicidin A in the upper trace and for N-acetyl desformyl Gramicidin A in part B (Szabo and Urry 1979). The difference between these two channel

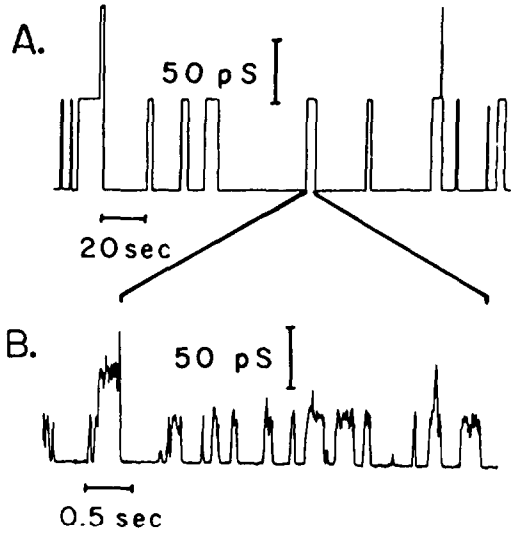

FIGURE 14

Single channel current traces in the presence of 1M RbCl with an applied potential of 100mV for Gramicidin A (A) and for N-acetyl desformyl Gramicidin A (B). The step increase and decrease is the turning on and off of a single channel. From Szabo and Urry 1979. Copyright 1979, American Association for the Advancement of Science.

structures is simply the replacement of a formyl hydrogen by a methyl moiety (i.e., acetyl for formyl), yet the effects on channel conductance and lifetime are dramatic. For the potassium ion, the single channel conductance is reduced from a most probable value of 45 pico Siemens (pS) to 27 pS and the channel mean lifetime is reduced from 3.3 seconds to 56 milliseconds. On a macroscopic basis, this has the effect of reducing conductance to 1%. As will be briefly argued below, even the reorientation of an amino acid side chain removed by some distance from the channel itself can have a similar dramatic effect in varying conductance.

This allows that even a most subtle binding effect of an opiate or other effector could either greatly increase or decrease effective conductance.

Mechanism of Gramicidin A Channel Transport

Once structure is known, the major components of determining the ionic mechanism of channel transport are to locate binding sites in the channel, if indeed they exist, to determine binding constants, and to estimate rate constants for each of the major rate processes. When this is adequately achieved, a complete free energy profile can be plotted and, when this is achieved for different temperatures, complete enthalpy and entropy profiles should be obtained. With such information, the single channel currents would be calculable over wide ranges of ion concentration, of applied potential, and of temperature. As this is achieved, all of the obvious and subtle control mechanisms will become apparent.

Location of Ion Binding Sites

From the conformation of the channel (see figures 1 and 2), it is apparent that, as the cation passes through the channel, it is coordinated laterally by the peptide carbonyl oxygens. Accordingly, if an appreciable occupancy occurs, it should be observable--using carbon-13 NMR--as an ion-induced carbonyl carbon chemical shift. This had previously been observed for the cyclic conformation correlate, the enniatin B-K+ complex (Urry 1976). However, to determine the ion-induced carbonyl carbon chemical shift for the Gramicidin A channel in lipid bilayers, it is necessary to synthesize Gramicidin A molecules wherein only the carbonyl carbon of a single residue is enriched with carbon-13. This has been done in 10 syntheses, i.e., for 10 different peptide carbonyl. For each synthesis, the channel is then packaged in lipid bilayers at 3 mM concentrations and the presence or absence of a cation-induced carbonyl carbon chemical shift is determined for one residue. The results, plotted as a function of carbonyl location in the left-handed single stranded β^{6}-helix, are given in figure 15 (Urry et al. 1982c). These cation-induced carbonyl carbon chemical shifts, when plotted with respect to other proposed structures, e.g., the double stranded β-helices (Veatch et al. 1974), can also be used to determine unequivocally the channel structure (Urry et al. 1983b) and even to determine the helix sense (Urry et al. 1982c). The axiom utilized in delineating structures is that helically (or structurally) equivalent carbonyls should show similar ion-induced chemical shifts when equally proximal to the ion.

Wire Model and Ion Binding Sites of the
Gramicidin A Transmembrane Channel

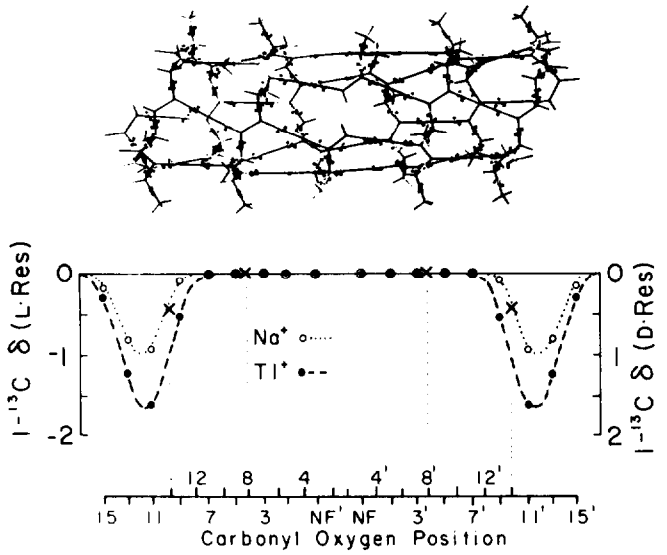

FIGURE 15

Location of ion binding sites using carbon-13 NMR to observe ion-induced carbonyl carbon chemical shifts. Adapted with permission from Urry et al. 1982c. Copyright 1982, Springer-Verlag, Inc.

Determination of Binding Constants

The ion-induced carbonyl carbon chemical shift can be used to estimate binding constants and also to clarify the nature of the binding process. Shown In figure 16 is the thallium ion-induced carbonyl carbon chemical shift followed with carbonyl carbons of the Trp and Trp residues and plotted as a function of log [Tl+]. What is apparent is that there are two binding constants--one for entry of the first ion (a tight binding process) and one for entry of the second ion which, due to repulsion from the first ion in the channel, is a weaker process. A tight and a weak binding constant can also be estimated using quadrupolar ion NMR and longitudinal relaxation time studies (Urry et al. 1980). Thus, in this twofold symmetric channel with two structurally equivalent binding sites, there two binding constants--a tight one for single occupancy and a weak one for double occupancy.

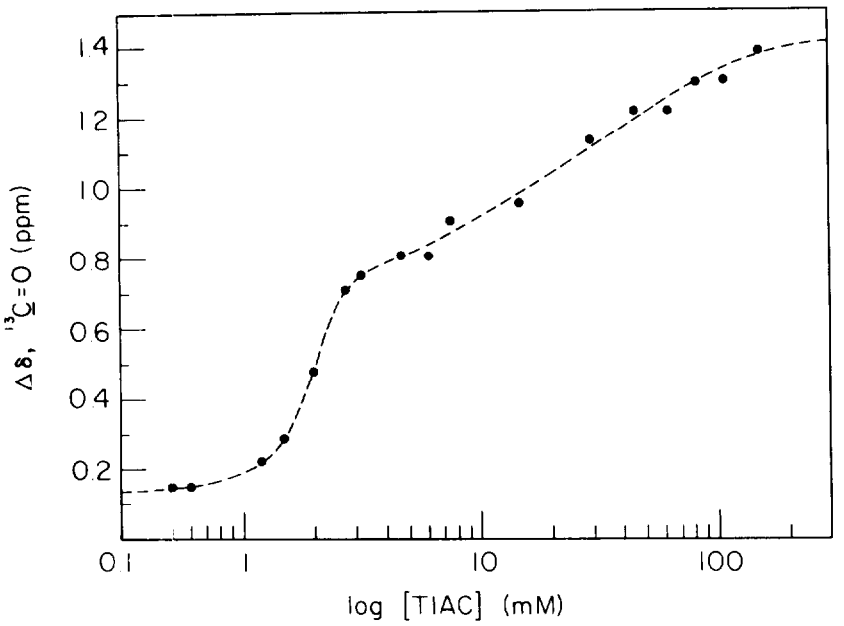

FIGURE 16

Plot of thallium ion-induced carbonyl carbon chemical shifts as a function of the logarithm of the ion concentration. This clearly shows tight and weak binding processes, respectively, for the entry of the first and second ions in the channel. From Urry et al., unpublished data.

Estimation of Rate Constants

Ion NMR linewidths plotted versus chemical shift for spin 1/2 ions or for situations that can so approximated and traverse relaxation time studies of the spin 3/2 quadrupolar ions can be used to estimate rate constants for ions leaving the channel (Bull 1972; Venkatachalam and Urry 1980). The latter approach, in particular, utilizes approximate expressions and arguable assumptions to convert experimental relaxation times into correlation times and finally to rate constants. Such estimates have been made for the sodium ion, and the obtained rate constants have been shown with the other data on binding constants and site location to calculate single channel currents over wide ranges of ion activity and applied voltage (Urry et al. 1980).

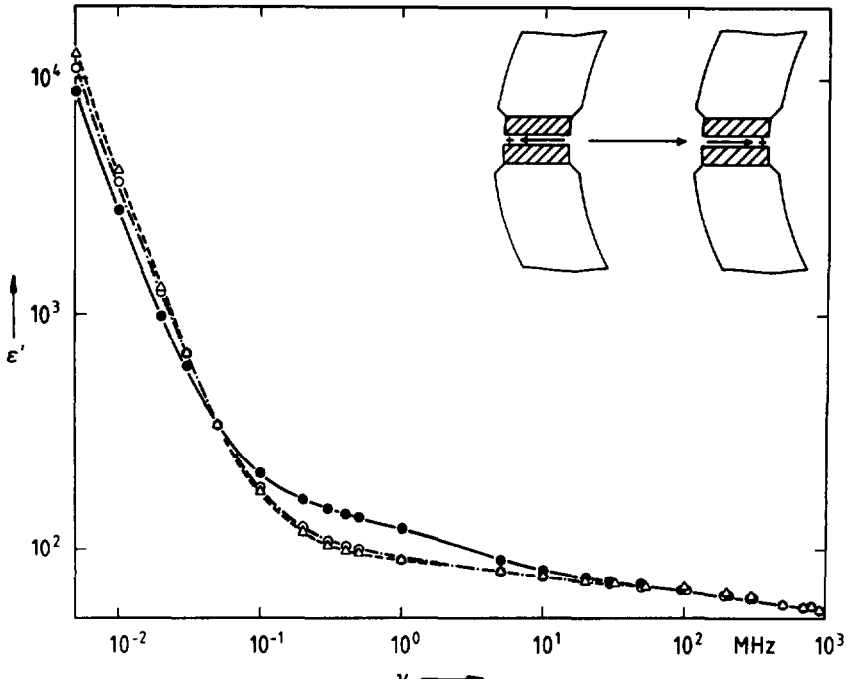

FIGURE 17

Dielectric relaxation spectrum of a suspension of malonyl-Gramicidin A channels. The solid curve showing the additional relaxation beginning just below 10 MHz is the case where there is one Tl+ ion in the channel. From Henze et al. 1982. Copyright 1982, Springer-Verlag New York, Inc. Inset: Representation of the dipole moment change attending an intrachannel ion translocation from one binding site to the other. From Urry 1985b. Copyright 1985, D. Reidel Publishing Company.

A particularly notable means, when useable, for obtaining a rate constant relevant to transport through the channel is dielectric relaxation. There is a large change in charge distribution when an ion jumps from one binding site to the other in the channel. This is depicted in the inset of figure 17. When the frequency at which the field reverses is faster than the ion can move, it does not contribute to the dielectric permittivity of the medium containing the channels. When the frequency is slower than the rate for ion translocation, the ion can jump back and forth as the field reverses and, as such, can contribute to the dielectric permittivity of the medium. This is seen for thallium ion in the channel in figure 17 where a relaxation is observed giving a rate constant for jumping between sites of

about 4 x 10^6/sec. (Henze et al. 1982). A particularly attractive aspect of this approach is that it requires no complicated theory with arguable assumptions to estimate the rate constant. Thus, dielectric relaxation has been seen to provide critical information above in the elastin structure-function problem and here in the development of the ionic mechanism of channel transport. It could also be used to provide information on the dipole moment of enkephalin molecules in solution.

Modulation of Single Channel Conductance and Lifetimes

Single channel conductance magnitudes and channel lifetimes are readily modulated. The dramatic effect of replacing a hydrogen by a methyl moiety, i.e., conversion N-formyl to N-acetyl, was noted above in relation to fiaure 15. This resulted in a 99% decrease in conductance. There are many examples of the marked sensitivity of the amino (formyl) end to modification, but there is a relative insensitivity to derivitization at the ethanolamine OH. For example, the O-pyromellityl derivative with its triple negative charge has only a small counterion cloud effect (Apell et al. 1977), whereas the N-pyromellityl derivative completely destroys activity (Barnberg and Janko 1977). More subtly, the D•Leu^2-GA analog results in a marked decrease in lifetime with essentially no change in conductance (Bradley et al. 1981a). As external effects. addition of the anesthetic halothane at the physiological dose reduces lifetime from 25% to 30% of normal (Bradlev et al. 1981b); raising calcium ion concentration decreases conductance (Bamberg and Laüger 1977) by competitive binding of calcium ion at the binding site even though divalent cations are not conducted (Urry et al. 1982c); and increasing membrane thickness decreases channel lifetime with no change in conductance (Hladky and Haydon 1972).

An interesting analog is the des L•Val^7-D•Val^8-Gramicidin A which is shorter by 3A due to the removal from the channel of two L-D dipeptide units, one in each monomer of the dimeric channel. The result, quite expectedly, is a shorter lifetime, but also, quite instructively, the conductance is decreased to almost one-half (Urry et al. 1984a). Since, by a diffusion-limited process, conductance would be expected to be increased by 30% for this extent of shortening, the result indicates the essential correctness of an Eyring barrier-type description and indicates further that the dominant conducting state is one with double ion occupancy.

Of particular interest with respect to control of conductance is the multiplicity of conductance states. When single channel conductance was mentioned above, the reference was actually to the most probably conductance state. As shown in the histogram of figure 18 by the dashed enclosure, while there is a most probable conductance state for Gramicidin A at 100 mV, 20 °C, and 1M KCl of 17 to 18 pS, there are other less probable states with lower conductance (Urry et al. 1984c; Venkatachalam et al. 1984).

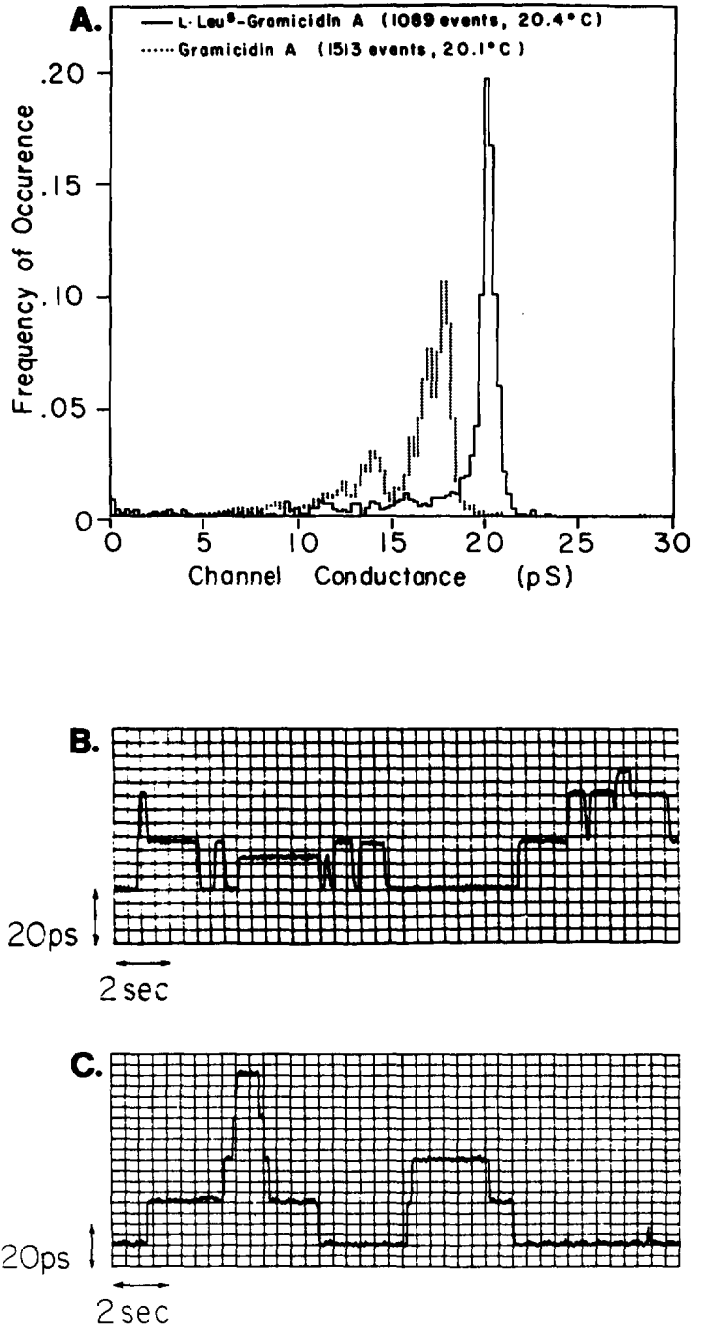

FIGURE 18

A. Conductance histograms for Gramicidin A (....) and L•Leu^5-Gramicidin A (-) at 100 mV, 1 M KCl and 20 °C in diphtanoyl lecithin/n-decane membranes. From Venkatachalam et al. 1984. Copyright 1984, John Wiley & Sons, Inc. Single channel conductance traces for Gramicidin A (B) and L•Leu^5-Gramicidin A (C) under these conditions.

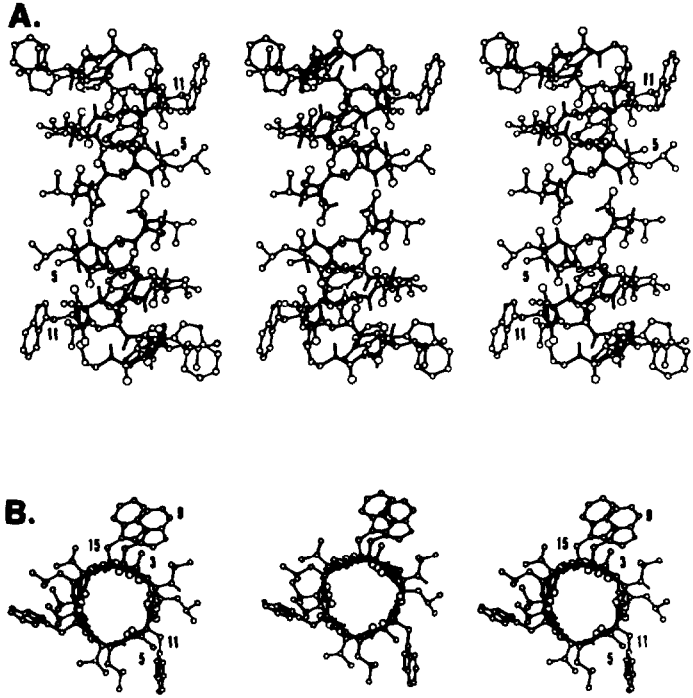

FIGURE 19

Stereo views of the sideview of the Gramicidin A channel (A) and of a channel view taken from the solution end of a monomer (B). For crosseye (close) viewing, cover the right-hand structures and use the left-hand pair; for walleye (distance) viewing, cover the left-hand structures and use the right-hand pair. These stereo views show the orientation of side chains for a favorable in vacuo distribution. This is one of a class of states with similar energy but with different side chain distributions. Adapted from Venkatachalam and Urry 1983. Copyright 1983, John Wiley & Sons, Inc.

This multiplicity of conducting states is a challenging issue to resolve in connection with abundant data arguing that the same channel backbone structure is responsible leading to the proposal that the differing conductance states arise from different side chain distributions with lifetimes on the order of seconds. One preferred side chain distribution is seen in figure 19 (Venkatachalam and Urry 1983). In an analysis of side chain mobility, it was found that the Trp side chain was especially free to occur in different rotameric states, partly because it is at the end of the **β-**helix but also because the residue on the adjacent turn of helix, residue-5, is an alanine residue with a small side chain. This allows the Trp side chain to reside

over the Ala side chain, much as the Trp side chain is seen in the distribution of figure 19 to lay up over the Ala side chain. Thus, it was reasoned that replacement of Ala by a residue with a bulkier side chain should restrict to a significant extent some of the rotameric states available to the Trp side chain and thereby decrease the multiplicity of states. To test this perspective, the Leu -GA analog was synthesized. As shown in figure 18, the probability of the most likely conductance state has been doubled. This provides a demonstration of a most intriguing and subtle means of modulation of conductance. Simply by altering or restricting the orientation of a side chain not directly involved in cation contact can markedly alter conductance. The mechanism is thought to involve the energetics of peptide libration. As the ion enters the channel, the peptide carbonyls must librate inward toward the channel center in order to coordinate adequately the cation. The energetics of this peptide libration are viewed as being dependent on the orientation of the side chain of the involved carbonyl. Accordingly, there are many ways to modulate the effective conductance of channels and it will be interesting to see if any of those which are exhibited by the Gramicidin A channel system will be analogous to the manner in which opioid peptides increase potassium ion conductance in nerve tissue.

Further Comments on Mechanisms at a Membranous Site

The single channel conductance traces in figure 18B and C were obtained using a cell with a teflon partition between two chambers and with a small hole in the partition over which a planar bilayer is formed. This is basically the Mueller and Rudin (1967) approach. In order to improve signal to noise and membrane stability to voltage ranges very small membrane areas are desirable. These small areas can be obtained by using a glass pipette drawn to a fine diameter of a few microns and by further preparing the tip to decrease noise and make it suitable for lipid bilayer coverage. This approach has been used by Andersen (1983) in studies on Gramicidin A and is essentially what has become known as the patch clamp technique so well developed by Sakmann and Neher (1983). This approach, which can carry the Gramicidin A problem to the important refinement of understanding side chain distribution issues, has found its greatest application to cells and to small membrane patches from cells. The mechanics of the process are schematically shown in figure 20 (Hamill et al. 1981).

This means that channels of suitable nerve and other cells can be studied much as the Gramicidin A channel has been studied and that the response of these channels to neurotransmitters and other effectors, such as the opioid peptides, can be assessed. It is not unreasonable to expect in the future that the patch clamp approach will provide more specific and informative assay systems for opioid peptides and will also provide simpler systems on which to dissect ionic transport mechanism.

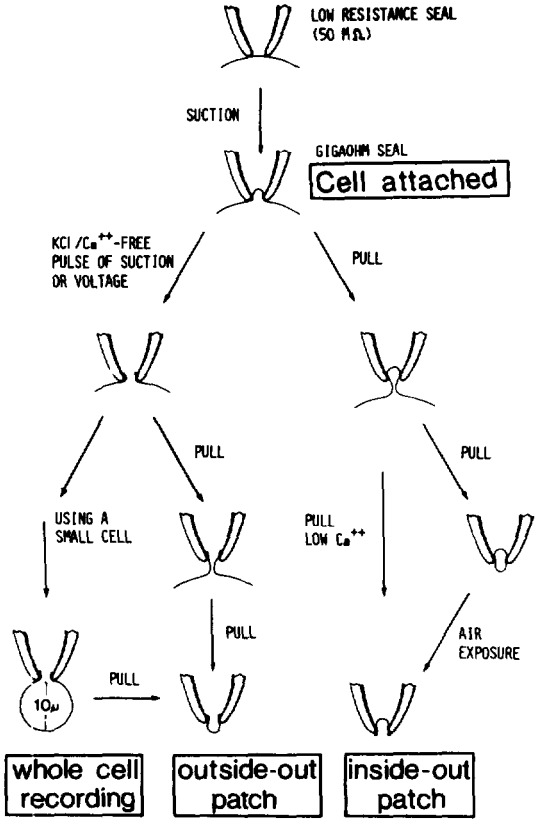

FIGURE 20

The patch clamp experiment (schematic representation). From Hamill et al. 1981. Copyright 1981, Pflügers Arch.

A particularly intriguing use of the patch clamp pipette technique, which has relevance to the neurotransmitter release activity of opioid peptides, is the study of chromaffin cells by Neher and Marty (1982). Patch clamp pipettes were used to measure the cell membrane capacitance of small adrenal chromaffin cells. Cell membrane capacitance was observed to exhibit step-like changes (2 to 6 x 10^{-14}+F) under conditions of enhanced release of chromaffin granules. Quoting directly from the Neher and Marty paper, "The size distribution of step-like capacitance changes is well compatible with the idea that steps of capacitance increase reflect individual events of exocytosis of chromaffin granules, whereas steps of the opposite polarity reflect the formation of vesicles or vacuoles by endocytosis."

It appears over the entire range, starting with the synthesis and conformational studies of analogs and spanning to the emerging means for studying mechanisms at membranous sites, that the study of the mechanism of action of the opioid peptides and opiates is entering a most challenging and productive period.

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Opioid Peptides: Analysis of Specificity and Multiple Binding Modes Through Computer-Aided Drug Design and Structure-Activity Studies

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INTRODUCTION

Analysis of the structure-activity data in the area of opioid peptides and opiates is severely compromised by the presence of multiple receptors, by incomplete pharmacological characterization of compounds in the literature, and by oversimplification of possible drug-receptor interactions. We will use our efforts to determine the receptor-bound conformation of morphiceptin and its relationship to enkephalin to illustrate both the approaches we have developed (Marshall et al. 1979) and the pitfalls which we have encountered. Morphiceptin is a synthetic opiofd peptide, Tyr-Pro-Phe-Pro-NH_2, with moderate opioid agonist activity and extreme selectivity for the mu opiate receptor. A mu receptor pharmacophore was proposed for morphfceptin based on the structural homology with enkephalin and other nonpeptide opioids. In order to simultaneously test the pharmacophore hypothesis and increase the potency and selectivity of morphiceptin, morphiceptin analogs were synthesized with potentially important chemical groups modified or eliminated, as well as conformationally constrained analogs. Mu receptor agonist and antagonist potency was determined in the stimulated guinea pig ileum (GPI) bioassay. Mu receptor affinity and delta receptor affinity were determined in receptor binding assays. The mu receptor agonist potency and affinity correlated well.

The structure-activity relationships of the morphiceptin analogs refined the pharmacophore hypothesis. The activity of the analogs with potentially important chemical groups, modified or eliminated, supported a three-group pharmacophore hypothesis. There was a strict requirement for the Tyr-1 nitrogen group, Tyr-1 phenolic group, and Phe-3 phenyl group. These groups are similarly required for activity in enkephalin and nonpeptide opiates. However, the Phe-3 phenyl of morphiceptin must either bind to a different subsite on the mu opiate receptor or to a similar subsite of the mu opiate receptor in a different orientation than the corresponding group (Phe-4) of enkephalin. Furthermore, there are strict conformational requirements for the activity of morphiceptin.

The study of the structure-activity relationships of morphiceptln by computer analysis of conformation and computer graphics further refined the pharmacophore hypothesis. The active and conformationally constrained analogs of morphlceptin can produce a common pharmacophore In three dimensions. Morphiceptin cannot interact with the mu opiate receptor when the pharmacophore groups are in the same relative orientation as the corresponding groups in the nonpeptide opiates. Morphfceptin can interact with the opiate receptor with the pharmacophore groups in the same relative orientation as the corresponding groups in enkephalin, but with a very different backbone arrangement. The results of these computer studies show that opioids with different overall constitution may interact with the mu opiate receptor in very different modes.

METHODS

Molecular Modeling

The molecular modeling system consisted of a VAX-11/750 and an NEC advanced personal computer as a color raster graphics terminal. The system was equipped with SYBYL (Trfpos Assoc., St. Loufs, MO), a computer-aided drug design software package.

The peptide models were assembled with standard amino acid fragments which were obtained by using the Empirical Conformation Energy Program for Peptides (ECEPP) (Momany et al. 1975; Nemethy et al. 1983) and a standard amide bond geometry Bendetti 1977). which represented averaged crystallographic data. Unusual amino acids and nonamino acid groups were constructed from standard fragments of chemical groups derived from crystallographic data, and their geometry was optimized using a molecular mechanics program with a standard force field (White 1978; Labanowskf and Marshall, in preparation).

The computer program SEARCH systematically determined all possible sterically allowed conformations of a molecule by incremently changing the torsfonal angles of freely rotatable bonds and discarding the conformations for which there were repulsive van der Waals interactions (Bosshard et al. 1972). A hard sphere potential model, with the van der Waals radii calibrated against protein crystallographic data, was used to calculate the repulsive van der Waals interactions (Marshall et al. 1974). The program SEARCH also simultaneously determined the specified distances that define each relative orfentatfon of specified chemical groups for each sterically allowed conformation of a molecule. The set of distances that define the relative orientations of the specified chemical groups produced by all the sterfcally allowed conformations of molecules was defined as an orientation map. The program SEARCH also determined the mathematical intersection of the orientation maps for a set of different molecules with the corresponding chemical groups, which represented the common orientations of the specified chemical groups for the set of molecules. The

orientation map was represented by a table of the observed maximum and minimum distances, a table of vectors which described the set of all possible distances, or three-dimensional plots of three of the possible distances. Conformations of molecules that produced the entries in orfentation map were retrieved and examined by molecular graphics.

The same SEARCH program also was able to determine all the sterically allowed conformations of a molecule, as described previously, and simultaneously output vectors produced by a specified pair of atoms relative to a fixed aggregate of atoms for each conformatfon of a molecule (Marshall et al. 1983). The vector map common to a set of different molecules could also be determined and it indicated that sterically allowed conformations existed where the specified aggregate and pair of atoms can be superimposed.

A molecular mechanics program MAXIMIN was used to minfmfze the energy of a set of molecules with the options to simultaneously superimpose specified aggregates of atoms common to a set of molecules; and it also was used to fix the relative orientation of aggregates of atoms within molecules (Labanowski and Marshall, in preparation). The parameters of MAXIMIN were derived from a literature valence force field (White 1978).

Radioligand Binding Assay

The saturation and competition binding assays for opiate receptors were performed by a modification of literature methods (Pert and Snyder 1973a; Chang et al. 1979; Chang and Cuatrecasas 1979; Gilian et al. 19791. The binding reaction was Initiated by the addition of freshly thawed homogenate to a solution of radfolfgand in the presence or absence of cold ligand in 50 mM Tris-HCl, pH 7.5 at 24 °C with a total reaction volume of 1 ml. The reaction was incubated for 60 minutes at 24 °C and was terminated by the addition of 4 ml of 50 nM Tris-HCl, pH 7.5 at 4 °C, and rapid filtration through a Whatman glass-fiber filter (GF/F). The filters were washed twice with 4 ml 50 mM Tris-HCl, pH 7.5 at 4 °C. The fflters were shaken in 5 ml scintfllatfon cocktail and the radioactivity determined with an efficiency of 38% to 42%. The assay was performed using rat brain homogenate (0.5 mg protein per ml assay volume), NMB cell homogenate (1.0 mg protein per ml assay volume), or NG108-15 cell homogenate (0.25 mg protein per ml assay volume).

Saturation binding isotherms were performed using NMB cell homogenate and the radiolfgand [^{3}H]-DADLE at eight concentrations, from 0.1 to 20 nM, in the presence and absence of 2.5 µM levorphanol. Saturation binding isotherms were also performed using NG108-15 cell homogenate and the radioligand [^{3}H]-DADLE at efght concentrations. from 0.1 to 20 nM, in the presence or absence of 2.5 µM DADLE. Similarly, saturation binding isotherms were performed using rat brain homogenate and

the radfoligand [^{3}Hl-DADLE at eight concentrations. from 0.1 to 20 nM, in the presence and absence of 2.5 µM levorphanol.

Competition binding isotherms were performed using NMB cell homogenate with the radfolfgand [^{3}H]-DADLE at a concentration of 1.0 nM in the absence or presence of 6 to 10 concentrations of cold competing lfgand and 2.5 µM levorphanol. Competition binding isotherms were also performed using NG108-15 cell homogenate wfth the radfolfgand [^{3}H]-DADLE at a concentration of 1.0 nM In the presence and absence of 6 to 10 concentrations of cold competing lfgand and 2.5 µM DADLE. Sfmflarly, competition binding isotherms were performed using rat brain homogenate and the radfolfgand [^{3}H]-DHM at a concentration of 0.24 nM in the presence and absence of 6 to 10 concentrations of cold competing lfgand and 2.5 µM levorphanol.

Nonspecific binding was defined as the binding in the presence of 2.5 µM levorphanol or DADLE, and specific binding was defined as the total binding minus the nonspecific binding. The receptor binding assays were performed wfth each combination of radfoligand and cold competing lfgand concentration in triplicate within an experiment, and experiments were performed in triplicate.

Receptor Binding Assay Data Analysis

Binding data from experiments wfth rat brain and NG105-15 cell homogenate were analyzed with LIGAND (Munson and Rodbard 1980). This program fits a mass action-based mathematical model for receptor binding, involving one or more lfgands and receptors, by nonlinear regression to radfoligand binding data. Binding affinity (Kq_3) and capacity (B_{max}) for [^{3}H]-DADLE and NG1D8-15 cells, or [^{3}H]-DHM and rat brain, were first calculated for each saturation binding isotherm individually. Similarly, Kd for each cold competing lfgand was calculated for each conpetition isotherm, using the previously calculated binding affinity of the radfolfgand. Nonspecific binding was treated as a fftted parameter. The variance model for weighting was for constant percent error. The data are reported for fits with a single binding site model. The arlthmatfc mean and standard error of the mean of B_{max} and the geometric mean and standard error of the mean of Kd were calculated for replicate binding isotherms.

The data for the binding isotherms using the NMB cell homogenate were treated in the traditional manner. Kd and B_{max} were calculated by linear regression of Scatchard plots for each saturation binding isotherm (Scatchard 1949). The IC_{50} for each cold competing lfgand was calculated for each competition binding isotherm using SIGMOID (DeLean et al. 1978). SIGMOID is a program which fits a four parameter logistic function to radfoligand displacement data by nonlinear regression. and interpolates the IC_{50}. The (Ki) for each competing lfgand, in each competition binding isotherm, was calculated according to

the Prusoff equation, from the IC_{50} of the competing ligand. the concentration of the radioligand, and the affinity of the radioligand (Cheng and Prusoff 1973).

Guinea pig ileum bioassay

Opiate activity of potential opioid compounds was determined in the coaxially stimulated GPI bioassay (Gyang and Kosterlitz 1966; Pert and Snyder 1973a, 1973b). Male albino guinea pigs with an average weight of 350 g were killed by a blow to the head and the small intestine was quickly removed. The distal 10 cm of the ileum and the duodenum were discarded and 3 cm strips of ileum were hung via silk in a 15 ml organ bath from an F50 myograph microdisplacement transducer with a 1 g resting tension. The organ bath contained Krebs-Ringer bicarbonate solution (120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$ 2.5 mM $CaCl_2$ 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$. 10 mM dextrose) with 20 µM choline chloride and 0.125 µM mepyramine maleate at pH 7.4 and 37 °C bubbled with 95% O_2/5% CO_2. The ileum was stimulated via silver electrodes transmurally using a Grass SD9 stimulator with supramaximal voltage (0.2 Hz, 1.5 ms). and the contractions were monitored with a Physiograph DMP-4B recorder. The tissue was allowed to equilibrate in the bath for 15 to 30 minutes.

The compounds to be evaluated were added to the bath in 10 to 100 µl aliquots, and were allowed to stay in the bath until maximal inhibition of contraction occurred. Then the bath contents were removed and the tissue was washed four times over 15 to 30 minutes. For a typical dose-response study of a compound, a minimum of four doses which produced 10% to 90% inhibition of contractions were used. Potency was defined as the IC i.e the concentration of an agonist which produced 50% inhibition of contractions. The IC was calculated by linear regression of the LOGIT transformation (Rodbard and Frazier 1972) of percent inhfbftion of contraction [LOGIT(%inhibition) = Ln(%inhibitionl(l%inhibition))] versus the log of the concentration of the compound, and interpolation to the concentration of the agonist that produces 50% inhibition of contraction. The IC for DADLE was determined in each tissue so that the %relative potency could be calculated for each compound tested in the same tissue. The % relative potency was defined as 100 x IC_{50} DADLE/IC_{50} compound. The geometric mean and standard error of the mean of the IC_{50} and %relative potency for each compound were calculated from dose-response studies with at least three tissues. For each compound that an IC_{50} could be determined, a supramaximal dose was added to the ba to demonstrate complete inhibition of contractions (i.e., full agonist activity) and 200 nM naloxone was added to the bath to demonstrate that the inhibitory activity was completely reversed. In addition, compounds which exhibited little or no inhibitory activity were checked at concentrations at which little or no inhibitory activity was observed for their ability to block or antagonize the inhibitory activity of DADLE.

The sensitivity of the opiofd agonist, morphiceptin, to naloxone antagonism was examined according to a modification of a method described by Kosterlitz and Watt (1968). A dose-response study was performed, and the IC_{50} calculated, for each agonist. both without and with three concentrations of naloxone (4.12, 20.6, 103, and 515 nM). The pA_2 Ke, and slope were calculated by linear regression of a Schild plot of the log (dose ratio - 1) versus the log ([naloxone]/nM), for each agonist. The arfthmatfc mean and standard error of the mean of the slope and pA_2. and the geometric mean and standard error of the mean of the Ke, were calculated for replicate experiments. The opiofd agonists used were Met^5-enkephalin, ethylketocyclazocine, and morphiceptin.

Chemistry of morphiceptin analogs

All solvents were HPLC grade and chemicals were reagent grade. Free and protected amino acids were purchased except for 2-aminoindane-2-carboxylate (Mauger and Ross 1962), trans-3-phenylproline (Cox et al. 1964; Sarges and Tretter 1973), and 2-methylproline (Ellington and Honigberg 1974) which were prepared by methods reported in the literature. Benzhydrylamine polystyrene 1%-crosslinked divinlybezene resin was synthesized by the method of Pietta et al. (1973); p-methylbenzhydrylamine polystyrene 1%-crosslinked divinylbenzene resin was purchased.

Melting points were determined on a Hoover capillary apparatus and microanalyses were performed by Robertson Laboratory of Florham Paris, New Jersey. Optical rotations were performed using a Perkin-Elmer 241 automatic polarimeter with a 1 dm cell. Proton magnetic resonance spectra were recorded at 60 MHz using a Perkin-Elmer T60 spectrometer or at 100 MHz using a Varian XL100 spectrometer. The IR spectra were recorded on a Perkin-Elmer 7108 spectrometer for compounds as a KBr pellet. Fast atom bombardment mass spectra were recorded on a Finfgan 3200 mass spectrometer equipped with a FACS fast atom bombardment gun using Xe gas at 6 to 8 Kev. Two to ten µg of each sample in 1 µl MeOH was applied to a glycerol matrix of the probe for analysts. Twenty five to fifty µg of each peptide was examined for homogeneity by thin-layer chromatography (TLC) using precoated silica plates. The peptides were visualized with iodine vapor, chlorox/starch-KI, and/or ninhydrin. All peptides showed a single spot on TLC. Hydrogenations were carried out on a low pressure Parr hydrogenator at room temperature. Selected protected peptides were purified by medium pressure liquid chromatography on Lobar silica columns (E. Merck) with various solvent systems.

For amino acid analysis, the peptides were hydrolyzed at 110 °C for 24 hours in deaerated tubes in 6N HCl and 0.5% phenol with Nle as an internal standard. Amino acid analysis was performed using one of the following methods: a procedure described by Benson and Hare (1975) and modified to include a Glenco MM amino acid analyzer equipped with a dual beam photometer (570 nm and

440 nm), and Hewlett-Packard 3390A and Spectra-Physics Minigrator integrators; or the procedure of Bohlen and Mellet (1979) using a Waters amino acid analyzer equfpped with a filter fluorometer optimized for o-phthalaldehyde amino acid derivatives, and a digital integrator; or a modified PTC procedure (Henrickson and Meredith 1984).

Selected analogs of morphfceptin were synthesized by the solid phase method. Some analogs were synthesized by this method because of the side reactions encountered in the initial solution phase synthetic strategies. The peptides were synthesized on the benzhydrylamine (Pietta et al. 1973) and p-methylbenzhydrylamine (Matsueda and Stewart 1981) polymers, which afford a C-terminal amide. Standard initial attachment and elongation protocols were used, with DCC for coupling, BOC groups for N-terminal protection, Bzl groups for side-chain protection of tyrosine, and TFA for BOC removal. Extended coupling times and double coupling cycles were used in the syntheses with the sterically hindered amino acids: 2-methylproline, N-methyl-D-alanine, N-methylphenylalanine, 2-aminoaminoindan-2-carboxylate, and cycloleucine. The final peptide was deprotected and cleaved from the polymer by either the high HF or high/low HF (Tam et al. 1983) procedures, and was worked up by standard procedures.

Selected analogs of morphiceptin were synthesized by solution methods, including those substituted wfth sterfcally hindered amino acids and C-terminal phenyl alkyl amides that are not easily incorporated by solid phase methods. Multiple synthetic strategies were chosen to take advantage of common fragments and afford the peptides in high yield and purity. The synthetic strategies used common coupling reagents and protecting groups. DCC with HOBT (Konig and Geiger 1970). EDCI with HOBT (Sheehan et al. 19651, PPA (Wissmann and Kleiner 19801, and DPPA (Hamada et al. 1977) were the coupling reagents used. EDCI and PPA were preferable because no purification other than the standard workup was usually necessary.

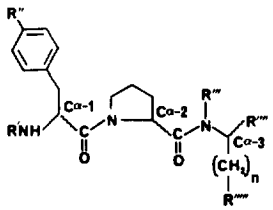
Preparative RP-HPLC is becoming a standard analytical and preparative technique that is extremely valuable in purification and analysis of synthetic peptides. The preparative techniques at the time this work was begun were either for large gram scale purifications using the Waters Prep-500 HPLC equipment (Rivier et al. 19841 and very expensive columns, or for the smaller mg scale purifications using the conventional HPLC equipment and small semipreparative columns. This prompted the development of an intermediate scale preparative RP-HPLC methodology. Twenty to eighty mg of each deprotected peptide was chromatographed on a 22 x 250 mM Zorbax 5 µM ODS column. The peptides were eluted with isocratic conditions at 5% B for 10 minutes, a concave gradient between 5% and 20% to 35% B over 10 to 30 minutes, and isocratic conditions at 20% to 35% B for 1 to 2 hours, at 8.0 ml/minute while monitoring absorbance at 280 nM. A was 1.1 N HCOOH in H_2O with the pH adjusted to 3.0 with TEA, and B was

MeOH. The homogenous fractions demonstrated by analytical RP-HPLC were subsequently desalted by chromatographing on a Waters 4.6 x 250 mM µBondapack ODS column. The peptide was applied to the column, the salts eluted with isocratfc conditions of 100% A, and the peptide eluted with a sharp linear gradient of 0% to 75% B, where A is 0.1 N CH_3COOH and B is MeOH. Lyophflization from dilute CH_3COOH afforded homogeneous peptides as acetate salts with yields of 30% to 90%.

RESULTS

Structure-Activity of Morphiceptin Analogs

The analogs prepared are shown in figures 1 and 2. Figure 1 concentrates on analogs useful in testing the pharmacophore hypotheses. Figure 2 shows compounds designed with different conformational constraints necessary to determine the receptor-bound conformation. The opiate activity of the synthetic morphiceptin analogs was measured in the stimulated GPI bioassay



#	R'	R''	R'''	R''''	R'''''	Cα-1	Cα-2	Cα-3	(CH2)n
1	-H	-OH	-H	-CO-Pro-NH_2		L	L	L	1
2	-H	-H	-H	-CO-Pro-NH_2		L	L	L	1
3	-H	-OH	-H	-CO-Pro-NH_2		D	L	L	1
4	-Ac	-OH	-H	-CO-Pro-NH_2		L	L	L	1
5	-H	-OMe	-H	-CO-Pro-NH_2		L	L	L	1
6	-H	-OH	-H	-CO-Pro-NH_2		L	L	L	1
7	-H	-OH	-H	-CO-Pro-NH_2	NO_2	L	L	L	1
8	-H	-OH	-H	-CO-Pro-NH_2		L	L	D	1
9	-H	-OH	-H	-CO-Pro-NH_2	NO_2	L	L	D	1
10	-H	-OH	-H	-H		L	L	-	0
11	-H	-OH	-H	-H		L	L	-	1
12	-H	-OH	-H	-H		L	L	-	2
13	-H	-OH	-H	-H	NO_2	L	L	-	3
25	-H	-OH	-Me	-CO-Pro-NH_2		L	L	-	1

FIGURE 1

Selected Structures of Morphiceptin Analogs The numbering scheme corresponds with that in the tables of biological data.

for each morphiceptin analog, DADLE and morphine. The analogs of isorphiceptin produced parallel dose-response curves in the bioassay, consistent with their interaction at a common receptor and full agonist activity (table 1).

The sensftfvfty of morphiceptin to naloxone antagonfsm was studied in order to determine whether the agonist activity of morphfceptin was mediated by the μ or κ opiate receptor. Typically, μ agonists are more sensitive to naloxone than κ agonists. but both are antagonized by high doses of naloxone, in the GPI bioassay (Lord et al. 1977). Dose-response studies were performed with Met-enkephalin, a μ agonist in this bfoassay; ethylketocyclazocine. a κ agonist in this bioassay; and morphiceptin, with and without three concentrations of naloxone. Parallel shifts in the dose-response curves were observed for morphfceptin with increasing concentrations of naloxone, i.e., the same slope and maxima, as well as Met-enkephalin and ethylketocyclazocine, and a Schild plot of the data were linear with a slope that was not significantly different from unity.

These results are consistent with naloxone acting as a competitive antagonist of morphiceptin (Lord et al. 1977). The pA_2, and Ke for naloxone antagonism of the agonist activity of morphiceptin were not significantly different-from that of Met-enkephalin ($P<0.05$), a μ agonist in this bioassay, but were significantly different from that of ethylketocyclazocine ($P<0.05$), a κ agonist in this bioassay. The activity of morphiceptin is mediated by a receptor that has a similar affinity for naloxone as the receptor that mediates the activity of Met-enkephalin, but a different affinity from the receptor that mediates the activity of ethylketocyclazocine.

A competition binding assay was used to determine the affinity of the synthetic morphfceptin analogs for the μ and 8 opiate receptors. The appropriate combinations of opiate receptor selective radioligands and sources of opiate receptor were used to obtain the most accurate measure of affinity for each receptor. μ-Receptor affinity was measured in a competition binding assay using [^{3}H]-DHM, a relatively specific μ ligand (Chang and Cuatrecasas 1979; Gillan et al. 1979), and rat brain homogenate, which contains a mixed populatfon of opiate receptors (Chang and Cuatrecasas 1979; Chang et al. 1981). δ-Receptor affinity has often been measured in a competitive binding assay using [^{3}H]-DADLE, a moderately δ-specific lfgand (Kosterlitz et al. 1981), and rat brain homogenate.

However, since [^{3}H]-DADLE has an affinity for the δ receptor that is only fivefold to tenfold greater than the affinity for the μ receptor at concentrations required for a reasonable signal in a competition binding assay, a significant proportion of the ligand occupies the μ receptor as well as the δ receptor at the concentrations required for the competition

binding-assay. As a result, competition binding assays with highly μ- specific competing ligands, such as morphfceptin. produce shallow or biphasic displacement curves and afford inaccurate estimates of the true δ-receptor affinity (Chang et al. 1981a, 1981b). Since more selective δ radiolfgands are not yet commercially available. an alternative technique was chosen. [^{3}H]-DADLE was used, but with NG108-15 murine neuroblastoma x rat glioma and NMB human neuroblastoma cell homogenates, both homogeneous sources of δ receptors (Chang et al. 1978; Simantov and Snyder 1976; Ard and Gottlfeb, in preparation).

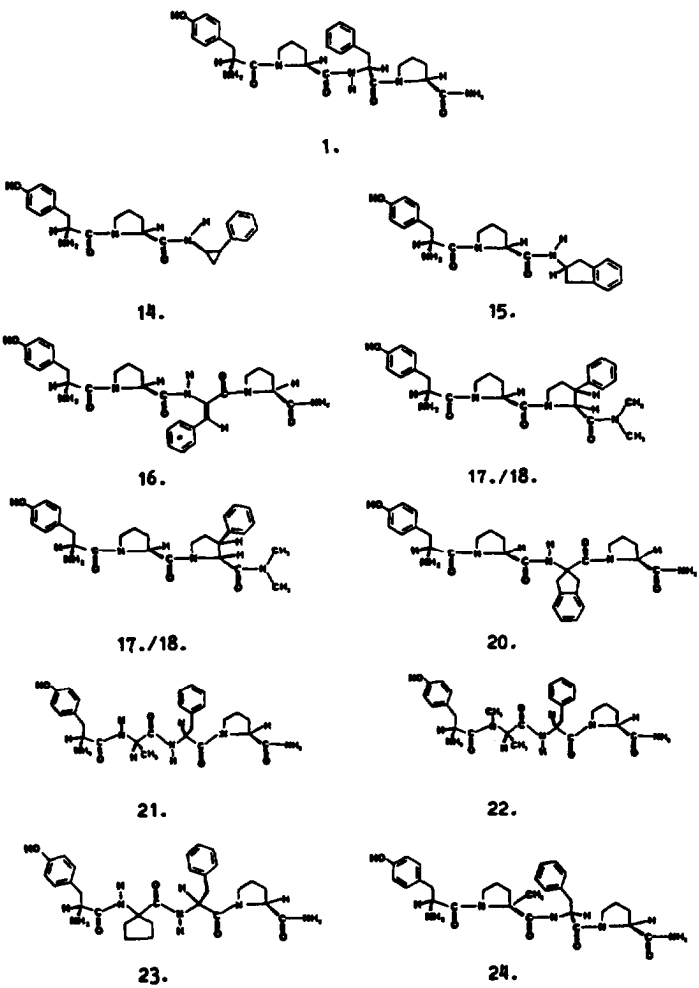


FIGURE 2
Selected Structures of Morphiceptin Analogs The nunbering corresponds with that in the tables of biological data.

TABLE 1

Summary of Biological Activity of the Morphiceptin Analogs as Determined in the Stimulated Guinea Pig Ileum Bioassay

Compound	IC_{50}/nM[a]	SEM	%Rel.Pot.[b]	SEM
1. Tyr-Pro-Phe-Pro-NH_2	136	0.6	100	11
2. Phe-Pro-Phe-Pro-NH_2	9,280	2,110	1.95	0.33
3. D-Tyr-Pro-Phe-Pro-NH_2	9,980	510	1.04	0.13
4. NAc-Tyr-Pro-Phe-Pro-NH_2[c]	>20,400	--	<0.49	--
5. Tyr(OMe)-Pro-Phe-Pro-NH_2	4,690	1,040	3.37	0.48
6. Tyr-Pro-Cha-Pro-NH_2[c]	>10,000	--	<1.61	--
7. Tyr-Pro-pNO_2Phe-Pro-NH_2[c]	9,280	2,110	1.95	0.33
8. Tyr-Pro-D-Phe-Pro-NH_2	143	18	165	8
9. Tyr-Pro-D-pNO_2Phe-Pro-NH_2[c]	>10,000	--	<3.2	--
10. Tyr-Pro-NH(benzyl)	3,160	810	4.51	0.34
11. Tyr-Pro-NH(phenethyl)	156	14	102	10
12. Tyr-Pro-NH(phenylpropyl)	9,770	1,820	2.16	0.24
13. Tyr-Pro-NH(pNO_2phenethyl)[c]	>10,200	--	<1.72	--
14. Tyr-Pro-NH(trans-2-phenyl-cyclopropyl)	170	20	153	22
15. Tyr-Pro-NH(2-indanyl)	24.6	3.6	668	102
16. Tyr-Pro-Z-ΔPhe-Pro-NH_2[c]	>14,900	--	<0.49	--
17. Tyr-Pro-Pro-tPhpro-$N(CH_3)_2$ *HPLC fraction 1	40.7	2.7	247	27
18. Tyr-Pro-Pro-tPhpro-$N(CH_3)_2$[c] *HPLC fraction 2	17,100	2,500	1.23	0.10
19. Tyr-Pro-NMePhe-Pro-NH_2	182	11	90.2	4.0
20. Tyr-Pro-Ain-Pro-NH_2	>10,000	--	<3.05	--
21. Tyr-D-Ala-Phe-Pro-NH_2	232	16	96.9	10.9
22. Tyr-D-NMeAla-Phe-Pro-NH_2	138	26	143	18
23. Tyr-Cleu-Phe-Pro-NH_2[c]	>10,000	--	<3.05	--
24. Tyr-D,L-2-MePro-Phe-Pro-NH_2[c]	>10,000	--	<3.07	--
25. Tyr-D-Ala-Gly-Phe-D-Leu	16.7	0.6	1029	--
26. Morphine	190	35	162	17

[a]All compounds for which the IC was determined were full agonists, and their activity was fully reversible by 200 nM naloxone.

[b]%Relative potency (%Rel.Pot.) was normalized to morphiceptin.

[c]Compounds for which an IC could not be determined were also ineffective in antagonizing DAE.The IC_{50} and %Rel.Pot. are expressed as the geometric mean and standard error of the mean (SEM). The IC_{50} and %Rel.Pot. were determined according to the described experimental methods.

Competition binding isotherms were performed with each morphiceptin analog, DADLE and morphine, using each combination of radfolfgand and receptor preparation. The Kd was calculated with the computer program LIGAND (Munson and Rodbard 1930) for each analog from the competition binding assays using [^{3}H]-DHM and rat brain homogenate, and using [^{3}H]-DADLE and NG108-15 cell homogenate. The competition binding isotherms were monophasic and were best fit by the single site model (data not shown).

The structure-activity relationships for morphiceptin analogs in the GPI bioassay and the μ-receptor binding assay will be discussed together since there was such a strong correlation between agonist potency and affinity. The correlation of the log of the %relative potency and the log of the %relative affinity for the morphiceptin analogs produced a correlation coefficient of 0.93. This suggests that the changes in agonist potency for this series are due to changes in affinity. In no cases were partial agonists or antagonists encountered in the bioassay. A partial agonist would be expected to show a decrease in potency without a decrease in affinity, and would lie off the line of correlation of affinity and potency. This correlation also suggests that the receptor in the bioassay and binding assay are very similar, even considering that they are from different species and different parts of the nervous system.

The structure-activity relationships for morphiceptin at the mu opiate receptor support the μ-receptor pharmacophore hypothesis stated previously for morphiceptin. The smallest fully active fragment of morphiceptin is Tyr-Pro-NH(phenethyl) [11], suggesting that the terminal Pro-NH_2 is not required for activity. This indicates that pharmacophoric groups are limited to this fully active fragment of morphiceptin.

The Tyr^1 tyramine group of morphiceptin is very important for activity. The elfmination [2] or methylation [5] of the phenolic hydroxyl and the N-acetylation of the nitrogen [4] results in a dramatic loss in activity. The effects of these modifications suggest that the phenolfc hydroxyl could be interacting with the receptor via a hydrogen bond or ionic interaction.

The Phe^3 phenyl group of morphiceptin is also very important for activity. Reduction of the phenyl group to a cyclohexyl derivative [6] results in a dramatic loss of activity, suggesting an important role for a flat hydrophobic and, perhaps, aromatic group. Nitratfon of the phenyl group [7] also results in a dramatic loss in activity, possibly as a result of negative steric or electronic interactions with the receptor. In additfon, nitration of the D-Phe [9] and truncated phenethyl amide [13] analogs of morphfceptin also resulted in a dramatic loss in activity, suggesting that even analogs with different possible conformational freedom can not fold to accommodate the nftro group on the phenyl ring. Clearly, the activity of

morphiceptin is particularly sensitive to modificatfons of the Phe^3 phenyl group.

The remainder of the morphiceptin structure besides these three critical groups, the phenol group of Tyr^1, the amine group of Tyr^1, and the phenyl group of Phe^3, probably serves as a linker and orients these three pharmacophoric groups relative to each other. The loss of activity for the D-Tyr^1 analog of morphiceptin [3] is interesting and probably represents a form of stereospeciffcity much like that seen with the classical opioids (Pert and Snyder 1973a, 1973b; Cox and Weinstock 1966; Kosterlftz et al. 1973). The relative diastereomeric selectivity for the α-carbon of the morphiceptin Tyr^1 is about 50. With a minimum three receptor subsites, the receptor would be expected to be able to recognize the chfralfty in a ligand.

The remainder of the backbone may interact with the receptor directly, but the direct interaction is not necessary for activity. This is difficult to prove, but a few analogs are consistent with this conclusion. The high activity of the D-$NMeAla^2$ [22], D-Ala^2 [21]. $NMePhe^3$ [19], and $tPhpro^3$ [17] analogs of morphiceptin suggests that the μ receptor is somewhat tolerant of changes in steric volume of the backbone of morphiceptin. Furthermore, the high activity of the $NMePhe^3$ [19] and $tPhpro^3$ [17] analogs of morphiceptin suggests that the amide nitrogen is not functioning as an important hydrogen bond donor in the interaction with μ opiate receptor. There is a requirement for a nontyramine phenyl group for the activity of morphiceptin similar to enkephalin and selected nonpeptfde opiates (Morley 1980; Fournie-Zaluski et al. 1981; Gacel et al. 1981; Simon et al. 1981; Ophefm and Cox 1976), suggesting that the nontyramine phenyl groups are also interacting with a common subsfte of the opiate receptor. However, the loss of activity upon nitration of the phenyl group in morphiceptin, as opposed to the enhancement of activity upon nitration of the corresponding phenyl group in enkephalin (Fauchere et al. 1983; Schiller et al. 1983), suggests that either the phenyl group of morphiceptin binds to a different subsfte of the μ opiate receptor than does the corresponding phenyl group of enkephalin, or the phenyl group binds in an entirely dfferent orientation to the same subsite of the μ opiate receptor. In addition, the nitration of the D-Phe^3 [9] and phenylamfde [13] analogs of morphiceptin also decreases activity. Since these analogs might be expected to have different conformational possibflfties that would allow a unique orientation of the phenyl group relative to the tyramine group, and a decrease of the repulsive interactions between the receptor and the nftro group. the inactivity of the nitrated analogs of morphfceptin is more likely a result of interaction with a unique receptor subsite. It would be interesting to examine the activity of the meta-NO_2 Phe^3 and ortho-NO_2Phe^3 analogs of morphiceptin and enkephalin to further test the possibility that the phenyl group of morphiceptin is interacting with a unique opiate receptor subsite.

Many constraints in conformation that were imposed in morphiceptin analogs by substitution for the Phe^3 also maintained or enhanced activity at the μ opiate receptor. Most importantly, the 2-aminoindanyl amide analog of morphiceptin [15] was much more active than morphiceptin. It is possible that this increase of activity is a result of the favorably constraint of the torsional angle equivalents of the x^1 and x^2 of the Phe^3 of morphiceptin, since the indan ring has limited flexibility. It is interesting that the 2-indanylamide analog of morphiceptin [15] was active, but the homologous Ain^3 analog of morphiceptin [20] was inactive. Ain would be expected to be able to mimic the 2-indanylamide. However, the extra Pro-NH_2 in the Ain^3 analog [20], which was not present in the 2-indanylamide analog [15], could be fixed in a region of space that sterically disallows interaction the opiate receptor. The trans-2-phenylcyclopropyl amide analog of morphiceptin [23] was also as active as morphiceptin. In this analog, the torsion angle equivalent to the x^1 of the Phe of morphiceptin is constrained by the cyclopropyl ring.

The $tPhpro^3$ analogs of morphiceptin turned out to be very interesting. One of the diastereomers was 200-fold more active [17] than the other [18]. Again, it is likely that the difference in the activity of these diastereomers is a result of the conformational constraints imposed. By analogy with proline, the torsional angle equivalents of morphiceptin at Phe^3, ϕ, and x^1 should be relatively fixed (Balasubramanian et al. 1971; Madison 1977; Delaney and Madison 1982). and tPhpro is likely to restrict the freedom of preceding torsional angle ψ of Pro^2 (Marshall and Bosshard 1972; Tonelli 1976). The two isomers should differ significantly in the position of the phenyl group relative to the tyramine group. Perhaps, the different allowed positions of the phenyl relative to the tyramine in the two isomers is the factor which determines activity. It is also possible that one isomer is inactive because of another repulsive interaction of a nonessential group with the receptor. Unfortunately, the absolute configuration of tPhpro in the active or inactive isomers is not yet known. Certainly, the active diastereomeric tPhpro analog of morphfceptin will be most important in defining the active conformations of morphiceptin by conformational analysis.

Only selected analogs of morphiceptin were examined for δ-receptor binding affinity using the two different sources of δ receptors. The major objective was to determine if the analogs of morphiceptin that had a high affinity for the μ receptor had an increased or decreased δ-receptor affinity, and therefore, decreased or increased μ selectivity, relative to morphiceptin.

The $tPhpro^3$ analog of morphiceptin [17]. which had hfgher affinity for the μ receptor, had lower affinity for the δ receptor than morphfceptin [1], and potentially increased selectivity for the μ receptor, although an exact measure could not be determined because of the extremely low affinity for the

δ receptors. It is important to recognize that few opiofds exhibit the extreme ectfvfty shown in table 2 for the mu receptor as the tPhpro[3] analog of morphiceptin [17], without the sacrifice of affinity and potency at the μ receptor. It will be important to demonstrate that this analog also exhibits a very low affinity for the κ opiate receptor. as well as low agonfst potency for the δ and κ receptors. This analog of morphfceptin could prove to be most valuable as a selective tool in the study of the role of the mu opiate receptor in mediating the biological responses to opiofds.

TABLE 2

Summary of Morphiceptin Analog Binding Selectivity for the μ and δ Opiate Receptor Sites

Compound	Ki[δ^c]/Kd(μ[a])	Kd [δ^b]/Kd(μ[a])	Ki(δ^c)/Kd(δ^b)
1. Tyr-Pro-Phe-Pro-NH_2	>49	>2,210	
15. Tyr-Pro-NH(2-indanyl)	366	338	1.083
17. Tyr-Pro-Pro(trans -β-phenyl)-$N(CH_3)_2$ *HPLC fraction 1	>558	>7,480	--
22. Tyr-D-NMeAla-Phe-Pro-NH_2		251	--
25. Tye-D-Ala-Gly-Phe-D-Leu[2]	0.196	0.358	0.547
26. Morphine	155	155	0.998

[a]DHM in rat brain homogenate.
[b]DADLE in NG108-15 cell homogenates
[C]DADLE in NMB cell homogenates

Molecular Modeling

Opioid agonists and antagonists are recognized and bound by the opiate receptors via important chemical groups, and in addition, agonists activate the opiate receptors. There is a particular bound conformation of an agonist or antagonist that is the most thermodynamically stable for the receptor complex as a whole, with noncovalent interactions between the receptor and the agonist or antagonist optimized. Binding energy may be consumed in the deformation of the agonist or antagonist conformation from the most stable solution conformation. Therefore, it would seem to be important to define this receptor-bound conformation in order to design agonists and antagonists with the receptor-bound conformation prestabilized in solution. The result should be an increase in the thermodynamic stabflity of the receptor-bound agonist or antagonfst complex and, thus, an increase in potency and affinity. Furthermore, it should be possible to design agonists and antagonists which take advantage of the subtle differences in the conformational requirements of the subtypes of opiate receptors and exhibit receptor-subtype selectivity.

It is also important to realize that it is difficult to compare noncongeneric molecules in terms of conformation, because their chemical constitution is so different. For this reason, it is important to define the chemical groups that are dominant in the interaction with the receptor, i.e., the pharmacophore. Then the noncongenerfc molecules can be compared in terms of the possible orientations of the pharmacophore groups, rather than their possible conformations. Again, it is likely that the different subtypes of receptors may preferentfally interact with different pharmacophoric groups and/or different relative orientations of the pharmacophoric groups.

For rigid compounds, the relative orientation of the pharmacophoric groups is envfronmentally independent, and, therefore, the relative orientation of the pharmacophoric groups in states for which conformational analysis can be performed (NMR, X-ray, etc.) will not differ from that in the receptor-bound state. However, for flexible compounds, e.g., opioid peptides and many nonpeptide opioids, there are many possible orientations of the pharmacophorfc groups populated to varying degrees, and the possible orientations are also environmentally dependent. Without knowledge of the environment or structure of the receptor site, it is difficult to choose from the many possible receptor-bound orientations of the pharmacophore groups, if, indeed. there is a single one. The most likely orientation(s) of the pharmacophoric groups in states where the conformational analysis is performed may not resemble that when bound to the receptor.

It was originally thought that structure-activity studies of morphiceptin would provide further insight into the recognition and activation requirements at the μ opiate receptor involving enkephalin, Tyr-Gly-Gly-Phe-(Leu,Met). Structural homology to enkephalin suggested that morphiceptin interacted with the μ receptor in the same way as enkephalin. If this was the case, the structure-activity studies of morphiceptin could be combined with those of enkephalin, further refining the pharmacophore model for the highly flexible enkephalin analogs. Alternatively, if morphiceptin did not interact with the opiate receptor in the same way as enkephalin, structure-activity studies would have to proceed independent of enkephalin.

Initially, a pharmacophore was proposed by considering the structural homology of morphiceptin to enkephalin, and possibly to the representative nonpeptide opioids, morphine, GPA1657 and PEO. The Tyr^1 tyramine of morphiceptin could potentially be equivalent to the corresponding Tyr^1 tyramine of enkephalin and the tyramine of morphine, GPA1657, and PEO. In addition, the Phe^3 phenyl of morphiceptin could be equivalent to the Phe^4 phenyl of enkephalin and perhaps the nontyramine phenyl of GPA1657 and PEO. The Pro^2 probably functions as a linker to orient the tyramine and the phenyl relative to each other in the same way as the Gly^2-Gly^3 of enkephalin, or the D-Ala^2-Gly^3 of the most potent enkephalin analogs--perhaps the same way as the core structures of GPA1657 and PEO. In order to determine if morphiceptin was simply an

enkephalin analog and whether the Pro^2 of morphiceptin was a surrogate for the $D\text{-}Ala^2\text{-}Gly^3$ segment of enkephalin. the model fragments N-acetylproline methylamide [I] and N-acetyl-D-alanine methylamide [II] were examined using SEARCH. Sterically allowed conformations were determined and, simultaneously, the vectors produced by the methylamide nitrogen and carbon of [I] and [II] were plotted relative to the fixed acetyl group. There was clearly no intersection of the vector maps produced by the model peptides, indicating that the N-acetyl and methyl amide groups of the model peptides [I] or [III] were not superimposable in any of the sterically allowed conformations. Clearly, the Pro of morphiceptin is not a surrogate for the D-Ala-Gly segment of enkephalin. This is consistent with the idea that if the tyramine and phenyl groups of morphlceptin and enkephalin are interacting with the μ opiate receptor in the same relative orientation, the backbones must be very different, or the phenyl and tyramine groups are interacting with the μ opiate receptor in a very different relative orientation.

To further address the issue of whether the tyramine and phenyl of morphiceptin and enkephalin can interact with the μ opiate receptor in the same relative orientation, SEARCH was used in the same way as for morphlceptin by creating orientation maps. Preliminary results suggest that it may be possible for enkephalin to adopt a conformation in which the relative orientation of the tyramine and phenyl are similar to the corresponding constrained morphiceptin analogs [15] and [l7/18]. However, further studies are required, especially with the more rigid analogs of morphiceptin.

The conformationally constrained analogs of morphlceptin. Tyr-Pro-NH(2-indanyl) [15], and Tyr-Pro-tPhpro-$N(CH_3)_2$ [17/18] exhibit high activity at the μ opiate receptor. It was assumed that the tyramine and phenyl groups of these analogs, like their parent compound, morphlceptin [1] were similarly important for activity and interact with the same subsites of the μ opiate receptor as morphiceptin [1]. In other words, it was assumed that these compounds have a common μ-receptor pharmacophore.

SEARCH was used to determine the common relative orlentations of the tyramine and phenyl groups produced by the sterically allowed conformations of [15] and [17/18]. The studies followed four possible pathways in order to consider the possibility for the cis/trans isomerization for the Tyr-Pro amide bond in both [15] and [17/18] (Liakopoulou-Kyriakiades and Galardy 1979; Deslauriers et al. 1979). As a result, there were four possible orientation maps describing the common relative orientations of the tyramine and phenyl groups produced by the sterically allowed conformations of [15] and [17/18].

The conformational analysis was simplified by assuming that the tyramine of morphiceptin interacts with the μ opiate receptor with the same relative orientation of the amine and phenol groups as for the rigid opioids, morphine, GPA1657, and PEO. This conformation of tyrosine does not invoke any strong repulsive van der Waals

contacts. A similar assumption was made in molecular modeling studies of enkephalin (Gorin and Marshall 1977) and was actually supported by the high activity of enkephalin analogs in which the tyrosine was substituted with conformationally rigid subpieces of morphine (Shaw and Turnbull 1978; Deeks et al. 1983; Cardinaux and Pless 1984). According to molecular mechanics calculations and molecular graphics comparisons, these constrained tyrosine substitutes do constrain the tyramine to a low energy conformation which places the phenol and amine in the same relative orientation as observed in morphine, GPA1657, and PEO.

Using SEARCH, four orientation maps were determined according to the four strategies. Six distances were used to define the relative orientations of the phenyl relative to the tyramine (figure 3). All four pathways produced orientation maps that were circumscribed by maximum and minimum distances that differed by one to five angstroms (table 3). The orientation maps were visualized by graphical plots of dimensions 1-3 and 4-6 where the axes represent the defined distances (figure 4). These orientation maps demonstrate that these analogs of morphiceptin may interact with the opiate in four possible sets of common relative orientations of the tyramine and phenyl groups produced by conformations that were energetically reasonable, i.e., no repulsive intramolecular van der Waals contacts. It is important to observe that the superimposed orientation maps 1 and 2, and similarly 3 and 4, for the different $tPhpro^3$ diastereomeric morphiceptin analogs did not intersect significantly, suggesting that the difference in activity between these analogs is a result of the different allowed orientations of the phenyl group relative to the tyramine group. Identification of the active configuration of tPhPro will determine which of the orientation maps describes the relative orientations of the tyramine and phenyl groups of morphfceptin required for activity at the μ opiate receptor.

Conformations of [l51] and [17/18] derived from each of the orientation maps were selected, and MAXIMIN was used to determine four possible conformations of [l5] and [17/18] in which the tyramine and phenyl groups were superimposed with the least energy of deformation. This is desirable because of the increment used in SEARCH which may not optimize overlap of pharmacophoric groups. Again, there were four unique possibilities because of the cis/trans Tyr-Pro isomerism and the ambiguity in the absolute configuration of the tPhpro of [17/18] (figure 5). Identification of the absolute configuration of tPhpro in [17/18] and the amide configuration which is required for activity at the μ receptor will determine which conformation or relative orientation of the tyramine and the phenyl is most important for activity at the μ opiate receptor.

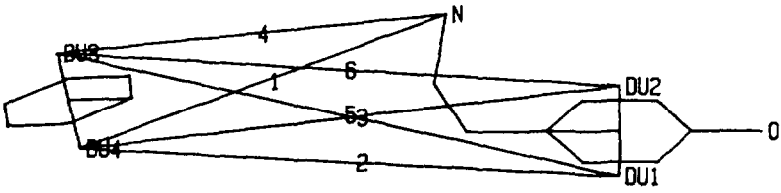


FIGURE 3

The Opiofd Pharmacophore and the Defined Orientation Map Distances 1-6

TABLE 3
Orientation Map Distance Ranges

Defined Distance Number	Distance Range (Minimum and Maximum in Angstroms) Orientation Map*			
	1	2	3	4
1	9 2 9 8	10 4-11 1	6.9-87	7 2 9 1
2	10.1-10.7	11.1-12.2	9.2-11.7	8.2-10.9
3	10.4-11.8	12.0-13.1	7.9-10.8	9.3-10.7
4	7.9-9.7	10.5-11.5	5.6-9.4	7.7-9.8
5	9.2-12.0	11.5-13.1	6.0-12.4	7.8-11.4
6	9.6-12.0	12.2-15.9	6.2-11.8	9.2-11.4

*Map 1 = Pro^2-trans, L-$tPhpro^3$; Map #2 = Pro^2-trans, D-$tPhpro^3$; Map #3 = Pro^2-Cis, L-$tPhpro^3$; Map #4 = Pro^2-Cis, D-$tPhpro^3$.

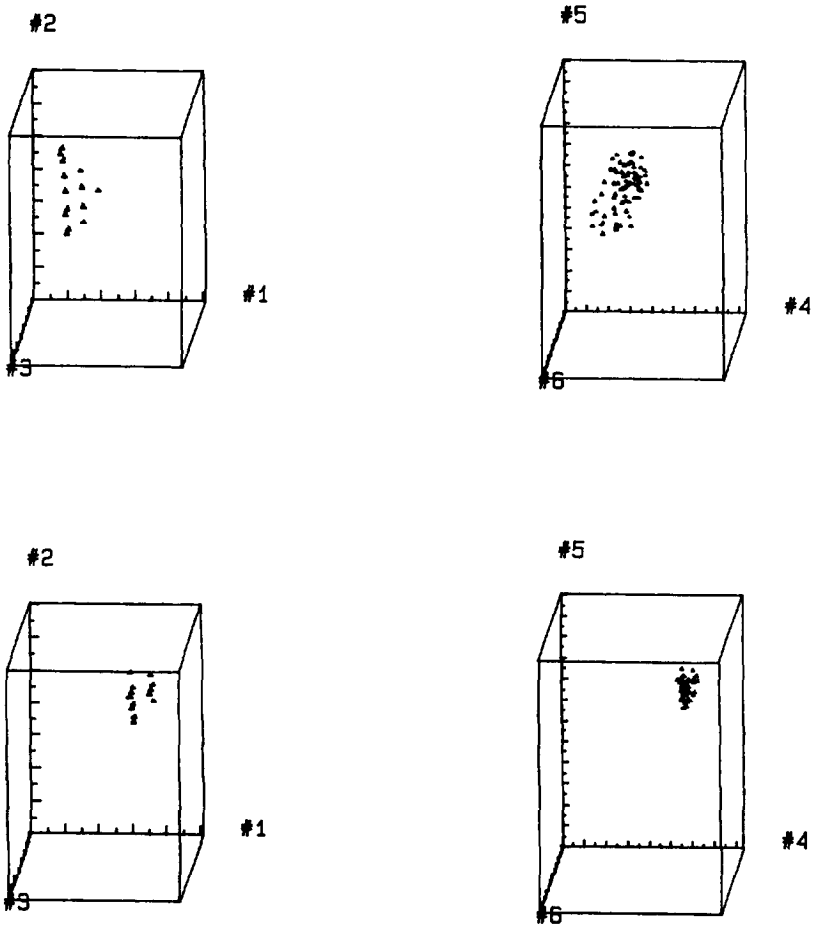


FIGURE 4

Orientation Maps for Morphiceptin Analogs

The orientation maps are plotted as distances #1-3 (left) and distances #4-6 (right). The top set of maps correspond to Map 2 of table 3 and the bottom set to map 1.

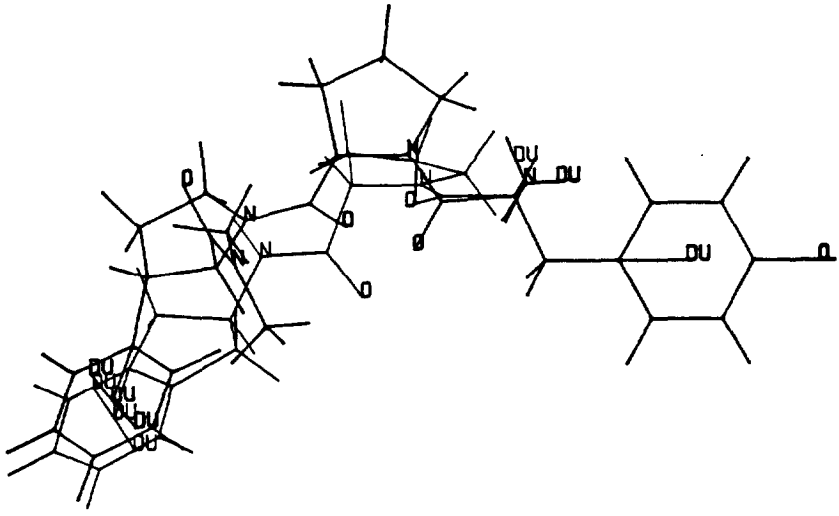


FIGURE 5

Conformations of Tyr-Pro-NH(2-indanyl) and Tyr-Pro-L-tPhpro-N(CH_3)$_2$, with the Tyr-Pro Amide in the Trans Configuration Superimposed

If one examines the four possible conformations of [15] and [17/18] previously calculated in relation to GPA1657 and PEO, it is possible to conclude that morphfceptin may not interact with the opiate receptor in the same way as enkephalin. Gorin and Marshall (1977) proposed that enkephalin interacted with the μ opiate receptor in a conformation in which the tyramine and phenyl groups were superimposed over the corresponding groups of GPA1657. Clearly, the constrained analogs of morphiceptin cannot adopt a conformation in which both the tyramine and phenyl are superimposed over the corresponding groups of GPA1657. This suggests that the tyramine of morphfceptin and enkephalin interact with the same subsites of the μ opiate receptor, and that the phenyl of morphiceptin and enkephalin may interact with a different subsite of the μ opiate receptor.

The suggestion that the corresponding tyramine groups of norphiceptin and enkephalin interact with a common primary binding site and that the corresponding phenyl groups interact with different secondary sites of the μ opiate receptor is also consistent with the previously described structure-activity relationships of morphiceptin and enkephalin. The activity of morphiceptin and enkephalin at the μ opiate receptor is generally sensitive to the chemical modification of the tyramine and phenyl groups. However, the activity of morphfceptin decreases dramatically with the nitration of the phenyl group, whereas the activity of enkephalin increases with the nitration of the phenyl group. It will be important in the future to actually see if the nitration of the constrained analogs of morphiceptin also decreases activity at the μ receptor in order to insure that these analogs themselves interact with the receptor in the same way as morphfceptin, an underlying assumption in all these molecular modeling studies. Also, it will be important to determine if nitration of the ortho and meta positions of Phe^3 or morphiceptin, perhaps, increases the activity of morphfceptin, as does paranftration of Phe^4 of enkephalin. This would suggest that the phenyl groups of morphiceptin and enkephalin interact with the same sites, but in a different orientation.

CONCLUSIONS

The structure-activity studies of morphfceptin clearly demonstrated that the minimal active structure of morphfceptin for μ-receptor activity, i.e.. binding affinity and agonist potency, was Tyr-Pro-NH(phenethyl). Furthermore, unmodified tyramine and phenyl groups were required for activity at the μ receptor. These requirements paralleled the similar requirements for the unmodified tyramine and phenyl groups in the enkephalin. the requirements for the unmodified tyramine in the nonpeptide opioids PEO and GPA1657, and the requirement of the phenyl group of PEO for superpotency. However, the activity of morphiceptin would not tolerate the nitration of the phenyl group, whereas the activity of enkephalin was enhanced by the nitration of the phenyl group.

The possibility of multiple divergent modes of interaction of molecules with opiate receptors has been described as accounting for the structure-activity relationships of the multitude of structurally dissimilar nonpeptide opiofds. It is possible that the phenyl group of these different opioids may bind to a variety of subsites or accessory sites of the μ opiate receptor, in order to confer significant activity through sufficient binding energy. Thus, a μ-receptor pharmacophore for morphiceptin analogs consisting of tyramine and phenyl groups oriented in one of several specific orientations relative to each other was suggested from the computer modeling studies of morphiceptin structure-activity relationships.

The computer-modeling studies of morphfceptin, although in the preliminary stages, helped to refine the pharmacophore hypothesis in three dimensions and suggested some possible relationships

between morphiceptin, enkephalin, and selected nonpeptide opiates. The systematic analysis of the conformations of the most conformationally constrained analogs of morphiceptin demonstrated that there were a limited number of possible common relative orientations of the phenyl and tyramine groups in which there were no intramolecular repulsive van der Waals contacts. The common orientations of these groups as determined by the conformational analysis are candidates for the receptor-bound orientation, i.e., the μ-receptor pharmacophore for morphiceptin.

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Mechanistic Structure-Activity Studies of Peptide and Nonpeptide Flexible Opioids: An Interdisciplinary Approach

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SUMMARY

In this chapter, results obtained using an interdisciplinary approach to investigate opioid narcotic analgesic agonism and antagonism are presented. In particular, this approach is illustrated by studies conducted on two specific classes of flexible opioids: peptides and 3-phenylpiperidines. For each class, the work discussed is a continuation of the two major themes of our past theoretical studies complemented by more recent experimental efforts involving medicinal chemistry and receptor and animal pharmacology.

One major focus of our past activities has been the use of theoretical chemistry techniques to characterize common molecular features among diverse classes of fused-ring and flexible opioids leading to high μ-receptor affinity and analgesic activity. A second major theme has been the identification of molecular determinants which modulate the extent of analgesic agonist and antagonist activity in a given opioid, and the design of analogs with mixed agonist/antagonist activity.

Continuing these themes, we report here theoretical calculations, analog synthesis, receptor binding, and in vivo animal studies of a series of 3-pheoylpiperidines and peptide opioids. In addition, we report, for the first time, preliminary attempts to ideotify peptide conformers leading to high affinity at δ-receptors.

THEORETICAL AND EXPERIMENTAL STUDIES OF 3-ARYLPIPERIDINES: DETERMINATION OF MOLECULAR FEATURES LEADING TO μ-RECEPTOR SITE AFFINITY AND MODULATION OF ANALGESIC AGONIST/ANTAGONIST ACTIVITY

The discovery of opioid analgesic properties in meperidine aroused interest in other classes of phenylpiperidines. In particular, 3-arylpiperidins congenere have been synthesized and tested in several laboratories (Kugita et al. 1964, 1965; Iorio and Casy 1978; Jacoby et al. 1981; Cheng et al. 1984). The known structure-activity data for two representatives of this class of compounds, 3R=CH_3 and $COOC_2H_5$-(β-meperidines),are summarized in table 1 (Kugita et al. 1965; Iorio and Casy 1978; Jacoby et al. 1981). As seen in this table, this class of opioids exhibits structure-activity profiles markedly different from those of the rigid opiates, with antagonist activity absolutely requiring a phenolic-OR, and being further modulated by 2-CH_3, and 3-R groups, without the requirement of N-substituent variation.

Using the semiempirical quantum mechanical method Perturbative Configurstion Interaction using Localized Orbitals (PCILO) (Diner et al 1969), we have calculated the energy-conformation behavior and electronic structure of analogs in this class of opioids in an attempt to explain their structure-activity profile in terms of fundamental opiate-receptor interactions.

The results of these calculations, also briefly summarized in table 1, together with their known activity profiles, led to postulated pharmacophores, i.e., conformations and orientations at the μ-receptor site that could initiate analgesic agonist and antagonist activity.

TABLE 1
Calculated Low Energy Isomers and Observed Pharmacological Activity for N-CH_3-3-Phenylpiperidines

			Activity (Agonist/Antagonist)		
3-R	2-R	Low Energy Conformer	No OH	(m-H)	(p-OH)
CH_3	H	ϕ_{EQ}, ϕ_{AX}, boat	ND	wk ag/ND	ND
	α-CH_3	ϕ_{EQ}, ϕ_{AX}, boat	ND	inact/ant	ND
	β-CH_3	ϕ_{EQ}	ND	inact/ant	ND
COOEt	H	ϕ_{EQ}	ag/inact	ag/ant	wk ag/inact
	α-CH_3	ϕ_{EQ}	ND	ND	ND
	β-CH_3	ϕ_{EQ}	ND	ND	ND

Specifically, for all the β-aeperidines studied, only one low energy conformer was found. This conformer is a piperldine-chair, with the pheoyl group equatorial. No stable conformer with the pheoyl axial or piperidine boat form could be found. Thus, as shown in figure 1, we have proposed that this conformer could initiate both analgesic agonist and antagonist activity by binding to a μ-receptor in two diffareot (bimodal) orleotations. In this figure, the proposed antagonist pharmacophore (I) satisfies our hypothesized minimum requirement for antagonism: an orientation in which its (m-OH)-pheoyl group can interact at the μ-opiate receptor in a manner similar to that of fused-ring opiates (shown in dotted lines for comparison). The agonist pharmacophore II satisfies the minimum proposed requirement for analgesic activity: interaction of the protonated piperidine amine group with an anionic receptor subsite as in fused ring opiates. In both of these pharmacophores, the ester chain can provide additional polar interactions with cationic receptor sites.

I. Antagonist

II. Agonist

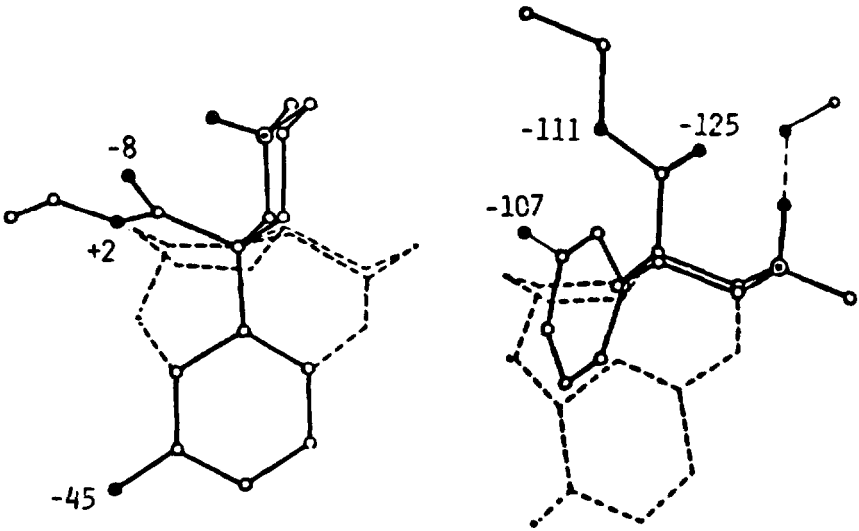


FIGURE I
Calculated Molecular Electrostatic Potential Minima (kcal/mol) Around β-Meperidine Oxygen Substituents in Proposed Agonist and Antagonist Pharmacophores

To model receptor interaction in the agonist and antagonist modes, molecular electrostatic potentials (Weinstein et al. 1973) in the vicinity of the (m-OH) and ester substituents were calculated with and without a protonated amine interaction with a model anionic receptor site. The negative potentials calculated in the plane of the phenol ring and in the ester group are a measure of the electrostatic energy of interactions with cationic receptor sites. The results shown in figure 1 indicate that without the initial protonated amine-model anionic receptor site interaction, postulated as crucial to agonist activity, the (m-OH)-phenyl group has the larger minimum negative potential and could, therefore, more effectively bind at cationic receptor sites. This result is consistent with the hypothesis that pharmacophore I leads to antagonism and that the (m-OH)-phenyl group contributes important receptor interactions for such antagonist activity.

The results also show that, in the presence of an initial interaction of the protonated amine with a model anionic receptor site, (OH), while both polar groups are activated, the augmented negative potential around the two ester oxygen atoms is larger than that around the phenol group, thereby favoring their binding to cationic receptor sites. These results are consistent with the hypothesis that pharmscophore II binding of β-meperidines at the receptor site could be responsible for their agonist activity.

Substitution of a 3-CH_3 group for the 3-$COOC_2H_5$ substituent of B-meperidine yields more flexible analogs. Specifically, extensive energy-conformation studies indicated the presence of three low energy conformers: two piperidine chair conformers, one with the phenyl-group equatorial and the other with the phenyl-group axial; and a piperidine boat conformer. While the two chair forms had the lowest energy, a boat form was also a stable conformation, with calculated activation energies of 9.5 to 16.5 kcal/mol and conformational energies of 5.5 to 6.5 kcal/mol, low enough to be a candidate conformation at the receptor site. Experimental values of chair-boat activation energies and conformational energy differences for the related ring system cyclohexane are in the same range as the calculated values, i.e., 9 to 11.5 kcal/mol and 4.8 to 6.0 kcal/mol, respectively (Komornicki and McIver 1973), confirming the validity of the calculated values.

Thus, for the 3-CH_3,3-phenylpiperidines, but not for B-meperidines, results of energy-conformation studies imply two possible modes of receptor binding which could initiate mixed agonist and antagonist activity. As indicated in figure 2, one is a bimodal interaction, similar to that proposed for the β-meperidines, in which antagonist activity is initiated by pharmscophore I and agonist activity is

initiated by pharmacophore II binding to the receptor. If this bimodal interaction is responsible for mixed agonist/antagonist activity, then N-substituent variation should not modulate such activity as it does in fused-ring opiates.

Pharmacophore I	Pharmacophore II	Pharmacophare III
		Possible Mixed
Antagonist	Agonist	Agonist/Antagonist

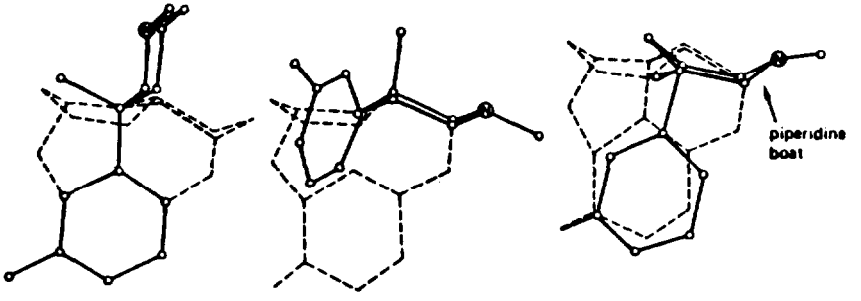


FIGURE 2
Postulated 3-Phenyl Piperidine Modes of Initiating Agonist/Antagonist Activity

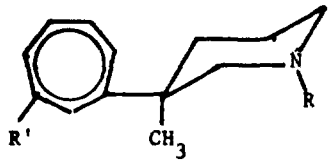
Alternatively, for these 3-methyl-3-phenyl compounds, the boat conformation of the piperidine ring in pharmacophore III can have simultaneous overlap of both the protonated amine group and (m-OH)-phenyl group with fused-ring opiates. If this is the mode of interaction at the receptor, N-substituent variation should modulate agonist/antagonist activity in a manner similar to fused-ring opiates.

To determine the effect of varying N-substituents, we undertook an experimental effort involving the synthesis, optical resolution, *in vitro* receptor binding and *in vivo* pharmacological activity of four N-R-substituted 3-methyl-3(m-hydroxyphenyl)piperidine analogs shown in table 2. The synthesis of racemic analog 1 followed a modified procedure of Kugita et al. (1964). The resolution of the enantiomers and their N-alkylation were reported for the first time by our laboratory and are described elsewhere (Cheng et al. 1984, 1985).

Opioid-receptor binding assays on rat brain homogenates were performed essentially as described by Pasternak et al. (1975). In the present studies, self- and cross-competition experiments were conducted for four labeled ligands: [H]naloxone, D-Ala^2-D-Leu^5-enkephalin (DADL), ethylketocyclarocine (EKC), and dihydromorphine (DHM) at two different concentrations of labeled ligand. This yielded a 4 by 4 "matrix" of competitive inhibition behavior. For each pair of enantiomers, inhibition of binding of each of the four labeled ligands was performed.

TABLE 2

Resolved Isomers of 3-Phenylpiperidines Studied



Compound (+) and (-)	R	R
1	H	m-OCH_3
2	CH_3	m-OH
3	Allyl	m-OH
4	Cyclopropylmethyl	m-OH
5	Phenethyl	m-OH

Data obtained from binding studies of each enantiomeric pair were analyzed simultaneously with that from the 4 by 4 matrix using a modified version of the program LIGAND (Munson and Robard 1980), which predicts the receptor binding affinities and capacities using weighted nonlinear, least squares regression analysis. The five-receptor-site model, shown in table 3, yielded a self-consistent set of receptor binding affinities and capacities that was statistically much more significant than a one-, two-, three-, or four-site model. A six-site model was not statistically more significant than the five-site model.

As can be seen in table 3, a site was identified which has high affinity for all the ligands, and was labeled μ_1. Sites "μ_2" and "δ" were labeled as those of high affinity for DHM and DADL, respectively. The fourth site with high affinity for EKC and naloxone, but low affinity for DHM and DADL, was labeled "κ." The fifth residual site with large capacity has apparent low affinity for all labeled ligands and could be a composite site.

Also seen in table 3, all 3-phenylpiperidine analogs have highest receptor affinity at the μ_1 site and in general are n-selective compounds. While there are not dramatic differences in affinity at μ between pairs of enantiomers, the N-allyl and N-cyclopropylmethyl analogs differ most in this respect. Relative affinities at the μ) site vary from about 2% to 10% of the values for DHM and naloxone.

TABLE 3
Receptor Affinities and Maximum Binding Capacities for a live-Receptor-Site Model

	K_D(nM)				
	"μ1"	"μ2"	"δ"	"κ"	5th Site
Naloxone	0.4	5.0	18	1.0	154
DADL	0.9	22	2.1	625	10,000
EKC	0.5	6.7	14	0.3	4,160
DHM	0.2	10	100	830	200
3-Phenylpiperidines					
(-)N-CH_3	114	1,170	30,000	625	8,330
(+)N-CH_3	132	1,000	15,900	714	19,200
(-)N-ally1	24	110	4,000	370	1,720
(+)N-ally1	100	370	4,000	137	3,700
(-)N-CPM	32	43	4,350	122	1,700
(+)N-CPM	120	256	2,700	83	833
(-)N-phenethyl	13	130	2,170	625	1,390
(+)N-phenethyl	5.0	6.2	588	179	26,300
B_{max}(pmol/g)	3.0	30.0	7.8	4.2	233

The similarity in receptor-site affinities between the enantiomeric pairs is in contrast to the striking differences obtained in in vivo activities. As noted in table 4, determination of agonism as shown by inhibition of mouse acetic acid writhing (Koeter et al. 1959), and antagonism of morphine-induced tail-flick analgesia (D'Amour and Smith 1941; Harris et al. 1969), revealed that all (-) isomers are- pure agonists, while (+) isomers are mixed agonist/antagonists.

TABLE 4

Analgesic and Narcotic Antagonist Potencies of Resolved 3-Methyl-3-m-hydroxypheaylpipcrldine with N-Substituent Variation

Compound	Mouse Writhing Test[a] ED_{50} (95% Con. limits) µmol/kg i.p	Mouse Tail-Flick Test[b] AD_{50} (95% Con. limits) µmol/kg i.p.
N-Methyl		
(-)-2	25.69 (14.68 - 44.96)	c
(+)-2	41.36 (21.55 - 79.42)	101.34 (71.35 - 143.90)
N-Ally1		
(-)-3	14.34 (8.96 - 22.94)	c
(+)-2	160.94 (106.58 - 243.02)	112.O2 (69.58 - 180.36)
N-Cyclopropylmethyl		
(-)-4	12.23 (2.63 - 21.41)	c
(+)-5	d	139.32 (83.93 - 231.27)
N-Phenethyl		
(-)-5	18.38 (11.08 - 30.52)	c
(+)-5	11.53 (7.21 - 18.44)	13.07 (8.71 - 19.58)
Morphine Sulfate	0.81 (0.51 - 1.29)	
N-Allylnormorphine Hydrochloride		2.04 (1.44 - 2.8 7)

[a]Inhibition of acetic acid induced writhing (Koster et al 1959)
[b]Antagonism of tail-flick inhibition induced by morphine sulfate (21.08 µmol/kg, s.c.) (D'Amour and Smith 1941, Harris et al 1969).
[c]Compounds showed no significant antagonist activity up to dose of SO mg/kg.
[d]Showed no significant agonist activity at a dose of 707.21 µmol/kg.

In general, the relative affinities of the analogs at the μ_1-receptor site correspond to their relative agonist activity, lending credence to the hypothesis of Pasternak et al. (1980) of a common high-affinity site for all types of opioids initiating analgesic activity.

The results presented are most consistent with the hypothesis that different receptor binding modes lead to agonism and antagonism. All (-) isomers are pure agonists and thus would seem to bind to n-receptors only in an orientation without a (m-OH)-phenyl overlap (pharmacophore II), preventing antagonist activity. By contrast, the (+) isomers appear to bind in a bimodal fashion with agonist activity initiated by binding in a pharmacophore II mode and antagonist activity initiated by binding in a pharmacophore I mode.

The N-R aubatituents do not modulate agonist/antagonist activity as in fused-ring opioids. In fused-ring opiates, N-methyl compounds rarely show antagonist activity, and N-phenethyl compounds have never been shown to possess antagonist activity. Also, the N-allyl and cyclopropylmethyl substitutions in this series do not increase antagonist activity as they do in the fused-ring opiates. Thus, pharmacophore III with simultaneous cationic amine and hydroxy-phenyl overlaps is probably not involved in the binding.

The surprising finding of strict etereoapecificity for antagonism, but not agonism, provides clues to additional spatial requirements for antagonist activity of 3-phenylpiperidines, other than (m-OH)-phenyl overlap with morphine at the μ-opioid receptor. Comparing the two different enantiomers in a pharmacophore I orientation (figure 3). it is seen that the amine-N of the piperidine ring differs in its spatial relationship to the crucial (m-OH)-phenyl group. Apparently, only one of the amine positions is compatible with local receptor subsite interactions.

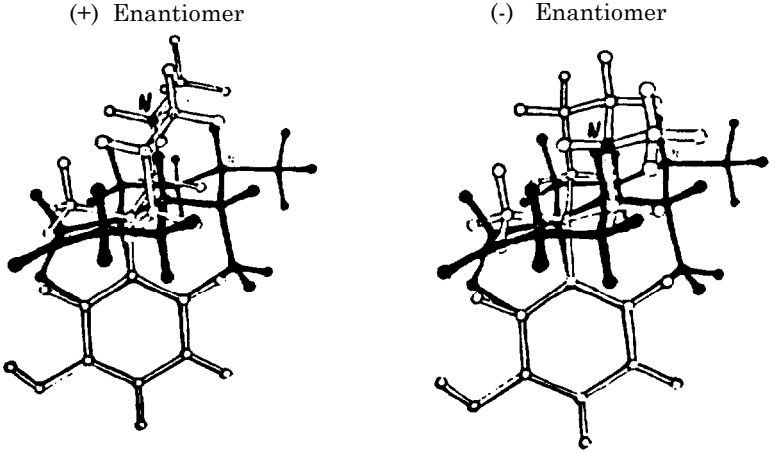


FIGURE 3

Proposed Antagonist Pharmacophore for the Two 3-Phenyl-piperidine Enantioners (m-OH)-phenyl Overlap with Morphine

Preliminary X-ray structure determination of the (+)-enantiomer of the m-OCH_3 nor-analog, 1 (Cheng et al. 1984), reveals that the series of active antagonists derived from this compound have an S-configuration, as shown in figure 3. Moreover, it was found, as shown, that the piperidine ring has a chair conformation and the phenyl ring is equatorial. These X-ray results support the conclusion that bimodal piperidine chair pharmacophores (I and II), rather than a boat conformation (pharmacophore III), are involved in receptor interactions. The X-ray determination of the absolute configuration of the active enantiomer further defines the relative position of the crucial amine and phenyl groups in the antagonist binding mode.

IDENTIFICATION OF OPIOID PEPTIDE CONFORMERS LEADING TO HIGH AFFINITY AT μ-RECEPTORS

Although the μ-site is traditionally associated with morphine and the fused-ring opiates, a number of peptides have also been found to show specificity for binding to the μ-site as measured by tissue-specific assays and in vitro receptor binding assays (Morley 1980). Of these peptides, the first found were analogs of enkephalins which had been synthesized in an attempt to produce opioids that were protected against proteolytic breakdown and thus increase in vivo activity. The structural modifications made in these analogs included substitution and modification of residues as well as lengthening and shortening of the peptide chain. A decrease in in vivo degradation did result, but at the same time, the μ-6 binding specificity of the peptide was often altered.

Initial energy-conformational studies made in our laboratory were designed to identify peptide conformations leading to μ-receptor affinity and focused on three of the first Met-enkephalin analogs synthesized as shown in table 5A (Pert et al. 1976, Walker et al. 1977). Subsequent studies were made of tetrapeptide analogs shown in table 5B. The tetrapeptide analogs were found to be even more potent than morphine as in vivo analgesics (Terenius et al. 1976, McGregor et al. 1978).

Of more recent interest are structure-activity studies of a series of analogs in which modifications have been systematically made in order to explore the structural requirements for specificity. In this regard, morphiceptin, an amidated tetrapeptide (Tyr-Pro-Phe-Pro-NH_2) fragment of β-casomorphin, a naturally occurring peptide in milk (Henschen et al. 1980), is of interest because it was found to have morphinelike physiological activity, as well as bind with high specificity to the μ-receptor (Chang et al. 1981). A series of morphiceptin analogs has subsequently been synthesized in which the second, third,, and fourth residues were modified, leading to

more potent, highly μ-selective analogs (Chang et al. 1983).

In a continuing effort to identify μ-selective opioid peptide conformers, energy conformation studies of the morphiceptin analogs, shown in table 5C, have begun in our group. Specifically, in this series we have examined the conformational effects of substitution of D-Pro for L-Pro in the second and fourth positions, and the addition of a methyl group to the backbone nitrogen of the phenylalanina residue at the third position.

TABLE 5

Three Types of μ-Selective Peptide Opioids Studied

A. Met-enkephalin Analogs (Met-Enk)

	Relative Affinity G.P.I.	Relative Affinity M.V.D.	Relative Affinity vs. Naloxone
Met-enk	1.0	1.0	1.0
$L\text{-}Ala_2\text{-}Met\text{-}enk$[a]	0.06	-	0.05
D-Ala-Met- enk[b]	6.3	5.0	5.0

[a]Pert et al.1974, [b]Walker et al. 1977

B. Enkephalin-type Tetrapeptides

Analog	Relative Receptor Affinity	Relative In Vivo Analgesia
Tyr-Gly-Gly-Phe-OH[c]	<0.01	-
Tyr-Gly-Gly-Phe-NH_2	-	-
Tyr-Dala-Gly-Phe-OH[d]	0.99	0.1
Tyr-Dala-Gly-Phe-NH_2[d]	1.14	2.5
Tyr-Dala-Gly-$N(CH_3)$phe-NH_2[d]	1.84	2.5
morphine	1.0	1.0

[c]Terenius et al.1976, [d]McGregor et al. 1978

C. Morphiceptin Analogs[e]

	IC_{50} (Binding)		(In Vitro) Activity	
	FK33824	DADL	GPI	MVD
Tyr-Pro-Phe-Pro-NH_2	63	30,000	318	4800
Tyr-Pro-$N(CH_3)$phe-pro-NH_2	37	10,000	225	2300
Tyr-Pro-$N(CH_3)$phe-D-pro-NH_2	5.5	10,000	34	240
Tyr-D-Pro-Phe-Pro-NH_2	10,000	-	-	-
Morphine	0.4	35	134	1300
DADL	22	32	4.8	141

[e]Data taken from Chang et al. 1981, 1983

In all of these studies, we have used the methods and procedures outlined in table 6 to identify μ-selective peptide conformers. Underlying these procedures are the assumptions that: (1) different conformations are associated with optimum binding at μ- and δ-receptor sites; and (2) differences in affinity between peptide analogs at each site are related to the relative energy required to obtain a favorable conformation.

TABLE 6

Methods and Procadures Used to Identify μ-Peptide conformations

I. SELECI A SET OF μ-ACTIVE/INACTIVE ANALOGS.
   USE:
   - Receptor affinity
   - Guinea pig ileum (G.P.I.) activity
   - Antinociceptive activity

II. PERFORM ENERGY-CONFORMATION CALCULATIONS.
   USE:
   - Different methods
     - Quantum mechanical
     - Empirical energy
   - Different search strategies
     - Aufbau: From single amino acids
     - Use standard secondary structures

III. SELECT A SET OF PUTATIVE ACTIVE CONFORMERS. CRITERIA:
   - Low energy in active analogs/High energy in inactive analogs
   - Relative energy parallels relative affinity in a series of analogs

IV. REFINE SELECTIONS USING RESIDUE SUBSTITUTION/ENERGY CALCULATIONS:
   - Active analogs "accommodated"
   - Inactive analogs "not accommodated"

V. COMPARE CANDIDATE CONFORMERS WITH μ-SELECTIVE, FUSED-RING OPIOIDS (MORPHINE-LIKE). LOOK FOR:
   - Crucial tyramine overlap
   - Other regions of overlap

At the time of our first study, extensive energy conformation studies had already been made of Met-enkephalin using the Empirical Conformational Energy Program for Peptides (ECEPP) (Isogai et al. 1977, Momany et al. 1975). In addition, a number of conformational comparisons with rigid opiates had been made by model building and comparison of overlaps of different assumed critical regions of Met-enkephalins and rigid opiates. Of particular interest were the p-OH phenethylamine (tyramine) moiety formed by the terminal amino group and the tyrosine residue (Bradbury et al. 1976; Horn and Rodgers 1976).

While valuable in describing numerous low and medium energy conformers of Met-enkephalin, none of the optimized conformers previously reported had significant spatial overlap with several functional groups in morphinelike opiates. On the other hand, the conformers proposed by overlap with rigid opiates were based on model building with no estimate of their relative energies. Deviations from minimum energy conformations of the isolated molecule could occur by an induced fit at the receptor site,allowing enhanced resemblance to rigid opiates, but with some energy required.

In order to determine the most likely conformers for interaction at the receptor site, we have made systematic energy-conformational studies by both empirical and quantum mechanical methods. The aim was to determine the energy of Met-enkephalin conformers with varying degrees of similarity to rigid opiates and select as most likely those conformers with modest energy expenditure and significant overlap with rigid opiates. In keeping with the protocol outlined in table 6, an additional requirement in our selection strategy was the accommodation of D-Ala^2, but not L-Ala , in the place of the Gly residue. This second criterion is consistent with the retention of activity when Gly is replaced by D-Ala (Walker et al. 1977), and with a large decrease of receptor affinity upon replacement by L-Ala (Pert et al. 1976).

The conformational energy calculations were performed using the ECEPP and the PCILO methods. While the empirical and quantum mechanical energy calculations did not give identical results, a number of likely candidates common to both methods were obtained.

As shown in table 7, both methods showed a trend toward higher energies for conformers with increasing resemblance to rigid

opiates. A conformer constructed to have maximum overlap with 7-[l-phenyl-3-hydroxybutyl-3]-endothenotetrahydrothebaine (PET), and not energy optimized, gave extremely high energies by both the ECEPP and PCILO methods. These high energies are due to the crowding of the terminal nitrogen and hydrogen atoms with the Gly^2 carbonyl carbon and oxygen atoms. Relaxation of the Tyr^1 and Gly^2 backbone angles to a local minimum decreased the energy significantly. Step-wise relaxation of a starting structure with w angles ~180° yielded a series of lower and lower energy structures by both methods.

TABLE 7
Energy of Met-Enkephalin Analogs with Increasing Overlap with Fused-Ring Thebaine Opiate (PET)

Description	ΔE (ECEPP) (kcal/mol)	ΔE (PCILO) (kcal/mol)
Gly^2, no tyramine overlap	0	0
D-Ala^2, no tyramine overlap	1.5	-0.5
D-Ala^2, tyramine overlap	9.6	2.3
D-Ala^2, minimal PET overlap	14.55	3.8
Increased PET Overlap (ω_1 = 180°)	25.2	14.4
Maximum Constrained PET overlap (ω_1 = 0°)	135.0	25.0

In another approach, it was assumed that a minimum viable overlap of Wet-enkephalin with rigid opiates should involve the tyramine (NH_2-C_α-C_β-ϕ-OH)moiety of both opiates. Imposition of this constraint on optimized Met-enkephalin conformers resulted in 22 conformers with relative energy 9 to 20 kcal/mol by ECEPP and 1.0 to 8.5 kcal/mol by PCILO, above the lowest energy conformer without the imposed overlap. The further condition that D-Ala^2 be accommodated more readily than L-Ala in place of Gly reduced the number of viable conformers from 22 to 12. These 12 conformers were optimized with the constraint of accommodating both the Gly and D-Ala residues and the tyramine overlap with morphine.

The results of this procedure were that the minimum energy con-

formers calculated by both the ECEPP and PCILO methods did not have any overlap with rigid opiates beyond the imposed tyramine overlap. On the other hand, the lowest energy conformer with significant additional rigid opiate overlap was the same by both methods: a G-GβII' bend with a hydrogen bond between the carbonyl group of tyrosine and the amine group of phenylalanine. This is a particularly viable candidate for interaction at the receptor site since it is only 3 kcal/mol above the minimum energy form as calculated by the PCILO method.

Figure 4 shows the overlap of this structure with the potent agonist PET. Not only do the important phenethylamine moieties overlap, but the phenylalanine side chain overlaps with the phenethyl C_{19} substituent of PET. In addition, the methionine backbone C=O group and side chain are in the region of the C_6 rethoxy group of PET. This conformer is quite similar to the one previously proposed by model building (Bradbury et al 1976) and is confirmed by these studies as a very likely candidate at the receptor site.

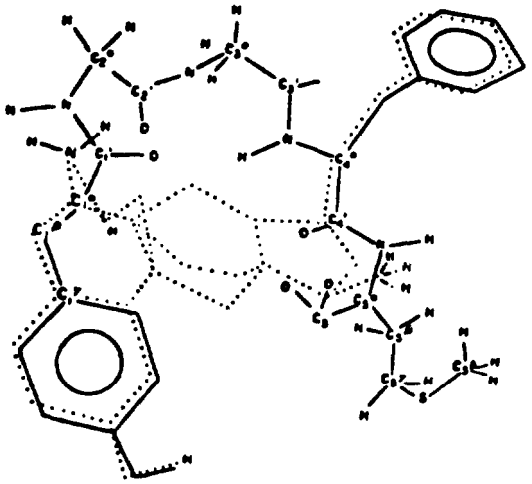


FIGURE 4
A Candidate Active Conformer for Met-Enkephalin Analogs: G-G βII' Turn

interact with the opiate receptor is provided by the first published X-ray crystal structure determination of Leu-enkephalin (Smith and Griffith 1978), shoving a G-G β bend stabilized by hydrogen bonding between the tyrosine and phenylalanine residues. While a subsequent crystal structure determination yielded extended structures (Karle et al. 1983), indicating the conformational flexibility of these peptides, a $\beta_{II'}$ turn remains

a viable candidate conformer.

In the second study, the conformational behavior of the five tetrapeptide enkephalin analogs given in table 5B were examined to continue identification of peptide opioid conformations leading to high and low affinity at the μ-receptor site.

Using the same empirical energy method (ECEPP) and semi-empirical quantum mechanical method (PCILO), conformational energies vere obtained for a large number of optimized geometries of each tetrapeptide. Two methods of selecting candidate active conformations from low-energy conformers mere used. In the first method, inactive conformers were designated as those having low energy in the very weak tetrapeptide, Tyr-Gly-Gly-Phe-OH, and high energy in the more potent ones. These candidate inactive conformers had geometries resembling **βV, βI,** or "random" peptide conformations. Conversely, candidate active conformers selected were low-energy conformations for both Tyr-D-Ala-Gly-Phe-NH_2 and Tyr-D-Ala-Gly-(NMe)Phe-NH_2, but not low-energy conformers for Tyr-Gly-Gly-Phe-OH. In the second method of selection, conformers with relative energies in the active and inactive peptides that followed the potency order Tyr-Gly-Gly-Phe-OH << Tyr-D-Ala-Gly-Phe-NH_2 ≤ Tyr-D-Ala-Gly-(NMe)Phe-NH_2 were chosen as candidate active conformers. By using both methods of selection, a **βII'** bend geometry, shown in figure 5, was found as the active conformer.

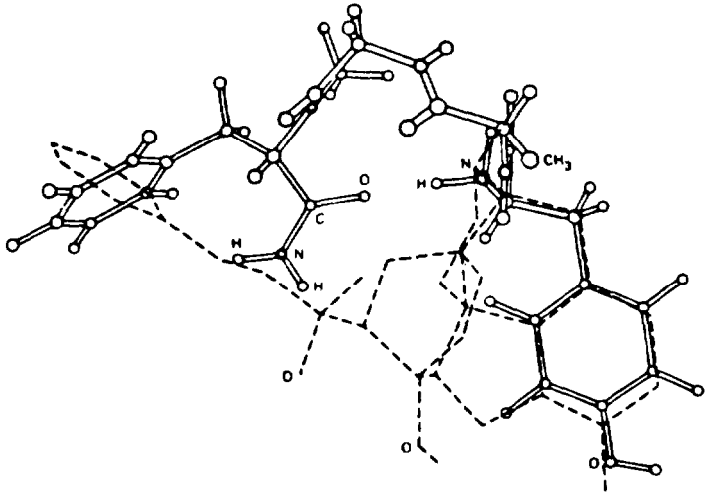


FIGURE 5
Candidate Active Conformer for Enkephalin like Tetrapeptides: A **β**II' Bend (---) Superimposed on PET

While very similar in structure to that found for the penta-peptides, this βII' conformer is not stabilized by a 1-4 hydrogen bond, but instead is stabilized by hydrogen bond between the tyrosine amine hydrogen atom and the phenylalanine carbonyl oxygen atom. Thus, our results show that such βII' conformers can exist as relatively low-energy forms for active peptides with an $N(CH_3)$ group on the fourth residues of the bend.

The candidate active forms have a greater degree of spatial overlap with the fused ring opiate PET than do the inactive ones, suggesting that the extent of this overlap may be important in high-affinity μ-receptor binding and in analgesic activity. The identification of a βI-type conformer as an inactive form of these peptide opiates is in agreement with proposed inactive conformation of Tyr-Gly-Gly-Phe based on crystallographic (Prange and Paecard 1979) and NMR data (Fournie-Zaluski et al. 1977).

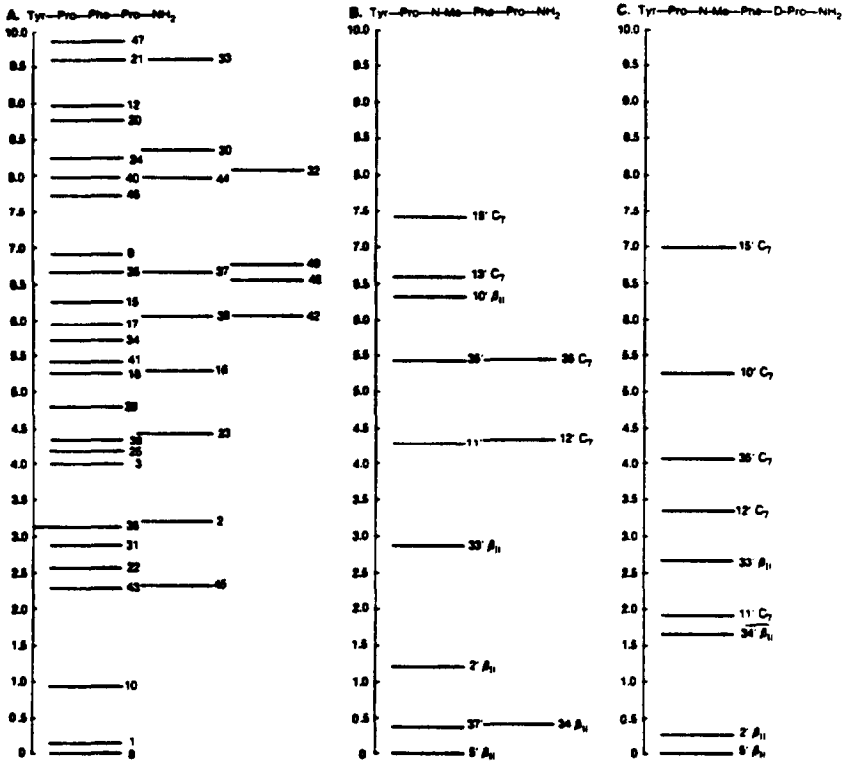
The morphfceptin analogs under current study, listed in table 5C, are among the most μ-specific linear peptides found and should, therefore, add considerable insight into the continued search for μ-selective opioid peptide conformers. In these energy conformation studies, we have used only the empirical energy method ECEPP and began with an "aufbau" search strategy. Starting with low energy derivitized single amino acid conformers, these were optimized and combined to form dipeptidee which were in turn optimized. A number of unique low energy conformers of the dipeptides were then combined to form tetrapeptide conformers which were then optimized. The results obtained for these analogs are summarized in table 8A-C.

Despite two proline residues, 38 different conformers of morphiceptin (Tyr-Pro-Phe-Pro-NH_2) vere found within 10 kcal/mol of the lowest energy conformer (table 8A). However, addition of a methyl group to the nitrogen backbone atom of the phenylalanine residue greatly diminished the flexibility, yielding only 12 conformers in this same energy range (table 8B). Moreover, while the low energy conformers found for morphiceptin varied widely, those for Tyr-Pro-NMe-Phe-Pro-NH_2 (NMePheMorphiceptin) were very similar to each other, falling into two basic classes: one class roughly equivalent to a βII turn, and the other shoving backbone ϕ and Ψ angles similar to those in the repeating C7 structure.

Since both of these analogs have similar μ-receptor affinities, it is reasonable to assume that similar peptide conformers would be involved in such receptor binding. Yet, there was no significant overlap between any of the low energy ($\Delta E \leq$ 10 kcal/mol) morphiceptin and NMePheMorphiceptin analogs found by the aufbau search strategy. Thus, a different strategy was used to find common conformations for the two peptides.

TABLE 8

Calculated Energies of Optimized Conformers for Three Morphiceptin Tetrapeptides Using an Empirical Energy Method (ECEPP)



In the second procedure, low energy conformers of each analog were examined for their ability to accommodate each other. The result was that no low energy conformer of morphiceptin could accomodate the N-methyl analog. The energy required to maintain the N-methyl analog in these conformers was of the order of hundreds of

kcal/mol and no constrained conformer vas a local minimum. By contrast, when morphiceptin was constrained to overlap with the low energy conformers of NMePheMorphiceptin, the energy required ranged from 4 to 14 kcal/mol. Some of these conformations were additional local minima for morphiceptin not found by the aufbau search strategy, while others were not local minima at all. Figure 6 shove the two analogs in an overlapping **β**II-type conformation (33′) which is a local minimum with relatively low energy for each.

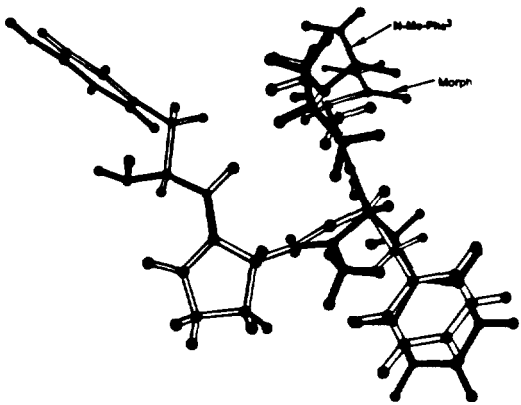


FIGURE 6
Overlap of Similar Optimized Conformers of Morphiceptin (**ΔE** = 6.5 kcal/mol) and N-Me-Phe^3-Morphiceptin (**ΔE** = 2.8 kcal/mol)

The morphiceptin analog Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017) is the most potent and μ-selective analog and can be considered the template for μ-specific morphiceptins. Thus, D-Pro^4 was eyetematically substituted into all the lov energy conformers of Tyr-Pro-N(Me)Phe-L-Pro-NH_2, given in table 8B. The relative energy of the D-Pro^4 conformers, optimized from this initial set and labeled by their initial similarity to the L-Pro analogs from which they were formed, are given in table 8C.

The effect of D-Pro^4 substitution was large on some conformers and small on others. Because the effect on activity was small, we eliminated as candidates those conformers which would not incorporate D-Pro in the fourth position without a large energy cost. The **βII-**type conformer 33′ best accomodated D-Pro^4 with very little geometry or energy change (figure 7) although it was not the conformer with lowest energy for NMePhe-Morphiceptin or its D-Pro^4 analog.

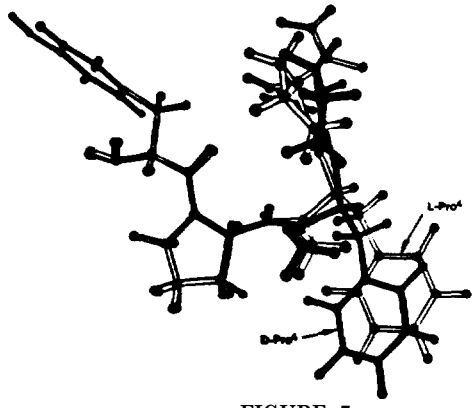


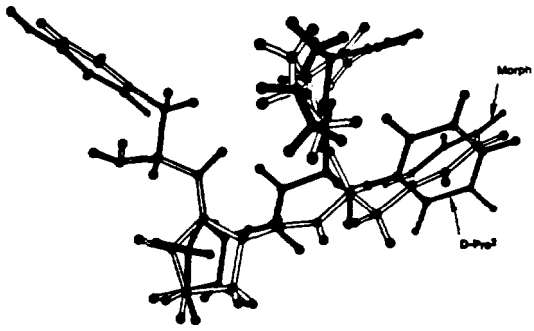
FIGURE 7
Overlap of Similar Optimized Conformers of N-Me-Phe[3] L-Pro (ΔE = 2.8 kcal/mol) and N-Me-Phe-D-Pro - Morphiceptin (ΔE = 2.4 kcal/mol)

As indicated in figure 6, it was also one of the conformers which best accommodated morphiceptin, and thus is our candidate conformation for morphiceptin like compounds.

Finally, we examined the effects of substituting a μ-Pro for an L-Pro in the second position of morphiceptin. In contrast to the fourth position, a D-Pro substitution produces an inactive analog (table 5C). Consistently, we found that forcing the Tyr-D-Pro-Phe-Pro-NH_2 analog to conform to any low energy conformer of N-Me-Phe-Morphiceptin required an energy of 25 to 400 kcal/mol compared to its lowest energy conformer. Moreover, none of these constrained conformations were local minims (figure 8A), nor did local minima of the D-Pro overlap with L-Pro[2]-morphiceptin (figure 8B).

In contrast to destruction of activity by substituting a D-Pro[2] residue in morphiceptin, a number of modified L-Pro[2] residues can be substituted in the 2 position without significant effect on μ-affinity and in vitro activity. Among these analogs, the most significant decrease in bioactivity was found with the substitution of a 4-hydroxy-proline into the second position of PL017. Conformational analysis revealed that there was virtually no energy cost for adding a 4-OH group to proline in any candidate conformers of PL017. This result indicates that the decrease in the observed bioactivity results from adverse electronic interactions at the receptor site rather than from conformational differences in these analogs.

A. D-Pro^{2}morphiceptin conformer constrained to overlap with L-Pro^2 analog (ΔE = 249 kcal/mol)



B. Optimized D-Pro^2 conformer(ΔE = 8.9 kcal/mol). Little overlap with L-Pro^2

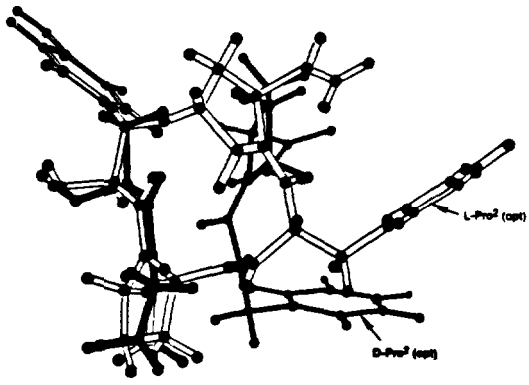


FIGURE 8
Comparison of L-Pro^2 and D-Pro^2 Morphiceptin Conformers

Conclusions and Future Directions

The three types of studies made thus far indicate a "universal" type of μ-selective opioid peptide conformer involving a **βII** or **βII'** turn. In enkephalin-type analogs, a **βII'** conformer is a likely candidate, while μ-selective morphiceptin analogs, appear to prefer a **βII** turn. As shown in figure 9, these two types of turns have a considerable overlap.

Studies are currently under way to investigate the effects of other subatitutions in the second position of morphiceptin analogs. Work is also currently proceeding on conformational analysis of the cyclic μ-selective compounds of Schiller et al. These authors have synthesized Leu-enkephalin analogs in which the aliphatic side chain $(CH_2)n$ of the second residue has been extended to various lengths (n=1,4) and cyclized with the C-terminal carboxyl group through an amide linkage (DiMaio et al. 1982). While the the degree of n-specificity varied in the four cyclic analogs of
this type synthesized, all shoved greater μ-specificity than their linear counterparts in binding assays, although diacrepanciea with tissue-specific assays did exist. The increased μ-specificity appeared to be due generally to a decrease in δ--receptor affinity.

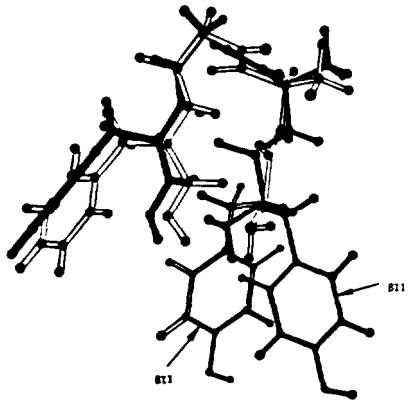


FIGURE 9

Tyr-D-Ala^2-Gly-Phe-NH_2 Shown in
βII and **βII'** Conformations

Comparing the candidate conformers of the most μ-selective and highest potency morphiceptin analog (PL017) with the lowest energy conformers of the most μ-specific cyclic compounds (with n=2,4), should provide a further probe of μ-selective opioid conformers. It will also be interesting to compare these conformers with the δ-selective linear and cyclic analogs which we are also currently studying.

PRELIMINARY STUDIES OF δ-SELECTIVE OPIOID PEPTIDE CONFORMERS LEADING TO HIGH AFFINITY ATδ-RECEPTOR SITES

Although the δ-receptor site was first identified by its preference for peptide compared to nonpeptide opioids, moat opioid peptidea synthesized have been found to bind and act with comparable or higher affinities at the μ-receptor.

Among the first molecules rationally designed for δ-specificity were the hexfpeptide Tyr-D-Set-Gly-Phe-Leu-Thr (DSLET), and its related $DThr^2$ (DTLET) analog (David et al. 1982). It was thought that this sequence would lead to δ-specificity since structure activity studies of peptidea had previously shown that: (a) Phe at position 4 plays a crucial role in μ- versus δ-selectivity; (b) lengthening of Met-enkephalin by Thr enhances MVD potency while decreasing GPI activity; (c) Leu-enkephalin shows increased MVD potency relative to Met-enkephalin; and (d) replacement of Gly^2 by a D-amino acid inhibits degradation and reinforces μ-selectivity if the residue is hydrophobic.

As summarized in table 9, GPI versus MVD studies indicate that DSLET and DTLET are 4 to 10 times more δ-selective than DADL, while binding studies indicate that they are only somewhat more δ-selective than DADL.

Thus, while the linear peptides DSLET and DTLET seem to be somewhat more δ-selective than DADL, the extent of this selectivity is unclear from the existing data. Since these linear peptidea are quite flexible, it is not surprising that they would not be overwhelmingly selective for a particular receptor.

As discussed above, Schiller and coworkers (DiMaio et al 1982) demonstrated that reduction of flexibility by cyclization could modulate the extent of μ- and δ-selectivity and lead to μ-selective Leo-enkephalin type peptides. These investigators have also synthesized another series of cyclic analogs baaed on a Tyr-Cys-Gly-Phe-Cys pentapeptide cyclized by a diaulfide bridge

TABLE 9
Receptor Binding and In Vitro Activity of
δ-Selective Opioid Peptides

Enkephalin Analog	IC_{50} nM [^{3}H]Nalox	IC_{50} nM [^{3}H]DADL	ED_{50} nM G.P.1	ED_{50} nM MVD
D-Ser^2-Leu^{5}Thr^{6a}	88	5.7	234	0.70
D-Thr^2-Leu^5-Thr^{6a}	36	6.4	100	0.58
[D-Pen^2-L-Pen^5][a]	3710	10	2720	2.5
[D-Pen^2-D-Pen^5][a]	2840	16	6930	2.2
[D-Pen^2-L-Cya^5][b]	178	12	213	0.32
[D-Pen^2-D-Cys^5][b]	157	26	1350	6.3
[D-Cys^2-L-Cya^5]	2.0[c]	0.8[c]	1.5[d]	0.80[d]
[D-Cys^2-D-Cys^5]	3.5[c]	1.6[c]	0.80[d]	0.30[d]
[L-Cys^2-L-Cys^5]			210[d]	950[d]
DADL[a]	16	4	24	0.72
Morphine[a] (normorphine)	23	27	91	541

[a]Mosberg et al. 1983a; [b]Mosberg et al. 1983b, [c]Schiller et al. 1981; [d]Schiller and DiMaio 1983.

between the second and fifth reaiduaa (Schiller et al. 1981, Schiller and DiMaio 1983). As shown in table 9, of thrae such analogs synthesized, all were relatively unselective. By analogy with oxytocin analogs, Mosberg and coworkers (1983a. 1983b) reasoned that gem-dimethylation of the cysteine β-carbons would greatly rigidify this cyclic structure. As shown in table 9, the resulting two analogs [D-Pen^2-L-Pen^5]enkephalin and [D-Pen^2-D-Pen^5]enkephalin, while retaining affinity and activity at δ-receptors, had greatly diminished activity at μ-receptors, yielding the most δ-selective peptides yet found.

We have begun energy conformation studies of both the linear peptide DTLET and the cyclic peptide [D-Pen^2-L-Pen^5]enkephalin, in order to identify, for the first time, peptide opioid conformers leading to high affinity at δ-, but not μ-receptors.

For the hexapeptide, DTLET, the aufbau search strategy was used with the empirical energy program ECEPP. Each of approximately 10 low energy derivatized single amino acid conformers was combined to give about 100 initial conformations for the Tyr-D-Thr and Phe-Leu dipeptides. Each of these dipeptide conformers were then optimized. To the optimized Tyr-D-Thr peptides were added low energy conformers of Gly to form tripeptides. A similar procedure was used to obtain Phe^4-Leu^5-Thr^6 tripeptidea. Finally, optimized tripeptide fragments were combined to form 64 initial hexapeptide conformers which were once again optimized. These 64 conformers spanned an energy range of 14 kcal/mol relative to the lowest energy conformer obtained. Fifty conformers had energies in the range of $\Delta E \leq 10$ kcal/mol.

This considerable effort resulted in characterization of the energy-conformational profile and indicated a high degree of flexibility of this linear peptide. This flexibility makes it difficult to identify a probable δ-selective conformer baaed solely on studies of linear peptides.

To help alleviate the problem of extensive flexibility, we have begun a systematically study of the more conformationally restricted [D-Pen^2-L-Pen^5] cyclic enkephalin. In order to perform this study, we have recently modified our structure-generating and empirical energy programs to incorporate and recognize diaulfide bonds between cyateinelike residues. The search strategy we are using involves:

1) Starting with the linear analog formed by breaking the diaulfide bond, and constructing a number of standard secondary structures as initial geometries.

2) Generating an initial cyclic structure by rotating side chain and backbone torsion angles to form a diaulfide bond with an S-S distance of 2.04 A.

3) Optimizing all initial structures.

Using this strategy, torsion angle optimization of an α-helical initial conformation was made, leading to a low energy minimum, the first geometry-optimized cyclic structure of this analog to be reported. This conformation of D-Pen -L-Pen was then used as a template to which DTLET was maximmly overlapped. Maximum overlap includes exactly matching the Tyr^1 and Phe^4 side chain angles of both the cyclic and linear analogs. Partial optimization of the DTLET conformation was then performed, allowing only torsion angles in DTLET, with no counterparts in the cyclic analog to vary. The resulting structure (figure 10) had an energy only 11

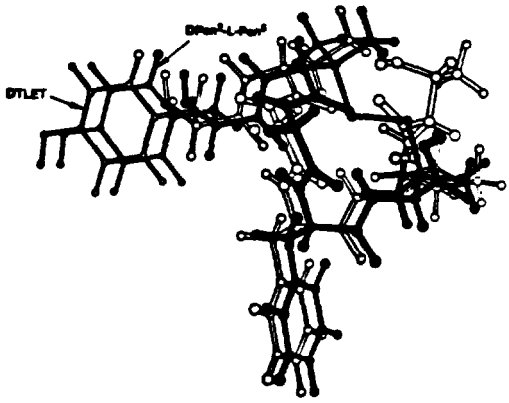


FIGURE 10

Overlap of Optimized (D-Pen^2-L-Pen^5)Enkephalin Conformer with Partially Optimized DTLET Conformer (ΔE DTLET = 11 kcal/mol)

kcal/nol higher than the lowest energy DTLET conformer obtained from the previoua aufbau search. Moreover, unconstrained optimization of this conformer caused only small departures from maximal overlap, as shown in figure 11, and resulted in a gain of 4 kcal/mol. Thus, the maximumly overlapped conformer of DTLET is near a local minimum of the potential energy surface, only 7 kcal/mol above the global minimum. Therefore, the conformer of DTLEI and [D-Pen^2-L-Pen^5]enkephalin shown in this figure are plausible candidates for a δ-selective peptide opioid conformer. This conformer of DTLET was not among the minima found from the aufbau search strategy and illuatratea the usefulness of a cyclic template in sorting out candidate δ-selective conformers.

We are continuing these studies, searching for additional low energy conformers of this and other cyclic analogs, to further aid in selecting the most probable conformer responsible for high-affinity binding at δ-receptors.

DESIGN OF μ-SELECTIVE OPIOID PEPTIDE ANTAGONISTS

The synthesis of opioid peptide antagonists has been repeatedly attempted since the initial discovery of the enkephalina. Moat previous attempts have, by analogy to fused-ring opiates, relied upon the addition of one or two N-alkyl moieties to Met- or

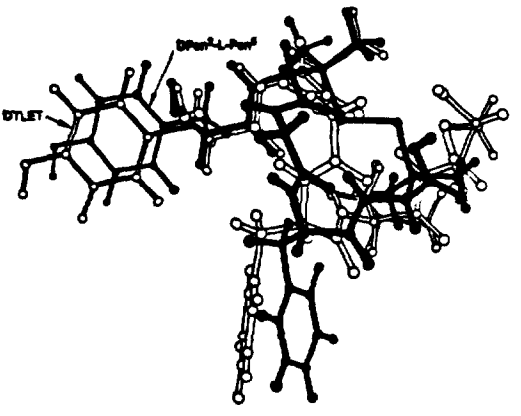


FIGURE 11

Overlap of Optimized DTLET with Optimized [D-Pen^2-L-Pen^5]Enkephalin Conformer (ΔE DTLET = 7 kcal/mol)

Leu-enkephalin analogs. Initial studies with Met- or Leu-enkephalina and their N-allyl analogs revealed alight antagonism to morphine's inhibition of amooth muscle contraction (Hahn et al. 1977), but no antagonism to analgesia after intracerebral ventricular (i.c.v.) administration (Pert et al 1977). The addition of N,N-diallyl to Leu-enkephalin analogs has produced potent δ-selective antagonists. ICI 154,129 (Shaw et al. 1982) and, more recently, ICI 174,864 (Cotton et al. 1984) have been shown to be Nghly selective in their inhibition of agonist actions in the field-stimulated MVD preparation. ICI 154,129 was also shown to inhibit the slowing of the head-turn time induced by etorphine, the model uaed to investigate δ-receptors in vivo by Gormley et al. (1982). These compounds, however, being δ-selective, showed no inhibition of morphine-induced analgesia.

Conformational studies of enkephalin analogs and comparisons with flexible, rather than fused-ring, nonpeptide opiates led us on a different path to peptide antagonists, and produced compounds with μ-selectivity. Previous energy calculations, described above, indicated that, in low energy conformations of Met-enkephalin, the amino-terminal phenethylamine moiety could overlap with the 3-phenylpiperidine class of flexible opiates (figure 12A). Moreover, these two types of flexible opioida share only a phenethylamine overlap with fused-ring opioida (figure 12B). Also

as diacuaaed in detail above, our own and previous atudiea of the 3-phenylpiperidinea indicate that they are moderately potent μ-selective opioid agonists, in which addition of an -OH group to the phenyl ring induces antagonist activity, and thus generates mixed agonist/antagonist compounds. A p-OH group produces very weak antagonism and an m-OH group greatly increases antagonist activity (Jacoby et al. 1981). Furthermore, in the 3-CH_3-3(m-OH)phenylpiperidine series, N-aubatitutiona did not influence relative agonist/antagonist activity as in fused-ring opiates (Cheng et al. 1984, 1985).

A. L-Tyr superimposed on 3-phenylpiperidine

B. L-Tyr superimposed on portion of morphinan nucleus

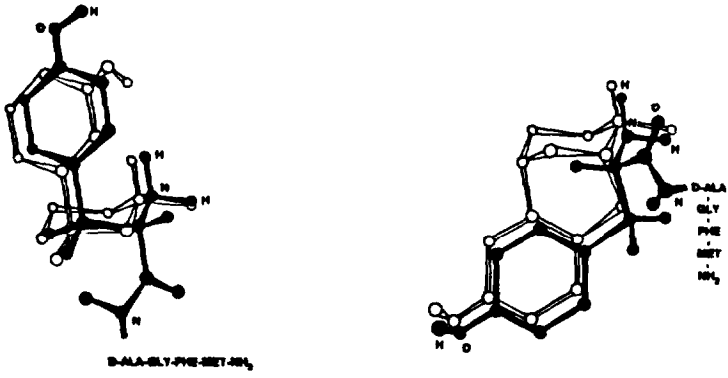


FIGURE 12

Comparison of 3-Phenylpiperidine and Peptide Opioids, A) With Each Other and B) With Morphinan

Thus, the two enkephalin analogs with modified tyroaine residues, showo in figure 13, were chosen for synthesis and testing along with D-Ala -Met-enkephalin amide. The synthesis of these peptides is described elsewhere (Loaw et al. in press). As shown in this figure, the modified tyroaine residues consisted of a m-0H tyroaine and a (m-OH) β-CH_3 tyroaine chosen to correspond to the (m-OH) and 3-CH_3 groups of the 3-methyl-3-(m-OH)phenylpiperidines, respectively. Binding affinities as well as in vivo, i.c.v. agonist and antagonist activity were determined for the three peptides.

As seen in table 10, the parent compound D-Ala^2-Met-enkephalin amide (p-Tyr) is a potent agonist in the tail-flick teat when administered i.c.v. in mice, being about one-third as potent as

morphine. This compound also showed significant antagonist activity in inhibiting a morphine-induced increase in tail-flick latency, but was only one-tenth as potent an antagonist as an agonist. Displacement of the hydroxy group on the tyrosine to the meta position produced striking changes in the relative activities

TABLE 10

Analgesic and Narcotic against and Antagoniat Potencies of (D-Ala[2], Met[5])Enkephalin Amide Analogs Evalueted in the Mouae Tail-Flick Teat

COMPOUND	AGONISM[a]		ANTAGONISM[b]	
	$K D_{50}$ µmol/kg	95% Conf. Lia. µmol/kg	$Ant.AD_{50}$ µmol/kg	95% Conf. Lia. µmol/kg
p-Tyr	0.214	0.074 ± 0.616	2.283	1.512 ± 3.453
m-Tyr	10.302	5.630 ± 18.854	1.684	0.785 ± 3.596
β-Ch_3-m-Tyr	2.394	1.203 ± 4.764	3.530	2.263 ± 5.507
Nalorphine	19.320	7.963 ± 46.754	0.322	0.184 ± 0.563
Morphine	0.063	0.026 ± 0.151	—	— —

[a]A modification of tail-flick teat of Li et al. (1978).
[b]Antagonism against 21.08 µmole/kg (s.c.) of morphine sulfate was measured 2, 5, 10, 20, 30, 45, 60, 90, and 120 min. after i.c.v. administration of enkephalin amide analogs.

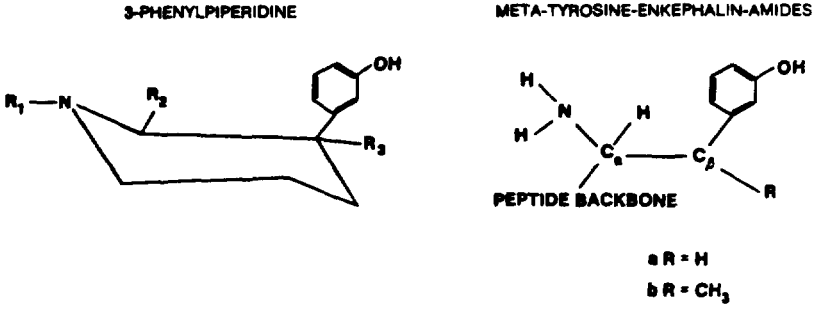


FIGURE 13
Peptides Selected for Synthesis

of the compound. The (m-OH)Tyr analog is 50 times weaker as an agonist, but slightly stronger as an antagonist, than its p-OH counterpart. This produces a compound six times more potent as an antagonist than as an agonist, and corresponds well with relative changes in potencies when 3-phenylpiperidines are modified from p-OH to m-OH (Jacoby et al 1981). The β-CH_3-m-Tyr analog has an activity profile between that of the other two compounds with approximately equal agonist and antagonist activity.

Competitive binding studies of these compounds were made using the four labeled ligands [H] naloxone, [E]DADL, [E]EKC, and [H]DHM. Also conducted were self- and cross-competitive binding of the labeled ligands themselves, as decribed above. The resulting 4 by 7 matrix of competitive inhibition experiments was analyzed simultaneously using the computer curve fitting program, LIGAND.

As shown in table 11, all of the peptides exhibited high affinity at the site designated as μ_1. Furthermore, the relative analgesic potency of the three peptides corresponds to their relative affinities at "μ_1-" The affinity at μ_1 and analgesic activity of the p-Tyr analog are 20 to 50 times higher then either of the two novel peptide analogs. This observation lends support to the evidence presented by Pasternak that the μ_1 receptor is responsible for opiate-induced analgesia (Pasternak et al. 1980). Also evident from table 11 is the n-selectivity induced by the (m-OH)Tyr. While the p-Tyr analog is very potent at δ receptors, both m-Tyr and β-CH_3-m-Tyr have quite low affinities at δ, consistent with the 3-phenylpiperidine templates.

Thus, it appears that (m-OH)Tyr and β-methyl-(m-OH)Tyr-Met-enkephalin-amide analogs are the first μ-selective enkephalin-type peptides with demonstrated antagonism to whole animal analgesia. Moreover, requirements for potent antagonism of Met-enkephalin analogs appear similar to that for 3-phenylpiperidines, possibly indicating similar binding conformations.

To continue to explore this similarity, we are in the process of synthesizing two more novel tyrosine analogs, an N-phenethyl-(m-OH)-tyrosine and a α,β-dimethyl-(m-OH)-tyrosine to incorporate into Met-enkephalin-amide peptides. If they behave as 3-phenyl-piperidines do, then both of these modifications should enhance antagonist potency.

TABLE 11

Receptor Affinities and Maximum Binding Capacities of (D-A1a^2-Met^5)Enkephalin Amide Analogs Assuming a 5-Receptor-Site Model

	K_D(nM)				
	Site 1 "μ1"	Site 2 "μ2"	Site 3 "δ"	Site 4 "κ"	Site 5
Naloxone	0.60	3.0	20.8	0.5	100
DADL	1.80	14.9	1.3	312	0,333
EKC	0.60	4.3	15.0	0.003	588
DHM	0.30	12.7	119	1.515	83
p-Tyr	0.53	5.0	0.40	1600	476
m-Tyr	13	10	50	5,260	435
β-CH_3-m-Tyr	19	50	200	370	179
B_{max} pmol/g	4.2	20	3.7	2.3	157

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Conformations of Opioid Peptides as Determined by Nuclear Magnetic Resonance and Related Spectroscopies

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INTRODUCTION

The peptides that possess opiate like biological properties are called "opioid peptides." Known members can be divided into three main groups according to their sources. 1) Endorphins: These peptides have been found to occur naturally in the central nervous sys tern. β-Endorphin is the largest in this group, followed by dynorphin, neoendorphins, and the enkephalins which are the smallest pentapeptides in the family (Hughes and Kosterlitz 1977). 2) Exorphins: These peptides are obtained as a result of partial enzymatic digestion of proteins derived from food stuffs (Loukas et al. 1983). For example, β-casomorphins have been isolated from the bovine milk protein, β-cesein. 3) Kyotorphins: These are synthesized small peptides (Takagi et al. 1979) whose opiate like properties do not arise from direct interaction vith opiate receptors,but perhaps act by releasing enkephalina and/or by inhibition of enkephalin degradation (Morley 1983).

Since the discovery of enkephalins, much attention has been dravn to this area. The enkephalins are distributed over wide areas of the CNS, specific parts of the brain, spinal cord, and in certain nerve plexi of intestines. Because of the interaction of enkephalina at the same receptor site as that of morphine, numerous structural analosa of nkephalins have been prepared and tested for their opioid activity, their propenaity to cause tolerance and physical dependence,their susceptibility to degradation by brain enzymes,and for their behavioral effects on laboratory animals and on schizophrenic patients. The isolation, identification, characterization, and structure-activity relationships of two naturally occurring enkephalins, namely H-L. Tyr-Gly-Gly-L·Met-OH([Met^5]-Enk) and H L·Tyr-Gly-Gly L·Phe-L·Leu-OH ([Leu^5]-Enk), are covered in detail in some recent reviews (Hughes and Koaterlits 1977; Miller and Cautrecasas 1978; Morley 1980; and Schiller 1984).

Structural similarities between the morphine derivatives and the enkephalins must exist if they share common receptors. Extensive research efforts have, therefore, been made to predict the enkephalin structures compatible with the rigid morphine structure. The main approaches utilized in these studies are: 1) speculation as to the conformation of the enkephalins at the opiate receptor resulting from model building and comparison with models of narcotic drugs, 2) conformations of enkephalins in solution as derived from various spectroscopic data, and 3) conformational energy calculations.

Recently, in an elegant review article, Schiller (1984) has exhaustively summarized all the previously reported conformation-activity relationships of enkephalin molecules. Data from various spectroscopic methods, nuclear magnetic resonance (NMR) in particular, have been discussed. In order to avoid repetition of the similar subject matters, a different approach will be taken in this review article as outlined in the folloving sections.

1. Definitions of various conformational states of a peptide/polypeptide backbone.

2. Descriptions of various NMR methods that are used in obtaining conformational information.

3. Use of such NMR methods in relation to A) backbone and B) side-chain conformations of enkephalin molecules.

4. Concluding remarks.

PEPTIDE CONFORMATIONS

The backbone of a polypeptide or protein is formed from a repeating peptide unit, as depicted in figure 1.

It can be seen that the overall conformational characteristics of the peptide unit are determined by rotations about the C^{α}-C, $N-C^{\alpha}$, C'-N, and C^{α}-R bonds (R is the side-chain of an amino acid). A convenient description of the conformation about each bond is in terms of the torsion angles ψ (C^{α}-C'), ϕ (N-C^{α}), ω (C'-N), and χ (C^{α}-R) as shown in figure 1: ϕ refers to the torsion angle of sequence of atoms $C_1^{\alpha}, N_2, C_2^{\alpha}, C_2'$; ψ to the sequence N_1, C_1^{α}, C'_1, N_2; and ω to the sequence $C_1^{\alpha}, C_1', N_2, C_2^{\alpha}$. Torsion angle is considered positive ($+\phi$) or negative ($-\phi$) according as when the system (figure 1) is viewed along the central bond N_2-C_2^{α} in the direction $N_2 \longrightarrow C'_2$ (or $C'_2 \longrightarrow N_2$), the bond to the front atom C'_1 (or C'_2) requires to be rotated to the right or to the left, respectively, in order that it may eclipse the bond to the rear atom C'_2 (or C'_1). Similarly, $+\psi$ and $-\psi$ can be determined by viewing along the central bond C_1^{α}-C_1'. Torsion angle ψ is either zero (for cis peptide bonds) or 180 (for trans peptide bonds).

Side-chain torsion angles designated by χ are specified by two (or three) superscripts, the first one (or two) indicating the central bond about which the angle is measured and the last one indicating whether the angle measured is relative to any branched chain atom. By the sequence rule (IUPAC-IUB 1970), when $\chi_1 = 0$, C^{γ} ($or\ C^{\gamma 1}$) is in the eclipsed position relative to N. Positive or negative angles can be generated by viewing along the central C^{α}-C^{β} bond in the direction $C^{\beta} \rightarrow C^{\alpha}$ and rotating C^{γ} ($or\ C^{\gamma 1}$) right or left, respectively, in order to eclipse with N. A fully extended polypeptide chain is characterized by $\phi = \psi = \omega = +180°$ (IUPAc-IUB 1970).

Rotations at the above mentioned bonds give rise to various conformations that are stabilized by intra- and/or inter-molecular hydrogen bond (H-bond) formations between the C=0 and N-H groups, as shown in figure 2.

An H-bonded ring between the N-H of an amino acid sequence number m and the C=0 of another residue of the sequence number n is designated as m $\rightarrow$ n which encompasses a certain number of atoms of the backbone chain. The possible H-bonded structuree in a system of 4 linked amino acids are shown in figure 2. A 2 $\rightarrow$ 2, 3 $\rightarrow$ 3, or 4 $\rightarrow$ 4 H-bond encompassee 5 atoms; the 3 $\rightarrow$ 1 or 4 $\rightarrow$ 2 contains 7 atoms; similarly, 2 $\rightarrow$ 3 or 3 $\rightarrow$ 4 and 4 $\rightarrow$ 1 H-bonds contain 8 and 10, respectively; while a 2 $\rightarrow$ 4 H-bonded ring consists of 11 atoms. These aforementioned intramolecular H-bonded rings are also indicated by C_5, C_7, C_8, C_{10}, and C_{11} conformations, respectively.

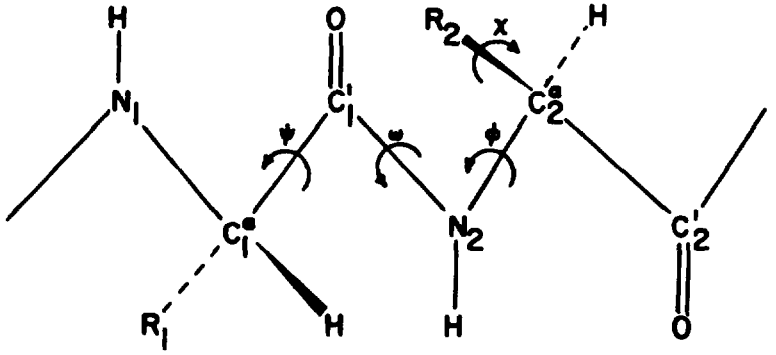


FIGURE 1

A Peptide Backbone Showing Various Torsion Angler

The conformation is extended and usually occurs in conjunction with an antiparallel ß-pleated sheet structure that is formed by intermolecular H-bonds, as shown in figure 3.

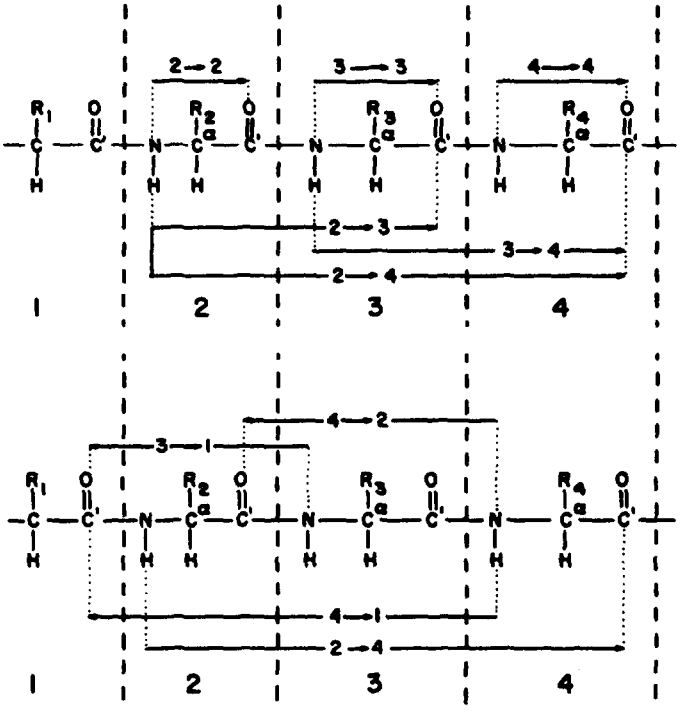


FIGURE 2
Possible Intramolecularly H-Bonded Rings in a Peptide System with Four Linked Amino Acids

Both the C_7 and C_8 conformations are also known as short-range interactions and usually occur with other dominant conformational features, such as helices or higher ring structures. For example, C_7 always coexists with C_{11}, as shown in figure 4. Following the ß-turn, which is a 10-membered H-bond, chronologically, an 11-membered H-bonded ring (figure 4) should be termed as γ-turn (Nemethy and Printz 1972; Matthews 1972). Unfortunately, however, controversy remains in literature regarding the designation of this structure. The structure in figure 4 happens to contain one 7-membered (C_7)ring which some research workers prefer to call γ-turn, while the C_{11} is referred to as ε-turn.

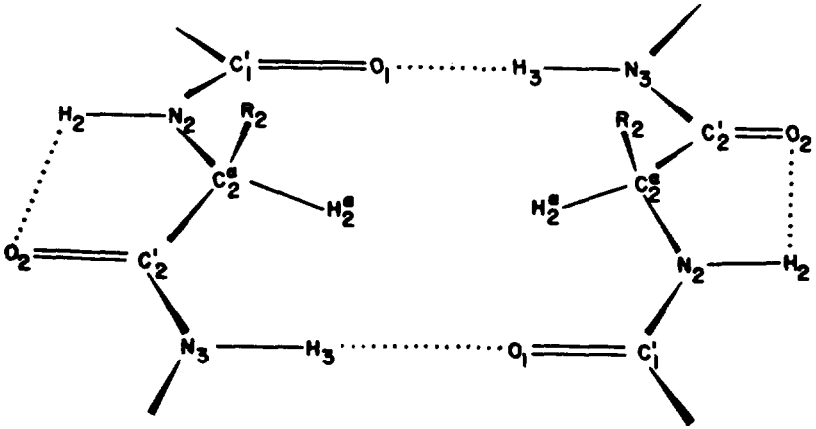


FIGURE 3
Antiparallel β-Sheet Structure with C_5 H-Bond

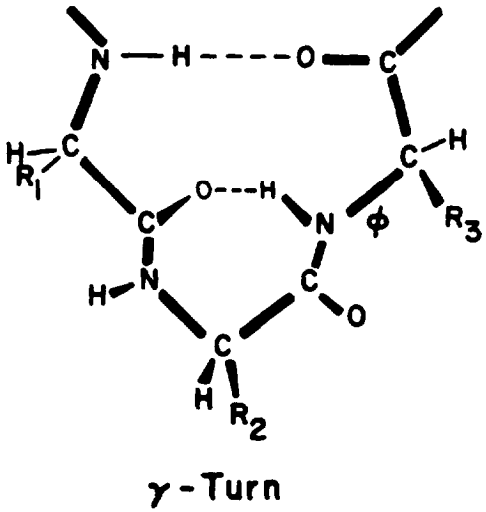


FIGURE 4
A γ-Turn Conformation in Peptides

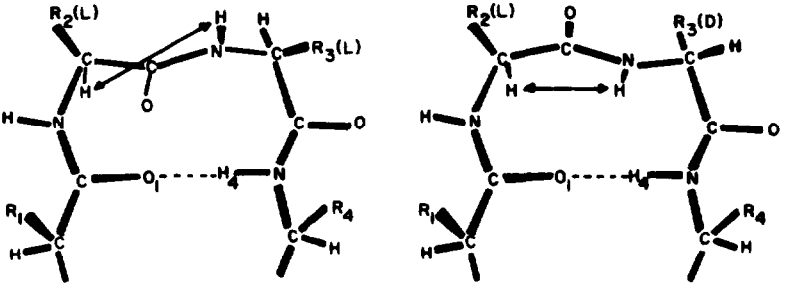
The β-turn, a 10-membered intramolecular H-bonded ring, just mentioned above, will be further discussed here since the occurrence of this conformational feature in enkephalin analogs is most abundant. The reader should, therefore, be adequately familiar with conformational properties of a β-turn. The main characteristic of a β-turn is to reverse the backbone chain, containing a suitable dipeptide moiety, in the same plane where it thus differs from a 3_{10}-helix. It was, therefore, previously termed as 4 → 1 intramolecularly H-bonded nonhelical conformation (Venkatachalam 1968; Benedetti et al. 1976). Conformstions related to β-turns have also been called earlier as: 3 (or 3-10) bond (Kuntz 1972), folded β-conformation or β-fold[10] (Shields et al. 1968), β-turn (Urry and Ohnishi 1970), β-bend Chandrasekaran et al. 1973), hairpin bend (Kuntz 1972; Chandrasekaran et al. 1973), β-loop (Kuntz 1972), 1 — 4 bend (Nemethy and Prints 1972), chain reversal (Lewis et al. 1973), U-bend (Chandraeekaran et al. 1973), lo-membered H-bonded ring (Deber 1974), 1,4 turn (Hruby 1974), β-twist (Benedetti et al. 1976), and tight turn (Richardson et al. 1978).

Various foldings of a peptide backbone chain into different forms or types of β-turns are depicted in figure 5. Venkatachalam (1968) classified the β-turns into conformational types: I, II, III, and their mirror images I', II', III'. The torsion angles ϕ and ψ that define these types are given in table 1.

It can be seen in table 1 that the β-turn type III is actually a helix. Because of sterical hindrance, it is necessary that the two residues within the H-bonded ring should be with L-configuration, i.e., L-L arrangement is preferred for a type I β-turn, while an L-D arrangement is preferred for a type II β-turn. The mirror images, D-D and D-L, are obviously required to form type I' and II' β-turns, respectively. There configurational arrangemente are clearly shown in figure 5 by the different projections of R_2 and R_3 on side-chains. A glycine residue can be conveniently accommodated in place of a D-substituted amino acid , particularly in type II and II' β-turns. A convenient way of distinguishing type I and type II β-turns is to consider the orientation of the peptide moiety between residues 2 and 3 (see figure 5). In type I β-turn, the α-H proton of residue 2 is oriented opposite to the N-H proton of residue 3, as indicated by a double-headed arrow in figure 5A. In type II β-turn, however, the same two protons are on the same side which is also indicated by a double-headed arrow in figure 5B. These are the simple descriptions of intra- and/or intermolecular H-bonded structures that will be mentioned frequently in section 3, which deals with the backbone conformations of various enkephalin analogs.

NUCLEAR MAGNETIC RESONANCE METHODS

NMR is the most powerful technique which can provide all the necessary information needed to describe a peptide conformation.



A. TYPE I B. TYPE. II

FIGURE 5

β-Turn Structures in Peptides

TABLE 1

Torsion Angles of Various Types of β-Turns

	Residues			
	2		3	
Type	ϕ_2	ψ_2	ϕ_3	ψ_3
I	-60	- 30	-90	0
I'	60	30	90	0
II	-60	120	80	0
II"	60	-120	-80	0
III	-60	- 30	-60	-30
III'	60	30	60	30

Resolution of NMR signals and their assignment to individual amino acid residues are the first steps in conformational analysis of peptides and proteins. Usually, the assignments are carried out from the inspection of the side-chain spin pattern of a particular amino acid residue by using spin decoupling technique. This is true when a molecule contains different amino acid residues occurring only once in the backbone with well-resolved NMR signals. However, it becomes more complicated when a molecule contains a recurring amino acid residue such as in enkephalin molecules where two glycyl residues are involved in positions 2 and 3. Selective isotope enrichment of one of such residues either by C, H, or N is the usual way for unequivocal assignments of NMR resonances. The two glycyl residues in [Leu^5]enkephalin were thus assigned by the partial C^{13} enrichment (Tancrede et al. 1978). Recently, however, a combined use of ^1H and ^{13}C resonances has been made to both ^1H and ^{13}C signals of a particular amino acid in peptide system (Khaled and Urry 1981; Khaled et al. 1981; Khaled and Watkins 1983). The basic principle of this technique is to selectively irradiate the α-H. and N-H. protons, across a peptide bond. These two protons are coupled to $\overset{\alpha}{C}=O$ carbon which also provide almost all the nuclear Overhauser enhancement (NOE) for this carbonyl carbon nucleus. Once two such protons are located and one of the amino acid residues is identified, either from its side-chain spin pattern or by perturbing the end groups in case of a linear peptide, the rest of the residues in the molecule can be assigned by stepwise irradiation of the ^1H resonances. Thin method ultimately allows the unequivocal NMR signal assignments without involving an expensive and time-consuming synthetic iaotope enrichment. After having assigned all the resonances, conformational analyses are carried out by using the well-established NMR methods as described next.

Identification of H-Bonds

The intra- and inter-molecular H-bonds are usually delineated from the temperature dependence of peptide N-H proton chemical shifts. An intramolecularly H-bonded peptide N-H proton has less temperature dependence compared to an intermolecularly H-bonded or an exposed N-H proton. The temperature coefficient $(\Delta\delta/\Delta T)$ of an intramolecularly H-bondgd N-H proton typically varies from 2×10^{-3} to 5×10^{-3} ppm/°C depending on the stability of the H-bond (Kopple et al. 1969; Ohnishi and Urry 1969). However, there exists a controversy regarding the temperature dependence of peptide N-H protons. Stevens et al. (1980) reported $\Delta\delta/\Delta T$ value of the order of 2.4 ppm/°C which they attributed to the N-H proton that is exposed to nonpolar solvents, such as chloroform. The $\Delta\delta/\Delta T$ ' values higher than 2.4 ppm/°C are interpreted as the indications of change of N-H protons from bound to free state, i.e., from H-bonded to exposed to nonpolar solvents.

The rates of peptide N-H proton exchange with the labile hydrogens or deuteriums in the medium can also provide information on the H-bonded and nonbonded N-H protons (Molday et al. 1972; Stern et al. 1968). This can be achieved either by adding a controlled quantity of D_2O into a protonated medium or by the saturation transfer of the labile proton of the solvent by a double resonance NMR method (Redfield 1978). The N-H protons which are exposed to the solvent experience reduced signal intensity according to the extent of their exposure to solvent. The difficulty with this method is that the proton exchange rates are the kinetic parameters which reflect the properties of the transition state of rate determining step in the protolysis reaction i.e., a state which may not resemble the preferred equilibrium state of the peptide. However, it is generally desirable to confirm intramolecularly H-bonded peptide N-H protons by as many methods as possible. There is, therefore, another method where the peptide N-H protons show differential dependence on the composition of a solvent mixture (Pitner and Urry 1972). The advantage of this method is that both the peptide N-H protons and peptide C=O carbons can be classified as an appropriate H-bonded pair, i.e., N-H...O=C pairing can be established. There is, however, one very essential requirement in this method which requires that the molecule should retain the same conformation(s) in both the solvents, which can be verified by coupling constants and temperature studies as stated above. Such an experiment is performed by monitoring the chemical shift changes of peptide N-H protons and peptide C=O carbons from a weak basic solvent, such as dimethyl sulfoxide, to a protic solvent, such as trifluoroethanol. Very recently , Khaled and Watkins (1983) have developed a new $^{13}C(^{1}H)$ NOE method to detect simultaneously an H-bonded pair, i.e., N-H...O=C, in peptides.

Estimation of Torsion Angles ϕ and ψ

A conformation is completely described when the angle of rotation about each bond of a peptide is specified (see figure' 1 and section 1). In peptides and amino acids, vicinal coupling between N-H and $C^{\alpha}H$ protons provides information about the peptide torsion angle ϕ using a Karplus relationship (1959) of the type

$$^{3}J = A\cos^{2}\theta + B\cos\theta + C \qquad (1)$$

where θ is the dihedral angle formed between the H-NC^{α} and N-C^{α}-H planes.

The dihedral angle θ is related to the peptide torsion angle by the equations

$$\begin{aligned} \theta &= \phi - 60 \quad \text{for L-amino acids} \\ \theta &= \phi + 60 \quad \text{for D-amino acids} \end{aligned} \qquad (2)$$

Empirically, Bystrov et al. (1969) have established the following equations:

$$^{3}J_{C^{\alpha}\underline{H}\text{-}N\underline{H}} = 9.4\cos^{2}\theta - 1.1\cos\theta + 0.4 \quad (3)$$

and for glycine residues

$$^{3}J_{C^{\alpha}H_{A}\text{-}NH_{X}} + {}^{3}J_{C^{\alpha}H_{B}\text{-}NH_{X}} = 9.8\cos^{2}\phi - 1.3\cos\phi + 15.0 \quad (4)$$

The above expressions give several possible angles for each experimental coupling constant. One of the possible angles would be approximately correct, if there were no torsional motion. Since, virtually in every case for a biological peptide, there would be some toreional flexbility, it is important to realize that the observed value is obtained from a summation of states properly weighted according to its energy dependent probability. Therefore, if the values ranging from 5.5 to 7.5 Hz were observed, then this could imply either a single state, ϕ_{t}, or nearly unrestricted rotation about the N-E bond. However, if a value of 4 Hz or 8 Hz is obrerved, then these values indicate substantially restricted angles. Similarly , if the residue is a glycine, then the greater the nonequivalent the $C^{\alpha}H_{A}\text{-}NH_{X}$ and $C^{\alpha}H_{B}\text{-}NH_{X}$ $N_{B}\text{-}NH_{X}$ coupling constants, the stronger the argument is for limited rotation about the N-C^{α} bond. If the coupling constants are equivalent, it may be due to essentially unrestricted rotation or, looking along the N- C^{α} bond, due to the N-H line segment bisecting the H-C^{α}-H bond angle. The same considerations may also apply to $C^{\alpha}H\text{-}C^{b}H_{2}$ for the determination of angle X, as described below.

For the ψ torsion angle of glycine residues, Barfield et al. (1976) have determined an expression for the geminal coupling, ^{2}J, which is also a function of the ϕ of equation 4.

$$^{2}J_{C^{\alpha}H_{A}\text{-}C^{\alpha}H_{B}}(\phi,\psi) = -13.91 - 1.55\cos^{2}\psi + 4.65\cos^{2}\phi - 2.80\cos^{4}\psi \quad (5)$$

For L- and D-amino acid residues, NOE can be used as long as the peptide moiety remains approximately planar, becaure the $C^{\alpha}H_{i}$ to NH_{i+1} interproton distance is a function of the angle ψ (Leach al. 1977).

Side-Chain Conformations (X)

The conformational properties of amino-acid side-chain sometimes restrict the allowed range of ϕ and ψ angles if there is an intramolecular interaction with the adjacent amino acid. For a complete understanding of the conformation of peptides it is important to determine the conformation about the $C^{\alpha}\text{-}C^{\beta}$ bond. A correlation has been observed between $^{3}J(HH)$ coupling constant and the dihedral angle θ for the CH-CH system using again a Karplus-like relation: $^{3}J = B\cos^{2}\theta - C$, were B + 10.4 for $0 < \theta < 90$, and B = 13.5 for $90 < \theta < 180$ and C = 0.

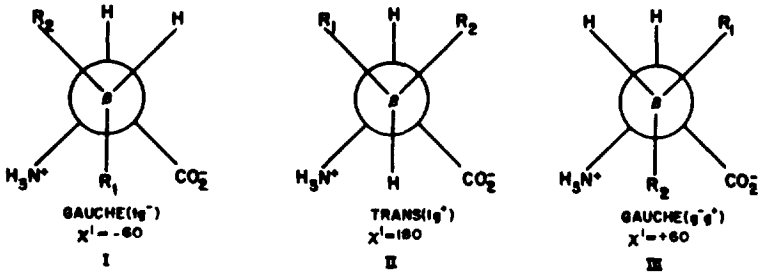


FIGURE 6

Possible Side-Chain Conformations of Amino Acids $R_1 = R_2 = H$ for Ala, $R_1 = R_2 = CH_3$ for Val, and $R_1 = H$, R_2 = aryl or alkyl group representing Phe, Tyr, Leu, etc.

For most amino acid residues,there is considerable rotational freedom about the $C^{\alpha}-C^{\beta}$ bond. It is assumed that the three staggered conformations (I, II, and III depicted in figure 6) have relative populations, P_I, P_{II}, and P_{III} such that

$$P_I + P_{II} + P_{III} \qquad (6)$$

For rotation about the $C^{\alpha}-C^{\beta}$ bond that is rapid on the NMR time scale

$$^3J(HH)_{obs} = J_tP_{II}+J_8P_I+J_8P_{III} \qquad (7)$$

assuming J_g has the same magnitude for conformers I and III.

For free rotation ($P_I - P_{II} = P_{II}$ the observed ^3J is given by

$$^3J(HH)_{free} = 1/3\ (J_t + 2J_g) \qquad (8)$$

From experimental coupling values and assuming J = 13.6 Hz and J = 2.56 Hz, obtained by Pachler (1964), the relative rotamer populations can be calculated for different amino acid side-chains, such as valine, leucine, phenylalanine, aspartic acid, and tyrosine. The torsion angles can also be estimated by using the equation of Abraham and McLauchlan (1962):

$$^3J_{C^{\alpha}\underline{H}-C^{\beta}\underline{H}} = \begin{matrix} 10.5\ \cos^2 X^1 - 0.28 \text{ for } X^1 = 0 - 90° \\ 10.7\ \cos^2 X^1 - 0.29 \text{ for } X^1 = 90 - 180° \end{matrix} \qquad (9)$$

The relative population of trans and gauche, isomers can be calculated, but it is not possible to distinguish between the two possible gauche conformers (I and III in figure 6) unless R_1 and R_2 have been unequivocally assigned, e.g., for valine

($R_1 = R_2 = CH_3$) and phenylalanine (R_1 or R_2 = H). For phenylalanine and tyrosine, the assignments of the two β-CH_2 protons could be made by stereospecific deuterium labeling. This has been accomplished in enkephalin molecules by Kobayashi et al. (1979, 1980). As mentioned above, a large or small coupling constant or nonequivalence, involving a CH_2 moiety , provides direct evidence for restricted torsion angles. Where it becomes important to resolve the ambiguity of midrange coupling constants and equivalent coupling to CH_2 moieties. synthetic enrichment with ^{13}C and/or ^{15}N can be carried out and the additional heteronuclear coupling constants can be used to resolve the ambiguity experimentally. This has recently been done for the $C^{\alpha}H-C^{\beta}H_2$ torsion angles of enkephalins (Stimson et al. 1979; Garbay-Jiurequiberry et al. 1982) and will be discussed further in section 3.

Nuclear Magnetic Relaxation Times (T_1 and T_2)

The longitudinal or spin-lattice relaxation time (T_1) and the trsnsversal or spin-spin relaxation time (T_2), particularly the T_1, have been used to study the dynamic behavior of enkephalin molecules by many research workers.

In practice, T_2 is measured from the line width of a single signal by using the equation

$$T_2 = \frac{1}{\pi W} \tag{10}$$

where W is the line width at half height of a signal.

Various molecular dynamics can be well characterized by a time factor called correlation time,which serves as a measure of the average time that two nuclei remain in a given orientation and is designated as T_c. For molecular rotation, the T_c is the average length of time that the molecule requires to rotate through an angle of a radian, while for translation, the T_c is the time it takes to move the molecule to one molecular diameter. The rotational process is related to the intramolecular relaxation, while the translational process is related to the intermolecular relaxation. In general, both processes depend on the viscosity of the medium and vary critically on the size and shape of the molecule involved. If the molecule being studied tumbles isotropically and behaves like a spherical rigid rotor (having only rotational motion), the equation to derive Tc from T_1 is given by (Levy and Nelson 1972).

$$\frac{1}{T_1} = \frac{1}{20}\frac{(\gamma_H\gamma_C\hbar)^2}{r_{CH}^6}\left[\frac{\tau_c}{1+(\omega_H-\omega_C)^2\tau_c^2}+\frac{3\tau_c}{1+\omega_C^2\tau_c^2}+\frac{6\tau_c}{1+(\omega_H+\omega_C)^2\tau_c^2}\right] \tag{11}$$

Where γ is the gyromagnetic ratio, w is the angular frequency, and r is the distance between the two coupled nuclei. This is

the relsxstion for one ^{I}H attached to a ^{I5}C; for ^{I3}C-^{I}H, the right-hand side of the equation will have to be multiplied by n (n is the number of protons attached to a carbon atom).

Nuclear Overhauser Effect

As mentioned in the preceding section, besides providing the estimate of torsion angle ψ, NOE can, in principle, be used in determining the spatial relationships between nuclei that are not covalently linked. It is the fractional change, f_i (j), in intensity by cross-relaxation of one resonance, i, when another resonance, j, is perturbed (Noggle and Schirmer 1971). Simple mathematical derivations of f_i (j) are given by Roques et al. (1980) and, therefore, the repetition of such expressions here is unwarranted. The change in intensity of the signal of spin i on saturation of spin j reflects the relative proximity of i and j. The NOE, f_i(j), is, therefore, a function of both the molecular geometry if a molecule and the molecular dynamics.

Two-Dimensional (2-D) NMR

In the event that the NMR spectra are too complex to be resolved in normal NMR experiments, 2-D NMR in such cases appears to be an invaluable tool. The basic principle of the experiment involves two successive precession periods, t_1 and t_2. The first time t_1, is called the evolution period and the second time, is characterized by two frequency coordinates, one with chemical t_2, is called the detection period. The spectral resolution shift information and the other with the coupling constant information. A large variety of experiments is possible, depending on the phase or frequency of Rf irradiation, decoupling level, and so on, that are applied to the nuclear spins during the intervals t_1 and t_2.

These experiments include 2D J-resolved spectroscopy, 2D correlated spectroscopy (COSY) and 2D cross-relaxation spectroscopy. In enkephslin systems both the 2D-J-resolved and COSY have been utilized by a few research workers (Gidley et al. 1981; Kessler et al. 1983, in press).

CONFORMATIONS OF ENKEPHALINS

Various physical methods, such as NMR, UV, CD, IR, and Laser Raman spectroscopy have been used to derive the conformations of enkephalin molecules. NMR, however, remains the most informative, as discussed. In this section, therefore, most of the NMR work will be discussed along with other spectroscopic methods. Since it is desirable to describe the backbone and side-chain conformations separately, this section is divided into two subsections.

Backbone Conformations

From the structural modifications of enkephalin,the stereospecificity and the structure-activity relationships of each residue have been established. Tyrosine at position 1 is the most sensitive one; a slight alteration, even in its aromatic side-chain, drastically reduces the biological activity. Clycine at position 2 can conveniently be replaced only by any D-substituted amino acid residue. Positions 3 and 4 are fixed for the glycine and L-phenylalanine residues, respectively, while position 5 is quite open to any amino acid residue with either L or D configurations. Based on these findings, Beddell et al. (1977) have suggested various H-bonded conformations of enkephalin whose general definitions and terminologies have already been described. All such possible H-bonded conformations of enkephalin molecules are described below. Each of these structures as observed by NMR and either supported or refuted by other physical techniques has also been discussed.

Phe^4 N-H → 0=C Tyr^1 (4 → 1 β-turn, Bradbury et al (1976) first predicted this β-turn, formed between the Phe^4 N-H and Tyr^1 C=0 groups from the application of the empirical rules for predicting secondary structure from amino acid sequence. Smith and Griffin (1978) also found this H-bond in crystalline state of [Leu^5] enkephalin grown from an aqueous methanol solution. In all the previously reported studies in solution, the occurrence of such H-bond could not be found. Only very recently Beretta et al. (1984), for the first time, have predicted the formation of this β-turn in complexes of [Met^5] and [Leu^5] enkephalin amides with 18-crown-6-ether in chloroform using high temperature dependence ($\Delta\delta/\Delta T$) of Phe^4 N-H proton resonance, measured at 500 MHz, as the indication of H-bond formation. As mentioned previously , there exists the controversy of using $\Delta\delta/\Delta T$ parameter; accordingly, this report is one such example. The lowest values reported in this study are for Leu^5 and Met^5 N-H protons which, based on the other criterion (see the indentification of H-bonds), should give rise to a β-turn involving the [Met^5] N-H and Gly^2 C=0 groups. This conformational feature is discussed next.

Met^5 or Leu^5 N-H → 0=C Gly^2 (5 → 2 β-turn), This is the most commonly observed conformational feature of enkephalins reported by many research groups. Roques et al. (1976) and Jones et al. (1976) were the first to report the occurrence of 5 → 2 β-turn in DMSO-d_6 based on the coupling constants and the low $\Delta\delta/\Delta T$ values of Met^5 or Leu^5 N-H proton. Almost at the same time , Bleich et al. (1976) reported a normal $\Delta\delta/\Delta T$ value for the Met^5 N-H proton and suggested that a folded conformation might be stabilized by a nonbonded interaction. Jones et al. (1977) later came out with an explanation for this discrepancy showing that the cationic and switterionic forms of enkephlin gave different NMR parameters. In all NMR studies involving ^{1}H, ^{13}C and ^{15}N nuclei in DMSO, the same 5 → 2

β-turn was observed (Khaled et al. 1977; Garbay-Jaureguiberry et al. 1977; Stimson et al. 1979; Marion et al. 1981; Zetta and Cabasai 1982). The controversy, however, remaineder for sometime on the type of the turn. This β-turn contains Gly^3 - Phe^4 peptide moiety within the 5→2 H-bonded ring. Since a glycine residue could be substituted for a D-amino acid residue, thus forming a D-L peptide moiety, this D-L configurational arrangement should then be designated by a type II' β-turn (see section 1) The ^{13}C NMR studies by Stimson et al. (1979), however, vere found to be beat compatible with a type I β-turn vith presumably uncharged terminal groups. Laser Raman studies (Han et al. 1980; Rapaka et al. 1984) supported a compact β-turn structure, but the exact type could not be delineated. The proton T_1 measurement by Niccolai et al. (1980) also revealed the occurrence of a type I β-turn. The unequivocal determination of the type of β-turn by NOE (Khaled and Urry 1976) was impeded by the complex spin pattern of the Gly^3 methylene protons. More recently, however, mearurement of $^3J1_{H(Gly3)\underline{15}N(Phe4)}$ by Garbay-Jaureguiberry et al. (1982) finally indicated the occurrence of a type II' β-turn which is also energetically more favorable, as previously proposed by Isogai et al. (1977).

Associated form. The associated form with an antiparallel cross β-structure was, first reported by Khaled et al. (1977) from combined ^1H and ^{13}C NMR data. In this structure, four intermolecular H-bonds were found to occur between the Gly^3 NH and Gly^3 C=0 groups and between the Met^5 or Leu^5 NH and Tyr^1 C=O groups. A "head-to-tail" interaction was also proposed and later supported by Anteunis et al. (1977). Occurrence of such associated form in crystalline state was first noted by Blundell and Wood (1982) but later completely described by Karle et al. (1983).

Conformational equilibrium. Enkephalins are small molecules and, therefore, it is likely that there could be a number of conformations that these molecules may adopt in solution. The variety of conformers discuessed above confirms the existence of such molecular flexibility. Using ^1H, ^{13}C, CD, and UV spectroecopies, Khaled et al. (1977) demonstrated that different conformations may occur, depending on the nature of solvents and concentration. They even proposed (Khaled et al. 1979) a conformation resulting from the inversion at central Gly^3 carbon atom which is similar to a V-conformation (Beddell et al. 1977). The results of subsequently performed ^{13}C and ^{15}N T_1 studies (Tancrede et al. 1978; Marion et al. 1981) supported such folding since Gly^3 residue was found somewhat more restricted than the Gly^2 residue. Using sterochemically deuterated Gly^2 and Gly^3 residues Fischman et al, (1978) also showed the averaging of Gly^2 and Gly^3 angles in both DMSO and D_2O solvents. In addition, Higashiiima et al. (1979) suggested that the zwitterionic form of [Met^5] enkephalin in DMSO at low concentration may exist in an equilibrium of

extended and folded conformations. Later, Miyazawa and Higashijima (1981) used IR absorption spectroscopy to distinguish cationic, zwitterionic, and anionic forms of N- and C-terminal free enkephelins in DMSO and once again supported the existence of a folded = extended conformational equilibrium. Although a folded form was stabilized by an electrostatic interaction between the free N- and C-terminals, the results from an NOE experiment ruled out the possibility of a 5 → 2 β -turn formation (Higashijima et al. 1979).

Many different conformational equilibria vere also predicted from the CD results as a function of temperature and pH (Spirtes et al. 1978; Rollosi et al. 1980). Soos et al. (1980) used the CD spectra of enkephalin to determine structure-activity relationship. Based on the increased negative ellipticity at 225 nm as indicative of folded conformation, they obtained a good correlation betveen the degree of folding and the potency in the GPI but not in the MVD, thus reflecting the requirement of folded form at the μ-receptor sites. Conformational flexibility of [Met^5] enkephalin amide was also observed in methanol and trifluoroethanol by using CD spectroscopy (Sudha and Balaram 1981). The Laser Raman spectroscopic results reported by Ran et al. (1980) also showed different conformers of [Leu^5] enkephalin in D_20 in zwitterionic form. Most recently, Rapaka et al. (1982) used Raman spectroscopy to show that the occurrence of β-turn in [Leu^5] enkephalin in vater is more predominant than in [Met^5] enkephalin. This interpretation was derived from the observation of a strong band at 1263 cm^{-1} for [Leu^5] enkephalin in comparison with a shoulder at 1265 cm^{-1} for [Met^5] enkephalin.

In an effort to minimize the conformational freedom, several cyclic analogs of enkephalin have been Synthesised with rertric ed backbone. Two sush cyclic analogs are: [D-Cys^2, D+Cys^5] and [D-Pen^2, D+Cys^5] enkephalinamides, cyclired via S-S bond. Pen stands for penicillamine and is equivalent to β,β -dimethylcyeteine. The former peptide showed the preference for the μ-receptor (Schiller et al. 1981), while the latter displayed significant δ-receptor specificities (Hosberg et al. 1982). The only difference between these two peptides is the side-chain of the amino acid at position 2. Recently the ^{1}H NMR studies by Mosberg and Schiller (1984) revealed a common conformational characteristic for both peptides. Although they did not indicate any particular conformational feature, it appears from the Δδ/ΔT parameter of the amide protons that the NH proton of the L or D-Cys^5 is the most shielded one, thus indicating the formation of a 5 → 2 β-turn. Another interesting group of cyclic analogs of enkephalin reported by Kessler et al. (in press) are the Tyr-Cyclo (-N-Xxx-Gly-Phe-Leu-) where Xxx = L-Orn, L-Lys, or D-Lys. The D-Lys^2 analog is 10 times more active in the GPI assay, i.e., μ-receptor specific, than the D-Orn^2-compound. Use of 2D ^{1}H and ^{15}N-^{1}H COSY, ^{3}J, and

$\Delta\delta/\Delta T$ parameters have all been used in order to understand the conformational properties of these peptides. The D-Orn^2-analogs showed a Leu^5 N-H $\rightarrow$O-C Gly^3 H-bond which they designated as γ-turn (please see the first section and note the discrepency) and an intramolecularly bifurcated H-bond between the Orn^2 N-H and Orn^2 C=0 groups. The D-Lys compounds, on the other hand, have been found to possess another bifurcated 10-membered intramolecular H-bond between the D-Lys N-H and the Gly^3 C=O groups which they called a β-turn (See the definition of β-turn that appeared above and note again the discrepancy). The D-Lys^2 compound is rather flexible and the μ-receptor specificity has been attributed to this reduced rigidity. However, contrary to this finding, Mosberg and Schiller (1984) correlated the increased flexibility of [D-Pen^2, L-Cys^5] enkephelinamide with the stronger preference for the δ-receptor sites.

Side-Chain Conformational (X)

An extensive effort has been made to elucidate the side-chain conformations of Tyr^1, Phe^4, and Met^5/Leu^5 amino acid residues in enkephalin molecules. NMR remained to be the sole spectroscopic method for providing information in this area. However, various ways of interpreting NMR data have made it difficult to reach any concensus on the ride-chain conformetions. As mentioned earlier (see the section on side-chain conformations), there are two ways of analyzing $^3J(\alpha\beta)$ couplings. The usual way is to obtain the weighted average of three populations (see figure 6) in case of free rotation along the C^α-C^β bond. If , however, there is any indication of restricted rotation either from the nonequivalent appearance of $C^\beta H_2$ protons or from the relaxation time measurements of the side-chain, the interpretation becomes more complicated. Some of these complications wil now be discussed in reletion to Tyr^1, Phe^4, and Met^5/Leu^5 side-chain conformations in enkephalins.

Tyr^1 Side-Chain (χ_1^1)

Since an unsubstituted tyrorine residue at position 1 is absolutely necessary for opioid activity in enkephalinlike molecules) the determination of its side-chain conformations drew considerable attention. Horn and Rodgers (1976) first suggested that the N-terminal of enkephalins may be serving the same role as found in several narcotics, such as levorphenol, metazocine, and profadol. All such drugs contain en important hydroxyl (OH) group and a tertiary amino group within the molecule. The distances between these two groups in various opiate agonist and antagonist molecules varies from 6.89 to 7.08 A. Loev and Burt (1978) attempted to gain insight into this problem by comparing enkephalin conformations vith rigid opiate 7-[1-Phenyl-3-bydroxybutyl-3] endoetbano-tetrahydrotbebeine (PET) and found that there is an excellent overlap of tyramine moiety of PET with the

Tyr[1] side-chain when χ_1^1 = -90°, although it was not the lowest energy conformation. In the first crystgllographic study by Smith and Griffin (1978), tvo χ_1^1 values (-43° and -86°) for Tyr[1] were reported. In a second X-ray study,Karle et al. (1983), hovever, reported four sets of χ_1^1 values, i.e., χ_1^1 = 177° 70°, 53°, and 169°. The χ_1^1 = -86° reported by Smith and Griffin (1978) for [Leu[5]] enkephalin in its folded form seems to bear a good resemblance with the postulation of Loew and Bert (1978).

The solution conformations of Tyr[1] side-chain remained most controversial and confusing. Following a Pachler-like (1964) analysis of the $^3J(C^{\alpha}H\text{-}C^{\beta}H_2)$, ^{1}H NMR data obtained in DHSO-d_6 (Jones et al. 1976; Garbay-Jaureguiberry et al. 1976, 1977; Robayashi et al. 1980; Zetta and Cabassi 1982) and in D_20/DMSO-d_6 (Anteunis et al. 1977) shoved that all three ride-chain rotamers are equally populated; in another word, the $C^{\alpha}\text{-}C^{\beta}$ bond is freely rotating. The problem associated in this kind of NMR data analysis in the averaging of the two $^3J(C^{\alpha}H_X - C^{\beta}H_A)$ and $^3J(C^{\alpha}H_X - C^{\beta}H_B)$ values. For example, one of the Tyr[1] $^3J(\alpha\beta)$ values is ca. 5 Hz and the other is ca. 8 Hz; the mean value is 6.5 Hz, which is essentially an indication of free rotatio Whereas, as discussed previously, this nonequivalency, i.e., $^3J_{AX}$ = 5 Hz and $^3J_{BX}$= 8 Hz, should be considered as indicative of some kind of restricted motion at the $C^{\alpha}\text{-}C^{\beta}$ bond. Bleich et al. (1976, 1979) reported the ^{1}H and ^{13}C relaxation parameters, confirming such restricted rotationlof the Tyr[1] ride-chain. They even concluded that the Tyr[1] side-chain alternates between the g+ and states at a slow rate of 400 sec. Careful analysis of $^3J_{C^{\beta}H}$ values of 2.7 and 1.0 Hz obtained by Stimson et al. (1979) gave a number of χ_1^1 values, out of which χ_1^1 = -60 or -90 has been considered most favorable. It appears from all the NMR studies that the Tyr[1] side-chain prefers a more populated tg conformation, reflecting one of the above mentioned χ_1^1 values ranging from -60° to -90°. Interestingly, however this side-chain conformation of tyroeine is more compatible with the tyramine moiety of an opiate.

Phe[4] Side-Chain Conformation(χ_4^1)

Folloving again the method of Pachler (1964), the three rotamer populations of Phe[4] side-chain were found to be equally preferred (Jones et al. 1976; Carbay-Jaureguiberry et al. 1976, 1977; Anteunis et al. 1977) both in DMSO-d_6 and D_2O. Kobayashi et al. (1979) and Zetta and Cabassi (1982), however, suggested the predominance of a tg conformation which is also strongly supported by Stimson et al. (1979). The ^{1}H and ^{13}C T_1 values, on the other hand, indicted a rapid internal motion relative to the peptide backbone (Bleich et al. 1976; Niccolai et al, 1980). It appears, therefore, that there still exist disagreements in the case of χ_4^1 for L-Phe[4] residue in enkephalin molecules.

Met^5/Leu^5 Side-Chain (χ_5^1)

This is the only residue in all enkephalin molecules that seems to enjoy a common agreement on its side-chain conformation, χ_5^1. ^{1}H NMR data analysis using the Pachler (1964) method gave a free rotating C^α–C^β bond both in DMSO-d_6 and D_3O (Jones et al. 1976; Garbay-Jaureguiberry et all 1976; Anteunis et al. 1977; Zetta and Cabasei 1982). The ^{1}H and ^{13}C relaxation parameters in this case are also in excellent agreement, i.e., they shov a rapid internal rotation (Bleich et al. 1976; Combrisson et al. 1976; Niccolsi et al. 1980). Leu^5 side-chain, to the contrary, adopts a conformation that exists predominantly in the tg state (Garbay-Jaureguiberry et al. 1977, 1982; Stimson et al. 1979).

CONCLUDING REMARKS

Numerous applications of theoretical and physical methods have at least established the fact that the enkephalins are extremely flexible molecules. By introducing the cyclization constraint in the backbone, it was possible to define a particular conformation. The question remains, hovever, why one molecule prefers a μ- and the other a δ-receptor vhile both the molecules seem to adopt a similar backbone conformation? Recently, Deek et al. (1983) have synthesized tvo analogs of [Leu^5] enkephalin, namely [(2-Amino-5-hydroxy-2-indanyl)carbonyl] and [(2-Amino-6-hydroxy-2-tetralinyl)carbonyl]Gly-Gly-Phe-Leu-OMe, with restricted Tyr^1 side-chain where the latter has been found to be 7 to 8 times more active than the [Leu^5] enkephalin at the μ-receptor site. Although no conformational studies have been reported, the Dreiding model of this constrained side-chain of Tyr^1 favors χ_1^1 = -90, which is compatible with the tyramine moiety of an opiate (vide supra). Possibly, this means that the tyroeine alone can mimic the opioid activity if appropriately carried to the receptor site(s). To test this hypothesis, L Tyr was linked with dodecalamine and its activity vas tested using a mouse seizure model (Khaled and Beaton 1985). Surprisingly, this molecule was found to possess analgesic activity similar to other opiates, as previously reported (Snead and Bearden 1982). These findings, therefore, resffirm the notion that the derivation of the actual bioactive conformation(s) of enkephalins will have to wait until such time as the receptor sites are identified, isolated, and characterized.

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Conformational Analysis of Opioid Peptides and the Use of Conformational Restriction in the Design of Selective Analogs

Peter W. Schiller, Ph.D.

INTRODUCTION

The discovery of the enkephalins by Hughes et al. (1975) was almost imediately followed by efforts to determine the conformation of there pentapeptider and to correlate it with the three-dimemlonal structurer of the more rigid opiates. The conformational analysis of small linear peptides like the enkephelins is not without problems. There is a strong possibility that rather than assuming a single preferred conformation in solution, they may exist in a conformational equilibrium. It is quite likely that their conformation(s) may be determined to a large extent by the molecular environment. Furthermore, the bioactive conformation may not preexist in solutlon, but may only be adopted when the peptide interacts with the receptor, perhaps in a step-wise binding process similar to the "zipper"-type model propored by Burgen et al. (1975). In the part 8 years, the conformation of the enkephalins and of other opioid peptides has been studied extensively by means of nearly all theoretical and experimental methods available to date. A wealth of information about conformational features of there peptide in the isolated state, in cryrtalline form, and in solution has been obtained and will be reviewed in the first part of this chapter. Ideally, the bioactive conformation should be studied with the peptide bound to a purified receptor. Since purified opioid receptors are still unavailable at this time, insight into the receptor-bound conformation of opioid peptides ten only be obtained indirectly through analysis of conformationally constrained analogs which are biologically active. Moreover, in the case of the opioid peptides, the situation is further complicated through the existence of multiple opioid receptors (μ, δ, κ, etc.) which differ from one another In their structurel requirements. Therefore, it will be necessary to determine a distinct receptor-bound conformation for each type of receptor capable of interacting with a particular opioid peptide. The pharmacologic and conformational characterization of conformationally rertricted opioid peptide analogs will be dercribed in the second part of this chapter.

A review covering the early literature on conformational studies of enkephalin has been published by Gorin et al. (1978). A more recent comprehensive review dealing with the conformational analysis of the enkephalins and with conformation-activity relationship has been written by Schiller (1984). Confonnational features of the endorphins have been reviewed by Yamashiro and Li (1984).

HYPOTHETICAL MODELS OF THE BIOACTIVE CONFORMATION OF ENKEPHALIN

Structural comparisons between opioid peptides and opiates require first of all the identification of structural elements which may play analogous roles in the interaction with opioid receptors. Subsequently, a pharmacophoric conformation of the peptide has to be postulated such that spatial overlap between corresponding functional groups in the peptide and in the more rigid opiates is achieved. The fact that corresponding modifications of the tyramine moiety in morphine and of the N-terminal tyrosine residue in enkephalin produce similar changes in the activity profile (cf. Schiller 1984) strongly suggeste that the tyramine moieties contained in the peptide and in the alkaloid are functional correlates. This correspondence formed the basis for nearly all hypothetical models of the bioactive conformation of enkephalin which have been proposed to date. Essentially two different proposals have been made concerning the functional role of the Phe^4 residue in enkephalin in relation to structural elements of morphine-related analgesics. Bradgury et al. (1976) suggested that the Phe^4 side-chain of [Met^5]enkephalin and the phenethyl substituent attached to carbon-19 of the potent morphine derivative PEO[1] might bind to an identical receptor subsite. Consequently, an appropriate pharmacophoric conformation of enkephalin would have to accossaodate the tyramine segment of Tyr^1 and the Phe^4 aromatic ring in a relative spatial disposition analogous to that existing between the corresponding moieties in PEO (phenol-phenyl separation of about 10 Å). Proposed confonnational models which fulfil this condition include a 4 → 1 hydrogen-bonded β-bend conformation (Bradbury et al. 1976), the 5 → 2 hydrogen-bonded β II'-bend (Clarke et al. 1978), and a bent conformation centered on Gly^2-Gly^3 lacking an intramolecular hydrogen bond (Hudson et al. 1980). In an alternative proposal, the atoms in <u>para</u> and meta position of the Phe^4 residue in enkephalin were considered as functional correlates of atoms C5 and C6 contained in the C-ring of morphine, again under the assumption of functional and spatial correspondence between the tyramine moieties contained in the two molecules. A systematic computer search performed with the active tetrapeptide H-Tyr-D-Ala-Gly-Phe-OH resulted in a single sterically allowed conformation in which the meta position of the Phe^4 aromatic ring nearly coincided with the spatial position of the carbon-6 atom in morphine. In the latter conformation, the intramolecular distance between the two aromatic rings is much shorter (about 5) than in the β-bend models (see above). Aside from these two major proposals, a

number of other suggestions concerning corresponding functional moieties in enkephalin and opiates have been made and, obviously, no general consensus has been reached to date.

CONFORMATIONAL ANALYSIS OF OPIATES

Theoretical Energy Calculations

The first conformational energy study of [Met^5]enkephalin performed by Isogai et al. (1977) using the ECEPP program resulted in a lowest energy conformation characterized by a type II-bend centered on Gly^3-Phe^4 and stabilized by a hydrogen bond between the tyrosyl hydroxyl group and the carbonyl group of Gly^3. This conformer permits substitution of Gly^2 by L-Ala but not by D-Ala and, therefore, it is unlikely to represent the receptor-bound conformation, since it is well known that D-Ala^2 analogs of enkephalln retain high potency and L-Ala analogs are only weakly active. Performing a different type of conformational energy calculation, DeCoen et al. (1977) obtained a number of different olded and extended structures as low energy conformers of [Met^5]enkephalin and it was suggested that an equilibrium of such different conformations might also exist in solution. The results of a study based on the use of the Monte-Carlo method in conjunction with a clustering analysis al so indicated that a limited number of different structural classes might best represent the conformational behavior of enkephalin in solution (Maigret and Premilat 1982). In a subsequent study dealing with enkephalin analogs, Humblet and DeCoen (1977) found a folded structure with a turn centered on residues 2 and 3 as lowest energy conformer of [D-Ala^2,Met^5]enkephalin which was considered as a likely candidate for the bioactive conformation of enkephalln. Based on the results of a semi-empirical conformational analysis of [Met^5]enkephalin, [D-Ala^2,Met^5]enkephalinand [D-Ala^3,Met^5]enkephalin, Balodis et al. (1978) proposed a 4 ➞ 1 hydrogen-bonded β I-turn as the receptor-bound conformation. Using the ECEPP program, Manavalan and Momany (1981) performed an analysis of seven enkephalin analogs and found that several different low energy conformers common to the group of analogs studied exist. Furthermore, it became obvious that conformers with significantly different backbone structures may still have a very similar spatial disposition of the side-chains in positions 1, 4, and 5 and, in fact, the existence of only three major categories of side-chain configurations was indicated by the results of this study. Loew and Burt (1978) used the ECEPP program as well as a quantum chemical method to determine the energy content of enkephalin conformers which display various degreea of spatial overlap with PEO. A β II'-bendlike structure showing spatial overlap of the Tyr^1 tyramine segment and the Phe^4 side-chain with the corresponding moieties in PEO was found to be only 3.5 kcal/mol higher in energy than the lowest energy conformer and thus represents a plausible candidate for the active conformation. More recently, Loew et al. (1982) used a similar approach to study N-terminal tetrapeptide analogs of enkephalin and obtained

two structures of the βII'-bend type, stabilized by a hydrogen bond between the N-terminal amino group and the Phe[4] carbonyl group, which showed good rpatial overlap with PEO. In a similar approach, Maigret et al. (1981) determined minimum energy conformers of H-Tyr-D-Ala Gly-Phe-OH by imposing a somewhat different rpatial overlap with structural elements of morphine and obtained a putative pharmacophoric conformation which differed significantly from that described by Loew et al. (1982).

Grystal Structure Determinations

In a first X-ray study. the interpretation of the diffraction pattern obtained with crystals of [Leu[5]]enkephalin grown from aqueous methanol led to the proposal of a β-bend structure stabilized by two antiparallel hydrogen bonds between the amino group of Tyr and the carbonyl oxygen of Phe and between the amide proton of Phe and the carbonyl group of Tyr (Smith and Griffin 1978). Moreover, the data indicated the existence of two different conformations of the Tyr[1] reasidue. Essentially the same crystal structure was recently obtained with the analog [4'-Br)Phe[4],Leu[5]]enkephalin as the result of an X-ray study carried out by Ishida et al. (1984). In the meantime. examination of improved diffraction patterns obtained with the original [Leu[5]]enkephalin crystals led to the detection of additional weak spots. indicating that the crystals balonged to space group A2 rather than C2, as had originally been assumed. The recently completed analysis of there patterns revealed the existence of four conformationally distinct [Leu[5]]enkephalin molecules per unit cell. Whereas the four molecules have very similar backbone conformations closely resembling the originally proposed β-bend structure, they differ from one another significantly in the orientation of the ride-chains (G.D. Smith, personal communication, 1983). Karle et al. (1983) performed an X-ray analysis with [Leu[5]]enkephalin crystals grown from DMF in the presence of water which turned out to belong to space group $P2_1$. Again, the unit cell contained four molecules of [Leu[5]]enkephalin. each having a distinct conformation, but all of them showing similar extended backbones, in sharp contrast to the folded conformations existing in the crystals belonging to apace group A2. The major differences between the four molecules are again observed in the orientation of the side-chains. Preliminary results of an X-ray study recently performed with [Met[5]]enkephalin also provided evidence for an extended backbone structure (Ishida et al. 1984). The folded and extended structures observed with the enkephalins in the crystalline state moat likely represent conformers of comparably low energy; however. there is no compelling reason to assume that they represent bioactive conformations (cf. Schiller 1984).

Conformational Studies in Solution

Numerous studies on the solution conformation of the enkephalins have been performed by spectroscopic techniques under various solvent conditions. These experiments provided

information concerning the aggregation behavior of enkephalin, the backbone conformation(s) of the peptide, intramolecular distances in analogs. and side-chain orientations.

Both Khaled et al. (1977) and Higarshijima et al. (1979) observed a concentration dependence of Shemica shifts in NMR studies performed with [Met^5]- and [Leu^5]-enkephalin in DMSO which was interpreted to indicate a molecular association process. In contrast to these results, Bleich et al. (1976), Stimson et al. (1979). and Marion et al. (1981) found no evidence for self-aggregation in DMSO. On the other hand, there is general agreement that enkephalin does not self-associate in aqueous solution (Bleich et al. 1976; Levine et al. 1979; Higashijima et al. 1979; Schiller et al. 1978).

Baaed on the results of NMR and other studies, several propoeals for a preferred backbone conformation of the enkephalins have been made. NMR data obtained with [Met^5]enkephalin in DMSO were interpreted to indicate the existence of a 5 ➞2 hydrogen-bonded β-I-turn stabilized by a hydrogen bond between the amide proton of Met^5 and the carbonyl oxygen of Gly^2 (Roques et al. 1976; Jones et a1. 1976). This type of conformation can accommodate the Tyr^1 residue and the Phe^4 side-chain in a relative spatial disposition similar to that existing between the corresponding moieties in PEO and, therefore, it was suggested that it might represent the bioactive conformation of [Met^5]enkephalin. However, the fact that the analog [Leu(NMe)^5]enkephalin is active indicates that a hydrogen bond involving the amide proton in position 5 is not an absolute requirement for the receptor-bound conformation (Schiller and St.-Hilaire 1980). Performing an NMR study with the cationic form of [Met^5]enkephalin in DMSO, Bleich et al. (1976) found no evidence for a 5 ➞ 2 hydrogen bond and suggested that a preferred conformation might be stabilized by solvent interactions. Based on the results of yet another NMR study in DMSO, Khaled et al. (1977) proposed a 5 ➞ 2 hydrogen-bonded β-turn model of the monomeric form of enkephalin containing a second hydrogen bond between the amide proton of Gly^3 and the carbonyl oxygen of Tyr^1 and a third one between the Tyr^1 hydroxyl group and the Gly^3 carbonyl group. Similar results were obtained by Zetta and Cabassi (1982). The conformation of [Leu^5]enkephalin in DMSO was also investigated by NMR spectroscopy. Originally obtained ^{1}H NMR data (Garbay-Jaureguiberry et al. 1977) led again to the proposal of a 5 ➞ 2 hydrogen-bonded β-bend model which was subsequently revised in favor of a 2-5 βII'-bend structure on the basis of ^{15}N NMR data obtained by the same group (Garbay-Jaureguiberry et al. 1982) The results of a ^{13}C NMR study performed by Stimson et al. (1979) were interpreted to be compatible with a type I β-bend centered on Gly^3-Phe^4. Based on NMR data obtained in D_20, several proposale for a preferred conformation of the enkephaline in aqueous solution were made. Roquee et al. (1976) and Jones et al. (1976) suggested that the 2-5 β-I-bend model they proposed for [Met^5]enkephalin in DMSO might also represent

the predominant conformer in water. The studies performed by Bleich et al. (1976). Levine et al. (1979), and Zetta et al. (1979) also suggested the existence of a preferred conformation in aqueous solution but did not provide any evidence for intramolecular hydrogen bonding. Finally, ^{1}H and ^{13}C NMR data obtained by Higashijima et al. (1979) were interpreted to indicate that [Met^{5} lenkephalin in D_20 predominantly exists in an extended conformation devoid of intramolecular hydrogen bonds. In contrast to the proposals in favor of a single preferred back bone conformation of enkephalin discussed so far, several other studies produced results which were more in tune with a conformational equilibrium involving a number of different conformers. Fishman et al. (1978) measured $^{3}J(H'-H^{\alpha'})$ coupling constants at positions 2 and 3 of the peptide sequence with two isomers of [Leu^{5}]enkephalin containing stereochemically deuterated glycine residues. The obtained results indicated that averaging of the Φ_2 and Φ_3 angles is taking place both in $^{2}H_6$-DMSO and in D_20 and, therefore, provide strong evidence against a single preferred backbone conformation in the central part of the peptide. NMR data obtained by Higashijima et al. (1979) with the zwitterionic form of [Met^{5}]enkephalin in $^{2}H_6$-DMSO were taken to indicate an equilibrium of extended and folded conformations. Analysis of CD spectra obtained with [Met^{5}]enkephalin in H_20 and trifluoroethanol (Spirtes et al. 1978) and of a Raman spectrum recorded with [Leu^{5}]enkephalin in D_20 (Han et al. 1980) also led to the conclusion that the enkephal in molecules exist as an ensemble of different conformers.

In a number of Studied, intramolecular distances between side-chains in enkephalin analogs were determined. A fluorescence energy transfer experiment performed with the active analog [Trp^{4},Met^{5}]enkephalin in H_20 resulted in an average intramolecular distance between the phenol ring of Tyr^{1} and the indole moiety of Trp^{4} of 9.3 ± 0.4 Å (Schilier 1977). This distance would be compatible with the Tyr^{1}-Trp^{4} separation observed in the various β-bend models and with the distance between the two aromatic rings in PEO. However, it is quite possible that the measured value represents an average of distances present in several different conformers participating in a conformational equilibrium and, in fact, the results of a Monte-Carlo type calculation were found to be in agreement with the latter interpretation (Demonté et al. 1981). In further energy tranefer studies, no gross difference in the average Tyr^{1}-Trp^{4} intramolecular distance was observed within a series of nine [Trp^{4}]lenkephalin analogs which differed widely from one another in their μ-receptor affinity (Schiller et al. 1978; Schiller and St.-Hilaire 1980). Interestingly, a much larger Tyr^{1}-Trp^{4} intramolecular distance (> 15 Å) was determined with the potent dynorphin analog [Trp^{4}]dynorphinI(1-13) (Schiller 1983a). This marked difference in the Tyr^{1}-Trp^{4} separation between the [Trp^{4}]analogs of enkephalin and dynorphin may be related to the different receptor selectivity profiles of the two peptides. A close proximity between the methyl groups of the

Ala[2] and Met[5] side-chain in [D-Ala[2],Met[5]]enkephalin was indicated by the results of an NOE[1] experiment (Niccolai et al. 1980).

Numerous studies on side-chain conformations in enkephalin have been performed (for a detailed review, see Schiller 1984). Side-chain rotamer populations were mainly determined by Pachler analysis of ^{1}H NMR data. There is some agreement that the tg$^-$ rotamer may represent the favored conformation of the Tyr[1] side-chain under a variety of conditions (Kobayaehi et al. 1979; Zetta and Cabassl 1982; Stimson et al. 1979). Interestingly. the tg$^-$ rotamer shows closer conformational resemblance with the tyramine segment in morphine than the other two side-chain rotamera (tg$^+$ and gg$^+$). Furthermore, the results of most spin-lattice relaxation time measurements indicated a fixed conformation of the Tyr[1] residue relative to the peptide backbone (Bleich et al. 1976; Combrisson et al. 1976; Garbay-Jaureguiberry et al. 1977). Evidence to indicate that the Tyr[1] hydroxyl group of enkephalin in H_20 or DMSO/H_20 mixtures does not participate in an intramolecular hydrogen bond with a backbone carbonyl group was obtained from a fluorescence study (Schiller 1977). a UV titration etudy (Spirtes et al. 1978). laser Raman experiments (Han et al. 1980). and a_4 ^{1}H NMR study (Zetta and Cabassi 1982). In the case of the Phe[4] side-chain, all three rotamers were found to be significantly populated according to the results of reveral studies, with the exception of that performed by Stimson et al. (1979) which indicated that in $^2H_6$-DMSO the Phe[4] tg$^-$ rotamer is exclusively populated. Furthermore, it has been well established that the Phe[4] side-chain in the natural enkephalins undergoes rapid internal rotation relative to the peptide backbone. In $^2H_6$-DMSO, all three rotamers of the Met[5] side-chain in [Met[5]]enkephalin were found to be populated to some extent, where as in [Leu[5]]enkephalin the Leu[5] side-chain appears to exiat exclusively in the tg$^-$ conformation (Garbay-Jaureguiberry et al. 1977; Stimson et al 1979). With [Met[5]]enkephalin, rapid internal motion of the Met[5] side-chain was again indicated by the reaults of spin-lattice relaxation time measurements.

CONFORMATIONALLY RESTRICTED OPIOID PEPTIDE ANALOGS

Insight into the receptor-bound conformation can be obtained from structural analysis of conformationally restricted analogs that retain biological activity. However, unambiguous conclusions cannot be drawn in the case of conformationally constrained analogs which are inactive, since the loss of activity could be due either to an incompatible conformation or to a lack of the necessary molecular flexibility required in a step-wise binding process. A further interesting aspect of introducing conformational constraints into biologically active peptides is the possibility of obtaining selective analogs in a situation of receptor heterogeneity.

Conformational constraints have been built into the peptide backbone of enkephal in by substitution of N^{α}- and C^{α}-methylamino acids in various portions (for a review, see Gorin et al. 1978). Local conformational restrictions of the peptide backbone through insertion of 5- or 6-membered cyclic structuree resulted in mostly inactive analogs (for a review, see Schiller 1984). Several enkephalin analoge containing conformationally restricted side-chains have been reported. Restriction of the rotational freedom of the Tyr^1 side-chain in enkephalin substitution of 2-amino-6-hydroxy-tetralincarboxylic acid in position 1 resulted in an active compound, whereas an analog with 2-amino-5-hydroxy-2-indancarboxylic acid in that same position was found to be inactive (Deeks et al. 1983). The relatively flexible tetralin residue can assume conformations corresponding to the tg^- or tg^+ rotamer of tyrosine but not to the gg^+ rotamer, whereas the latter rotomer is the only one accessible to the rigid indan structure. This observation is of interest in view of the fact thst the tg^- rotamer shows resemblance with the conformation of the tyramine moiety in morphine. Conformational restriction of the Phe^4 side-chain was achieved through substitution of l-aminoindan in H-Tyr-D-Ala-Gly-Phe-OH (Gorin et al. ,1980) and of dehydrophenylalanine with Z-configuration in [$D\text{-}Ala^2$,Met^5]enkephalimide (Chipkin et al. 1979). In both cases, the obtained analog showed good activity. More drastic restrictions of the overall conformation of opioid peptides has been achieved through synthesis of cyclic analogs. Cyclic enkephalin analogs obtained through direct head-to-tail cyclitation between the terminal amino and carboxyl group (Kessler and Holzemann 1981) or through linkage of the termini via a glycyl residue or a 2-aminoethyl group as bridging element (Hudson et al. 1980) were found to be either insoluble or inactive.

In an alternative approech. cyclic opioid peptide analogs were synthesised through ring closure between a side-chain group and the C-terminal carboxyl group or through covalent linkage of two side-chains. A common characteristic of these analogs is an exocyclic Tyr^1 residue. A first family of cyclic enkephalin analogs was obtained through substitution of a D-α, ω-diamino acid residue in position 2 of enkephalin and cyclication of the ω-amino group to the C-terminal carboxyl group (DiMaio and Schiller 1980; DiMaio et al. 1982a) (figure 1, compounds I-IV). Analogs were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and of the MVD^1, which are representstive for μ- and δ-opioid receptor interactions, respectively (table 1). In comparison to [Leu^5]enkephalin, the prototype of this series, H-Tyr-c[-N^{γ}-D-A_2bu-Gly-Phe-Leu-] (II) was shows to be 17 times more potent in the GPI assay and 7 times less potent in the MVD assay. Therefore. this cyclic analog shows preference for μ-receptors over δ-receptors, as indicated by its IC50(MVD)/IC50(GPI) ratio of 5.77. No such preference is shown by the corresponding open-chain analog, H-Tyr-D-Abu-Gly-Phe-Leu-NH_2 (IIa), which is distinguished from its cyclic counterpart II merely by the

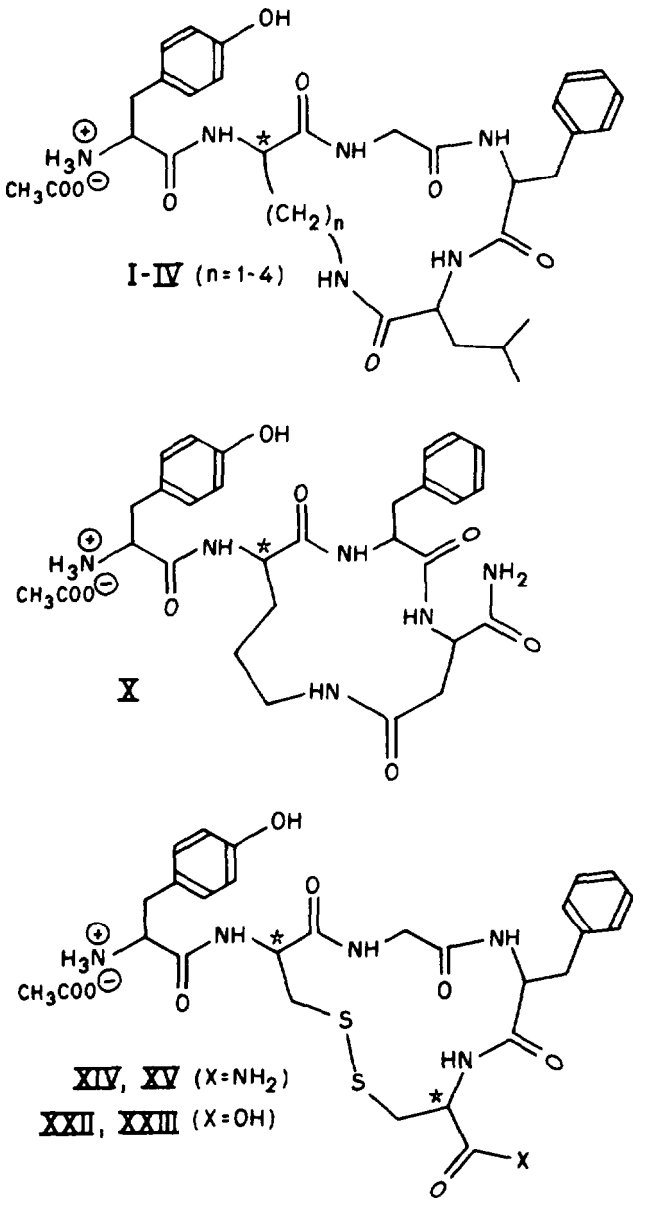

FIGURE 1

Structural Formulas of Cyclic Opioid Peptide Analogs

TABLE 1

Potencies of Cyclic Enkephalin Analogs in the GPI and MVD-Assaya

	Compound	GPI ICSO [nM]	MVD IC50 [nM]	MVD/GPI IC50-ratio
I	H-Tyr-c[-N$^{\beta}$-D-A_2pr-Gly-Phe-Leu-]	23.4 ± 4.2	73.1 ± 14.5	3.12
II	H-Tyr-c[-N$^{\gamma}$-D-A_2bu-Gly-Pht-Leu-]	14.1 ± 2.9	81.4 ± 5.8	5.77
IIa	H-Tyr-D-Abu-Gly-Phe-Leu-NH_2	28.7 ± 1.3	45.6 ± 9.1	1.59
IIb	H-D-Tyr-c[-N$^{\gamma}$-D-A_2bu-Gly-Phe-Leu-]	3'230 ± 760	330 ± 102	0.102
IIc	H-Tyr-c[-N$^{\gamma}$-L-A_2bu-Gly-Phe-Leu-]	1'450 ± 350		
III	H-Tyr-c[-N$^{\delta}$-D-Orn-Gly-Pht-Leu-]	48.0 ± 4.3	475 ± 99	9.90
IV	H-Tyr-c[-N$^{\varepsilon}$-D-Lye-Gly-Phe-Leu-]	4.80 ± 1.79	141 ± 28	29.4
IVa	H-Tyr-D-Nle-Gly-Phe-Leu-NH_2	24.6 ± 1.6	25.2 ± 7.9	1.02
IVb	H-Tyr-c[-N$^{\varepsilon}$-D-Lye-Gly-Phe-D-Leu-]	2.39 ± 0.57	17.5 ± 5.7	7.32
IVc	H-Tyr-c[-N$^{\varepsilon}$-D-Lye-Gly-Phe-Met-]	1.20 ± 0.26	34.3 ± 5.5	28.6
IVd	H-Tyr-cc-N$^{\varepsilon}$-D-Lye-Gly-Phe(p$N0_2$)-Leu-]	0.504 ± 0.138	13.4 ± 6.3	26.6
IVe	H-Tyr-c[-N$^{\varepsilon}$-D-Lye-Gly-Leu-Phe-]	3.36 ± 0.04	41.6 ± 9.4	12.4
IVf	H-Phe-c[-N$^{\varepsilon}$-D-Lye-Gly-Phe-Leu-]	105 ± 19	4'070 ± 200	38.8
	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.0463

[a] Mean of three determinations ± SEM.

opening of a single carbon-nitrogen bond. This result indicates that the μ-receptor selectivity of cyclic analog II is exclusively due to the conformational constraint introduced through ring closure, and, furthermore, permits the unambiguous conclusion that μ- and δ-opioid receptors differ in their conformational requirements (Schiller and DiMaio 1982). Within the series of cyclic analogs I-IV, the number of methylene groups in the 2-position side-chain increases from 1 to 4 and, therefore, subtle variation in the degree of conformational restriction is achieved. All members of this series were found to be μ-receptor selective and the IC50(MVD)/IC50(GPI) ratio increased gradually with lengthening of the side-chain in position 2, reaching a value of 29.4 with H-Tyr-c[-N^{ϵ}-D-Lys-Gly-Phe-Leu-] (IV) (table 1). The latter analog was 51 times more potent than [Leu^5]enkephalin in the GPI assay and 12 times less potent in the MVD assay. The corresponding open-chain analog (IVa) again showed no selectivity. Structure-activity studies with linear enkephalin analogs had shown that L-configuration in position 1 and D-configuration in position 2 are required for activity, whereas in position 5 either L- or D-configuration is tolerated. The results of potency determinations performed with cyclic analogs IIb, IIc, and IVb (table 1) indicate that the cyclic analogs have the same configurational requirements in positions 1, 2, and 5 as linear enkephalins. Furthermore, introduction of a nitro substituent in para position of the Phe^4 aromatic ring in cyclic analog IV (compound IVd) results in approximately a tenfold potency increase in both essays, as is the case with noncyclic enkephalins (Schiller et al. 1983). Based on these results, it can be assumed that the mode of binding to opioid receptors is the same for cyclic and linear enkephalin analogs. Using cyclic analog IV as parent compound, further structure-activity studies were undertaken. In most cases, corresponding modifications in the cyclic analog and in linear enkephalins had the same effect on potency (cf. analogs IVc and IVd). However, two interesting exceptions were noticed. Transposition of the Phe and Leu residues in the cyclic peptide (compound IVe) resulted in a small potency increase in both assays, whereas that same modification in the corresponding open-chain analog lowered potency quite drastically (DiMaio of al. 1982b). Even more interestingly, omission of the Tyr^1 hydroxyl group in the cyclic analog results in a compound (IVf) still more than twice as potent as [Leu^5]enkephalin, whereas linear enkephalin analogs containing Phe in position 1 show very low potency. These two observations may reflect a difference in the binding process between cyclic and linear analogs. It is possible that the semi-rigid cyclic analogs may bind to the receptor according to Fischer's "lock-and-key" model, whereas a zipper-type interaction may take place in the case of the more flexible linear analogs. Certain moieties may no longer be absolutely required for efficient binding in a lock-and-key-type interaction. Whereas in a zipper-type interaction certain modifications (e.g., Phe^4-Leu^5 transposition) may increase the step-wise binding process. The results obtained with the cyclic enkephalin analogs in binding assays based on

displecement of [^{3}H]naloxone as a μ-receptor-selective radioligand and of [^{3}H][D-Ala^2,D-Leu^5]enkephalin as a δ-receptor-selective radiolebel from rat brain membrane preperations were in general agreement with the activity profiles determined in the GPI- and MVD-asssys. However, unexpected results were obteined in a comparison of the cyclic analogs with their linear correlates in the GPI bioassey and the [^{3}H]naloxone binding assay, which are both representative for μ-receptor interactions. For example. cyclic analog IV is five times more potent than its corresponding open-chain analog IVa in the GPI assay. whereas en almosf txectly reversed potency relationship is observed in the [^{3}H]naloxone binding essay (DiMaio et el. 1982a). This discrepancy can be interpreted to indicate that the conformational restriction introduced in IV through ring closure slightly reduces μ-receptor affinity, but significantly increases the ability of the bound peptide to activate the receptor ("efficacy" enhancement). High resistance of cyclic analog II to enzymatic degradation was demonstrated in vitro (DiMeio and Schiller 1980), and intracerebroventricular administrstion of compound IV (20 nanomoles) produced a long-lasting analgesic and catatonialike effect in rats (Schiller et al. 1981a). The rigid 13- and 14-membered ring structures contained in cyclic analogs I and II exclude formation of a 4-1 or 5 → 2 hydrogen-bonded β-bend and are also incompatible with the crystal structures described by Smith (personal communication. 1983) and by Karle et al. (1983). Kessler et al. in press) investigated the conformations of cyclic analogs III and IV by performing a ^{1}H NMR study in [$^2H_6$]DMSO. In the case of compound III, the obtained data indicated a rigid conformation stabilized by two transannular hydrogen bonds (Leu^5-NH→ OC-Gly^3 and Orn^2-NH→ OC-Orn^2). On the other hand, cyclic peptide IV showed a somewhat more flexible conformation than III,and evidence for only a siqnle intramolecular hydrogen bond between the $N^{\varepsilon}H$ group of D-Lys^2 and the carbonyl group of Gly^3 was obtained. Since analogs III and IV are both μ-receptor selective, these interesting results suggest that at least two different backbone conformations of enkephalin are tolerated et the μ-receptor, provided that conformational changes of these semi-rigid analogs do not occur upon binding. It should be noted, however, that compound IV is about 10 times more potent then analog III in the GPI assey (DiMaio et al. 1982a).

A family of side-chain to side-chain cyclized opioid peptide analogs was obtained through substitution of an α,ω-diamino acid and a glutamic or aspartic acid residue in appropriate positions followed by amide bond formation between the side-chein amino and carboxyl groups (e.g., compound X, figure 1) (Schiller and Nguyen 1984). Potencies of such annalogs are presented in table 2. Compound V WAS found to be 250 times more potent then [Leu^5]enkephalin in the GPI assay, but turned out to be non-selective since it showed high potency on the MVD as well. Inversion of the configuration in position 2 (compound Va) again produced a drastic drop in potency. Transposition of the Glu

TABLE 2

GPI and MVD Assay of Side-Chain to Side-Chain Cyclised Analogs [a]

Compound		GPI IC50 [nM]	MVD IC50 [nM]	MVD/GPI IC50-ratio
V	H-Tyr-D-Lys-Gly-Phe-Glu-NH_2	1.13 ± 0.14	0.648 ± 0.132	0.573
VA	H-Tyr-L-Lys-Gly-Phe-Glu-NH_2	1'580 ± 420	10'600 ± 300	6.71
VI	H-Tyr-D-Glu-Gly-Phe-Lys-NH_2	42.7 ± 6.5	699 ± 146	16.4
VII	H-Tyr-D-Asp-Gly-Phe-Lys-NH_2	24.1 ± 2.8	256 ± 36	10.6
VIII	H-Tyr-D-Lys-Phe-Glu-NH_2	2.93 ± 0.30	5.21 ± 0.88	1.78
IX	H-Tyr-D-Glu-Phe-Lys-NH_2	7.99 ± 0.78	101 ± 18	12.6
X	H-Tyr-D-Orn-Phe-Asp-NH_2	43.8 ± 4.4	4'690 ± 1'020	107
XI	H-Tyr-D-Asp-Phe-Orn-NH_2	631 ± 123	10'400 ± 4'300	16.5
XII	H-Tyr-D-Orn-Gly-Glu-NH_2	8'750 ± 2'520	> 40'000	> 4
XIII	H-Tyr-D-Ala-Lys-Phe-Glu-NH_2	55'000	20'600 ± 4'700	0.375
	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.0463

[a] Mean of three determinations ± SEM.

and Lye residue in V resulted in an analog (VI) which was 6 times more potent then [Leu^5]enkephalin on the GPI but 60 times less potent on the MVD and, therefore, is μ-receptor selective. The difference in potency and receptor selectivity between compounds V and VI may be due to the different position and direction of the side-chain connecting amide bond which is likely to produce dissimilar transannular hydrogen bonding and thereby different ring conformations. Shortening of the side-chain in position 2 of analog VI results in a twofold to threefold potency increase with little change in selectivity (compound VII). The des-Gly^3 analogs of V and VI (analogs VIII and IX) show activity profiles similar to those of their perent peptides. Extraordinary μ-receptor selectivity is displayed by H-Tyr-D-Orn-Phe-Asp-NH_2 (X), which is 6 times more potent then [Leu^5]enkephalin on the GPI and 400 times less potent on the MVD. Its IC50(MVD)/IC50(GPI) ratio of 107 is comparable to that of the highly μ-receptor selective ligand H-Tyr-D-Ala-Gly-MePhe-$NH(CH_2)_2OH$ (DAGO) (Kosterlitz and Peterson 1981). Transposition of the Orn and Asp residues results in a less potent and less selective analog (XI). Compound XII showed week potency, in contrast to the significant activity which had been reported for the related linear analog H-Tyr-D-Met-Gly-NH-$CH(CH_3)$-CH_2-$CH(CH_3)_2$ (Roques et el. 1979). Analog XIII was also found to be weakly active, which is surprising in view of the relatively high potency observed with 1 linear [D-Ala^2]enkephalin analogs containing a side-chain with L-configuration in the 3-position.

A second type of side-chain to side-chain cyclized enkephalin analog was prepared by substitution of a D-Cys residue in position 2 and of a D- or L-Cys residue in position 5 followed by oxidative disulfide bond formation (figure 1; anologs XIV, XV, XXII, and XXIII) (Schiller et al. 1981b). The cyclic enkephalinamide analogs XIV and XV were found to be highly potent in both the GPI and MVD assays and, therefore, showed no significant receptor selectivity (table 3). The receptor binding mode of these analogs again seems to be similar to that of linear enkephalins, since they showed the same configurational requirements et the individual residues (cf. analogs XV, XVI, XVII, and XIX). This assumption was again supported by the potency increase observed upon substitution of p-nitrophenylalenine in position 4 of XV which resulted in a compound (XVIII) having five times the potency of dynorphin A-(1-13) in the GPI assay and an IC50 value of about 20 picomolar in the MVD assay. Substitution of Phe in position 1 and of Trp in position 4 of compound XV produced Active analogs (XX and XXI) showing considerable μ-receptor selectivity. In contrast to the nonselective cystine-bridged enkephalinamide analogs (XIV and XV), the corresponding analogs with a free C-terminal carboxyl group (XXII and XXIII) were shown to be moderately δ-receptor selective. The same difference in receptor selectivity is in general observed between linear enkephalins and enkephalinamides. Thus, cyclic enkephalins XXII and XXIII display about the same δ-receptor selectivity as [Leu^5]enkephalin but are about two orders of magnitude more

TABLE 3

Potencies of Cystine-Containing Enkephalin Analogs in the GPI and MVD Assay[a]

Compound		GPI IC50 [nM]	MVD IC50 [nMl	MVD/GPI IC50-ratio
XIV	H-Tyr-D-Cys-Gly-Phe-L-Cys-NH_2	1.51 ± 0.03	0.760 ± 0.086	0.503
XV	H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2	0.780 ± 0.010	0.298 ± 0.037	0.382
XVI	H-Tyr-L-Cys-Gly-Phe-L-Cys-NH_2	210 ± 58	950 ± 160	4.52
XVII	H-Tyr-D-Cys-Gly-D-Phe-L-Cys-NH_2	135 ± 13	891 ± 139	6.60
XVIII	H-Tyr-D-Cys-Gly-Phe(pNO_2)-D-Cys-NH_2	0.0351 ± 0.0100	0.0187 ± 0.0023	0.533
XIX	H-D-Tyr-D-Cys-Gly-Phe(pNO_2)-D-Cys-NH_2	483 ± 112	582 ± 113	1.20
XX	H-Phe-D-Cys-Gly-Phe-D-Cys-NH_2	48.6 ± 6.05	456 ± 159	9.38
XXI	H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2	0.842 ± 0.185	14.1 ± 1.1	16.7
XXII	H-Tyr-D-Cys-Gly-Phe-L-Cys-OH	3.06 ± 0.52	0.190 ± 0.035	0.0621
XXIII	H-Tyr-D-Cys-Gly-Phe-D-Cys-OH	1.48 ± 0.18	0.122 ± 0.016	0.0824
	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.0463

[a] Mean of three determinations ± SEM.

potent then the netural peptide. Substitution of a half-penicillamine (Pen) residue for the helf-cystine residue in position 2 and/or 5 of the cystine-bridged enkephelin analogs resulted in compounds with high preference for δ-receptors over μ-receptors (Momberget al. 1983). A conformational comparispn of corresponding Pen^2- and Cys^2-containing cyclic analogs by ^1H NMR spectroscopy in D_20 indiceted a somewhet enhanced rigidity of the ring structure contained in the [Pen^2]-analogs as compared to the [Cys^2]-analogs, which may be the cause of the observed selectivity difference (Mosberg and Schiller 1984). The results of fluorescence studies performed with analogs XV and XXI in H_20 indiceted a close interaction between the Tyr^1 aromatic ring and the disulfide bridge, and an average intramolecular distance between the Tyr^1 and Trp^4 aromatic rings in XXI of 9.7 ± 0.2 Å, which is close to the corresponding distance of 9.5 ± 0.3 Å determined with the linear analog H-Tyr-D-Ala-Gly-Trp-Met-OH (Schiller 1983b).

CONCLUSIONS

The results from theoretical energy calculations, crystal structure determinations, and spectroscopic studies in solution indicate that the enkephelins art flexible molecules which can exist in several different conformations of comperatively low energy. It appears that the molecular environment is likely to have an important influence on the conformation(s) of these peptides and there is good evidence to indicate that at least in aqueous solution they exist in a conformational equilibrun. The molecular flexibility of the enkephalins complicates the task of determining their bioactive conformation(s) and presumably is the cause of their lack of specificity in the interaction with different opioid receptor types. In view of this situation. the principle of conformational restriction has emerged as a promising new tool in opioid peptide analog design. In particular, synthesis of cyclic enkephelin analogs during the pest few years has resulted in potent, stable, and highly selective μ- and δ-receptor agonists. More extensive studies on the conformation of these cyclic analogs can be expected to lead to a better definition of the conformational requirements of the different opioid receptor types in the near future.

FOOTNOTES

[1] Abbreviations: CD, circular dichroism; ECEPP, empirical conformational energy program for peptides; GPI, guinea pig ileum; MVD, mouse vas deferens; NMR, nuclear magnetic resonance; NOE, nucleer Overhauser enhancement; PEO, 7-α-(1-(R)-hydroxy-1-methyl-3-phenylpropyl)-6,14- endo-ethenotetrehydrooripavine.

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Stereochemically Constrained Enkephalin Analogs Containing α-Aminoisobutyric Acid and l-Amino-Cyclopentane-I-Carboxylic Acid

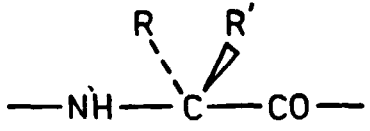
Raghuvansh Kishore and Padmanabhan Balaram

INTRODUCTION

Small biologically active acyclic peptides like enkephalins are conformationally very flexible. As a consequence, It is difficult to establish unambiguously the "biologically active" conformation which is recognized by the in vivo receptor. The relationship between stable solution conformations and structures bound to receptors is often not clear. The synthesis of conformationally restricted analogs and an evaluation of their receptor affinities or biological activity afford a possible approach to the delineation of bioactive conformations. Imposition of backbone and side-chain comformational constraints must, in principle, be accomplished by covalent modifications, which do not directly influenced interactions with receptors, but modulate peptide-receptor binding indirectly, by limiting the range of accessible tide conformetions (Spatola 1963; Marshall et al. 1976: This chapter briefly reviews an approach to restriction of peptide backbone conformational flexibility, by introduction of achiral α,α-dialkyl residues (figure 1) in enkephalins la place of Gly residues at positions 2 and 3.

Conformational Restrictions by α-Allsylation

Two degrees of torsional freedom about the N-C^{α} (ϕ) and C^{α}-CO (ψ) bonds determine the backbone conformations of individual residues in peptides. The peptide bond is generally limited to a trans geometry (ω = 180°). As a consequence, peptide conformations are conveniently mapped on the two-dimensional Remachandran (ϕ,ψ) plot (Ramachandran and Sasisekharan 1968). Figure 2 illustrates the theoretically computed ϕ,ψ maps,for L-ALa and D-Ala residues. The ϕ,ψ map for the Aib residue is shown in figure 3. It is evident that substitution at C^{α} limits the energetically accessible regions of ϕ,ψ space. In the case of symmetrical α,α-dialkyl residues, exemplified by Aib, only a very limited region of conformational space is, indeed, sterically and energetically favored (Marshall and Bosshard 1972: Prasad and Balaram 1984).

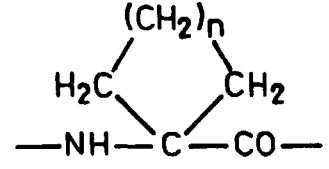


R = Me, R' = Me Aib

R = Me, R' = Et Iva

R = Et, R' = Et Deg

R = n Pr, R' = n Pr Dpg



n = 0 Acc^3

n = 2 Acc^5

n = 3 Acc^6

FIGURE 1

Structures of α,α-dialkylated residues having acyclic and cyclic sidechains.

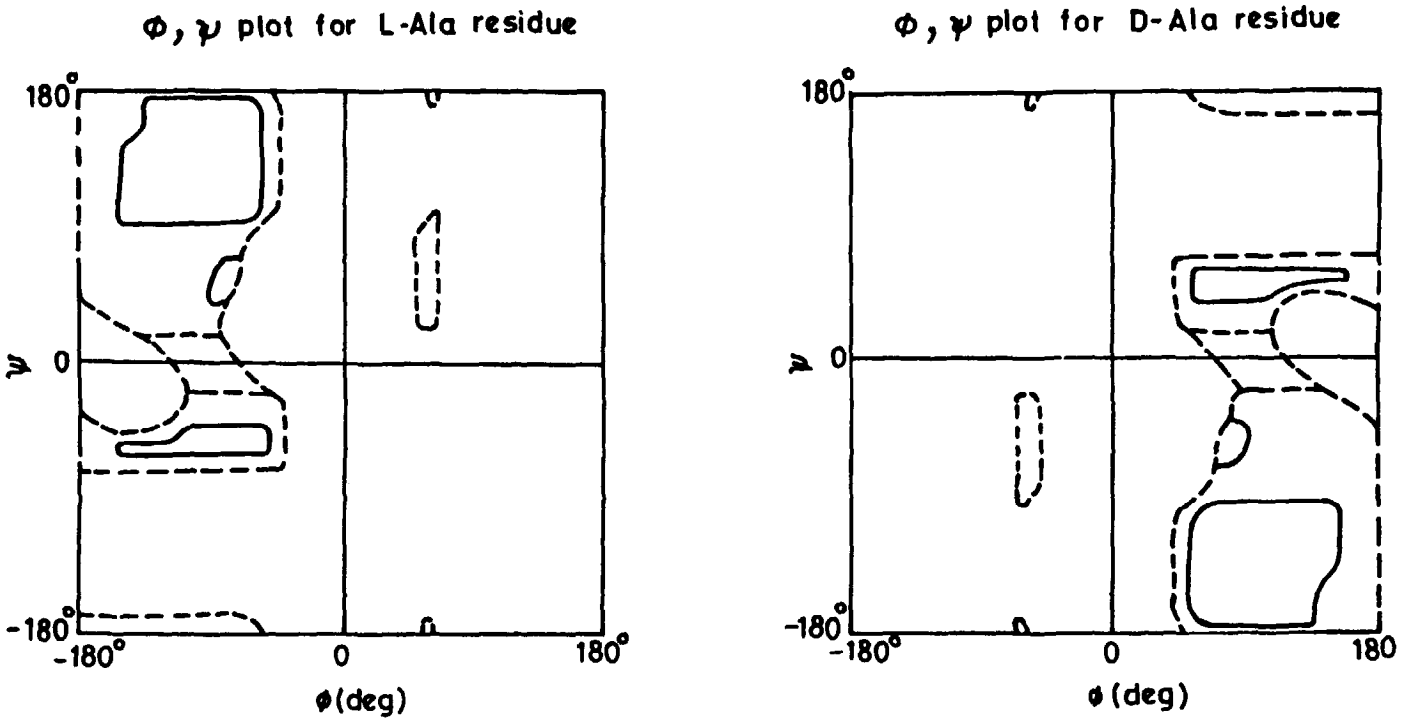


FIGURE 2

Pamachandran (Steric) map for N-acetyl-L-Ala-N'-methyl-amide and N-acetyl-D-Ala-N'-methylamide. The continous and broken lines enclose regions allowed by normal and extreme contact limits, respectively.

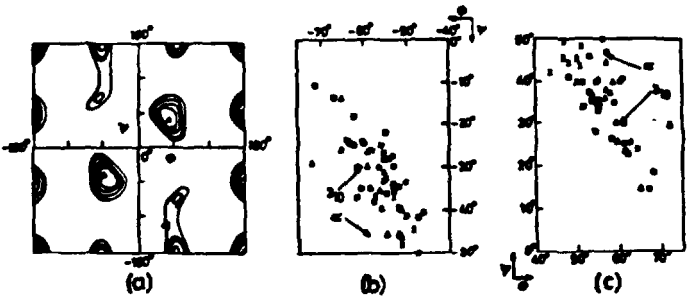
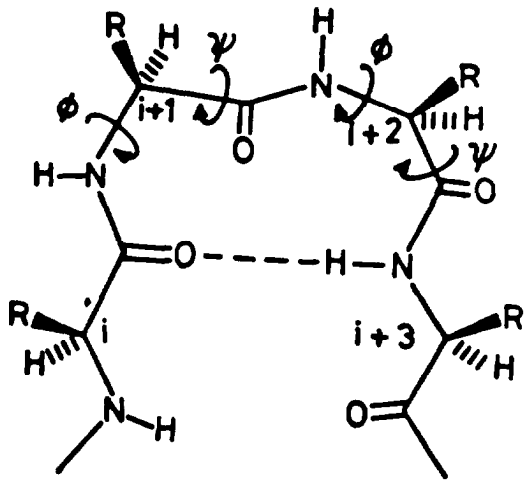


FIGURE 3

a) Potential energy map for N-acetyl-Aib-N'-methylamide. The contours are drawn with respect to the innrmost contour enclosing the minimum. The ideal 3_{10} (□) and α- (△) helical confomations are marked. These conformations are compatible with Aib residues in Type I or III β-turns. Crystal structure observations (●) of Aib residues in nonhelical regions are marked. b,c) Cryrtallographic observations for Aib residues in peptides in the right (b) and left (c) handed helical regions of the φ, ψ map. Tips of arrow indicate ideal 3_{10} and α-helices. △N-terminal residues in protected peptides □ Nonterminal residue x C-terminal Aib present as ester or acid.

Extensive conformational studies of Aib-containing peptides in the solid state and in solution have been reported. The conformational angles determined by X-ray diffraction for Aib residues in a large number of peptides are summarized in figure 3. With very few exceptions, Aib residues adopt conformations in the region of ±60° ±20° and ψ = ±30 ±20°. These values of φ, ψ are compatible with the presence of Aib residues at positions i+1 or i+2 of Type I(I') or III (III') β-turns or at position i+2 or a Type II (II') β-turn (Venkatachalam 1968; Smith and Press 1980). β-Turn conformations in peptides are generally, though not always, stabailized by intramolecular 4⟶1 hydrogen bonding between the C=0 group of residue i and the NH group of residue i+3 (figure 4). The conformational parameters for the idealized Type I-III β-turns are listed in figure 4. The introduction of Aib resides into Acylic peptides thus enhances the probabilty of β-turns formation involing Aib-X or X-Aib sequences. This tendency is amply demonstrated in crystal structures of several Aib-containg



	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
Type I	-60°	-30°	-90°	0°
Type II	-60°	120°	80°	0°
Type III	-60°	-30°	-60°	-30°

FIGURE 4

Schematic representation of a β-turn conformation stabilized by a 4⟶1 (C_{10}) hydrogen bond. Conformational angles for idealized Type I, II and III β-turns are indicated. The enantiomeric β-turns (Types I, II, and III') are generated by reversing the signs of al ϕ, ψ angles.

model peptides (Nagarj and Balaram 1981; Prasad and Parlaram 1983. 1984) and also in nuclear magnetic resonance (NMR) studies of proteceted peptides in organic solvents (Nagaraj et al. 1979; Iqbal and Balaram 1981). The presence of successive Aib residues in a sequence, i.e., an Aib-Aib-X segment, or alternated Aib residues,

i.e., an Aib-X-Aib-X segment, generally leads to formation of consecutive B-turn structures of the Type III-III or III'-III' astegories. Such conformations are incipient right- or left-handed 3_{10}-helical structures (Iqbal et al. 1981; Vijayakumar and Balaram 1983a, 1983b).

The 1-aminocycloalkanecerboxylic acids (figure 1) are structuolly analogous to Aib. Theoretical studies of the cyclopentane (Acc^5) and cyclohexane (Acc^b) containing N-acetyl-aminoacid-N-methyl amides suggest that these residues also strongly prefer right- or left-handed 3_{10}/x-helical conformations (Prased, unpublished results). The extended regions of ϕ, ψ space are, in fact, even less favorable than in the case of Aib. These conclusions are borne out by two recent crystal structrures of model peptides containg Acc^6 residues. In Aib-Acc^6-$NHMe^6$, a type III (III')B-turn conformation was observed with ϕ = +68.4°, ψ = ±15° for the Acc^6 residue. Molecules of both-handedness were present in the achiral crystals. In Boc -Aib-Acc^6-OMe, where an intramolecular 4⟶1 hydrogen bond is not possible, a semiextended conformation is observed at Aib (ϕ = +62.2° ψ = ±143°), but the Acc^6 residue had ϕ = ±48°, ψ = ±42.6°. strongly supporting the notion that the cyoloalkyl side-chain imposes even greater restrictions on conformational freedom (Bardi et al., in press). Intristingly, recent studies by Italian and English groups suggest that the use of α,α-dialkyl residues like α,α-diethylglycine (Deg) or α,α-di-n-propylglycine (Dpg) can result in a stabilibation of extended peptide conformations (Barone et al., in press [1984a, 1984b]) may thus be possilble to modulate the folding tendency of acyclic peptides by introduction of Aib or 1-aminocycloalkanecarboxylic acids on the one hand or the higher α,α-dialkyl amino acids on the other. The intrduction of each α,α-dialkyl residues into biologically active peptides could then provide a means of selectively stabillzing certain backbone conformations. In principle, the use of schiral amino acids could be most appropriate when Gly residuee present in the natural sequence are replaced in the analog structure.

Two complicating factors must be considered when analyzing structure-activity data generated by using such analogs.

1) Enhanced hydrophobicity of the analog peptides may influence binding to apolar receptor surfaces.
2) The alkyl substituents may affect peptide-receptor interact ions by virtue of the new stereochemical contacts involved.

The replacement of Gly residues by Aib imposes a considerable restriction on backbone flexibillty. The replacement

of hydrogen atoms by methyl groups at C^{α} may be viewed as a minimum steric perturbation. Steric effects at receptor binding sites may be more significant when bulky cycloalkyl groups like Acc[5] or Acc[6] are used If the analog peptides retain full biological activity or exhibit enhanced activity, then it is likely that the conformations possible in the stereochemioally constrained analogs are indeed recognized by the receptor. Furthermore, any direct effect of the substituent (noted above) is minimal or promotes receptor-peptide association. If the analog peptide is inactive, then the conformations favored in the analog are not recognized by the receptor. Alternatively, the substituent has a direct inhibitory effect an receptor interactions by impeding close contact of the peptide with the binding site.

STUDIES ON ENKEPHALIN ANALOGS

In the case of the enkephalins (Tyr-Gly-Gly-Phe-Met/Leu), the presence of the Gly-Gly segment results in considerable conformational flexibility (Fishchman et al. 1978). Solution spectral studies have, therefore, generally failed to provide evidence for a dominant conformtion in the native sequences, although evidence for folded Roques et al. 1976; Jones et al. 1976) and extended (Bleich et al. 1977; Khaled et al. 1977) structures have been presented. The backbone conformational flexibility of the enkephalins is also dramatically illustrated by X-ray diffraction studies, where one crystal form has yielded a Gly^2Gly^5 Type I' β-turn structure (Smith and Griffin 1978; Blundell et al. 1979), while another is composed of enkephalin molecules in an extended B-sheet conformation (Karle et al. 1933: Carmeman et al. 1983).

Conformations Determined by NMR

The Gly residues at positions 2 and 3 in Met- and Leu-enkephalin amides have been separately or similtaneously replaaed by Aib or Acc[5] residues and the conformation of the resulting enkephalin analogs have been inferred from NMR studies in $(CD_3)_2SO$ (Sudha and Balaram 1983; Kiahore and Balaram 1984). The presence of intramolecularly hydrogen-bonded conformations is established by delineating solvent-exposed and -shielded NH groups, using temperature coefficiant of NH of chemical shifts (d8/dt) in $(CD_3)_2SO$ as a diagnostic criterion. The $d\delta/dT$ values <0.003) ppm/k may be generally considered to represent strongly solvent-shielded NH groups, while values <0.005 ppm/K are characteristic of solvent-exposed NH groups. Intermediate $d\delta/dT$ values (0.003 to 0.005 ppm/K) are harder to interpret definitively, but are often indicative of moderately solvent-shielded NH groups (Ravi et al. 1933; Iqbal and Balaram 1981).

The chemical shifts (δ) and temperature coefficients ($d\delta/dT$) of nine enkephalin analogs incorporating Aib or Acc[5] residues are summarized in table 1. The corresponding

TABLE 1

NMR Parameters of NH Groups[a] in Enkephalinamide Analogs in Dimethylsulfoxide

Peptides	Chemical Shifts (δ ppm)				$d\delta/dT \times 10^3$ ppm/K		
	X[2]	Y[3]	Phe	Met[5]/Leu[5]	Y[3]	Phe	Met[5]/Leu[5]
Tyr-Aib-Gly-Phe-Met NH_2	7.15	8.21	7.93	7.98	5.5	2.8	4.6
Tyr-Gly-Aib-Phe-Met NH_2	8.50	8.43	7.86	7.63	6.5	4.6	1.8
Tyr-Aib-Aib-Phe-Met NH_2	b	8.28	7.78	7.81	6.5	1.8	1.8
Tyr-Alb-Gly-Phe-Leu NH_2	b	8.24	7.94	7.76	4.5	1.9	3.9
Tyr-Gly-Aib-Phe-Leu NH_2	b	8.42	7.78	7.62	5.9	3.5	1.8
Tyr-Aib-Aib-Phe-Leu NH_2	b	8.13	7.78[c]	7.71[c]	6.3	2.00	2.3
Tyr-Aco[5]-Gly-Phe-Leu NH_2	b	8.12	7.80	7.85	4.5	1.8	4.9
Tyr-Gly-Aco[5]-Phe-Leu NH_2	b	8.44	7.78	7.57	5.9	3.7	1.9
Tyr-Aco[5]-Aco[5]-Phe-Leu NH_2	b	8.09	7.67[c]	7.61[c]	6.2	1.9	2.1
Tyr-Gly-Gly-Phe-Met NH^d_2	8.24	8.13	8.15	8.05	4.6	4.9	5.6
Tyr-Gly-Gly-Phe-Leu NH_2	8.20	8.05	8.10	7.90	a	3.7	4.6

a) The NH of residue 2 generally broadene on heating in $(CD_3)_2SO$; $d\delta/dT$ was therefore not measured.
b) NH resonance obscoured due to overlap with aromatic protons or due to broadening.
c) Not assigned due to $C^{\alpha}H$ overlap.
d) Values taken front Higashijima et al. 1979.

Parameters for Met[5]/Leu[5] enkephalinamide are also listed. the following features of the NMR data are noteworthy.

1) The analogs with Aib/Acc[5] residues at position 2 show low $d\delta$/dT values for the Phe NH, oharectieristiac of a solvent -shielded proton. All other NH groups appear to be solvent exposed.

2) In the position 3 analogs, the Met NH has a very low $d\delta$,/dT value, indicative of its sting shielding from the solvent. Other NH groups appear to be solvent exposed; although in the Aib and Acc[5] analogs of Leu-enkephalinamide, the Phe NH has a moderate $d\delta$,/dT value, suggestive of a moderate degree of shielding from solvent.

3) In the analogs vhere both Gly residues are replaced by Aib/Acc[5] residues, both Phe and Met NH resonsnces have very low $d\delta$/dT values, characteristic of solvent-shielded NH groups.

4) All NH group in the Gly-Gly analogs have relatively high $d\delta$,/dT values.

In small acylic peptides, involvement of NH groups in intramolecular hydrogen bonding generally results in their shielding from the solvent. Direct steric shielding without hydrogen bonding is unlikely. The NMR results therefore lead to the following inferences.

1) The Phe NH is intramolecularly hydrogen bonded in position 2 analogs, incorpoating Aib or Acc[5] residues.

2 The Met NH is hydrogen bonded in the position 3 analogs.

3) Both Phe and Met NH groups are hydrogen bonded in analogs substituted simultaneously with Aib or Acc[5] residues at positions 2 and 3.

The strong tendency of Aib residues to promote β-turn formation, together with the NMR results, suggests that intramolecularly hydrogen-bonded conformations are stabilized in the Aib analogs. The conformations compatible with HMR data and the known stereochemical preferences of Aib residues are illustrated in figure 5. It thus appears that conformations involving a β-turn centered at positions 2 and 3 (residues i+1 and i+2, respectively) are highly populated in $(CD_3)_2SO$ for the Aib(2) analogs, while a β-turn at positions 3 and 4 is sugested for the Aib(3) analogs. In the Aib(2)-Alb(3) analogs, a consecutive Type III-III β-turn structure is compatible with the spectral data. Such conformations are also likely to be favored in the Acc[5] analogs, in view of the similarities in the spectral characteristics of the Aib and Acc[5] analogs. Furthermore, model building and theoretical energy calculations suggest that Aib and Acc[5] residues have similar stereochemical preferences.

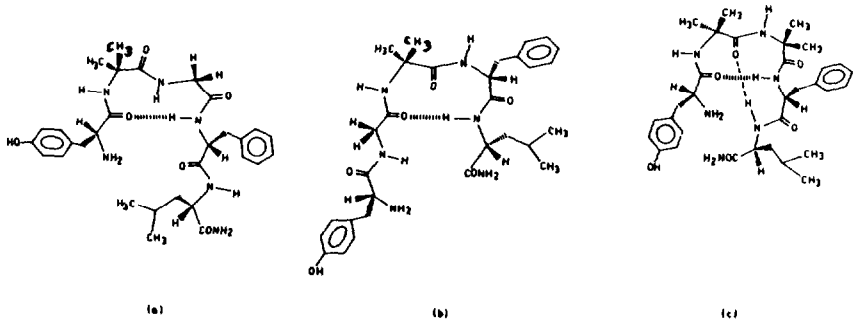


FIGURE 5

Proposed backbone conformations of Aib analoga of Leu-enkephalinamide a) Tyr-Aib-Gly-Phe-Leu+NH_2 b) Tyr-Gly-Aib-Phe-Leu-NH_2, c) Tyr-Aib-Phe-Leu-NH_2.

The crystal structures of the protected fragments Boc-Aib-Phe-Leu-OMe (Prasad, unpublished) and Boc-Aib-Aib-Phe-Met-NH_2 (Prasad et al. 1983) provide further support for theee conclusions. In the former, a Type I Aib-Phe β-turn is observed (figur 6), whlle the latter adopts a consecutive Type III-III (inciplent 3_{10} helical) conformation in the solid state (figure 6).

Circular Dichroism (CD) Studies

CD studies of Met(5)-enkephalinamide, Boc-Tyr-Gly-Gly-Phe-Leu-NH_2, and the fragment Boc-Gly-Gly-Phe-Met-NH_2 demonstrate a pronounced solvent dependence of spectra in solvents like 2,2,2-tri-fluorethanol (TFE) and methanol, indicative of structural transitions (figure 7). Replacement of the Gly residues by Aib results in an abolition of the solvent-dependent sign reversal of the CD band at 215 to 220 nm (Sudha and Balaram 1981). These observations have been interpreted by the authors as indicaitve of the role of Aib residues in stabilizing specific folded conformations in these sequences. In the above study, protected tetrapeptide fragments were used to examine the effect of Aib residues on solvent-dependent conformational transitions. This approach has subsequently been extended to a study of the enkephalin analogs themselves. The CD spectra of the three Aib analogs of Leu(5)-enkephalinamide in MeOH, H_20, and dioxane are shown in figures 8 to 10, respectively. The spectra of the corresponding Acc^5 analogs in MeOH and dioxane are shown in figure 8 and 9, respectively.

The following CD spectra classes may be recognized:

A) Positive CD band at ~220nm,
B) Negative CD band at ~,215 to 217 nm,
C) Two negative CD bands at 230 nm and 220 nm.

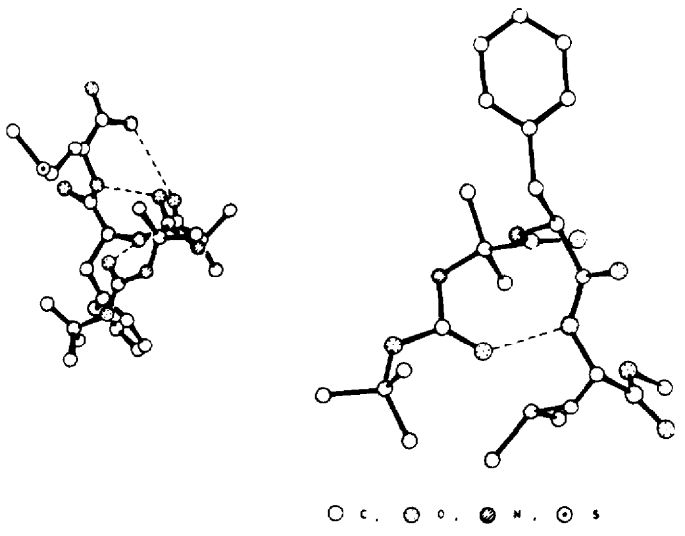


FIGURE 6

Perspective view of the molecular conformations or two peptide fragments related to Aib analogs of enkephalins. Left: Boc-Aib-Aib-Phe-Met-NH_2 (Prasad et al. 1983). Note 3_{10} helical structures formed by repetitive 4⟶1 hydrogen bonds. Right: Boc-Aib-Phe-OMe (Presad unpublished). A Type I β-turn with ϕ_{Aib} = -58.3°, ψ_{Aib} = -29.8°, ϕ_{Phe} = -84.7°, ψ_{Phe} = 1.3°

Class A CD spectra are exhibited by the position 2 analogs (Aib or Acc^5) in MeOH and H_2O. These spectra are characteristic of a β-turn conformation, tentatively assignable to either Type I' or Type III' categories, consistent with the class C' spectra of Woody (Woody 1974; Smith and Peace 1980; Crisms et al. 1984). Such spectra have been assigned to Type II B-turns in model peptides with Pro-X sequences (Criama et al. 1984; Rao et al. 1983). However, the occurrence of Aib or Acc^5 residues at position i+1 of a Type II β-turn (ϕ = -60°, ψ = 120°) is sterically unfavorable. Is is, therefore, more appropriate to aasign the class A spectra to Type I' or III' β-turns. The class B ectra obtained for the Gly(2)-Aib/Acc^5(3) or Aib/Acc^5 (2)-Aib/Acc^5(3) analogs in MeOH can be assigned to the consecutive β-turn (Type III-III' structure). For the Gly-Aib (Acc^5) analogs in H_2O both the negative band at ~217nm and the positive band at ~224 nm are observed, suggesting an equlibrium mixture of conformations yielding both class A and class B spectra. interestingly, in the Aib-Aib-(Acc^5-Acc^5)

analogs, there is a rather dramatic change in CD patterns; a class B spectrum is observed in MeOH, whereas a class A spectrum is seen in H_2O (figures 8 and 10). The NMR evidence suggests that a consecutive β-turn conformation (figure 5c) is favored in polar solvents like dimethyl sulfoxide. This conformation is expected to be adopted in MeOH also and could account for the class B spectrum In H_2O, one of the β-turns--probably Aib(3)-Phe(4)--may be destabilized, resulting in a single Type I' (III') Aib(2)-Aib(3) β-turn. This would be similar to the structures proposed for the Aib (2) (Acc^5(2)) analogs and may account for the observed class A CD pattern. In the relatively apolar solvent, dioxane, all six analogs exhibit disinctly different CD spectra as compared to MeOH or H_20. An unusual feature of these spectra is the presence of a rather long wavelength negative band at 230 nm. A definative conformational asignment in dioxane is not feasible at pressnt. Aggrgation effects may also play a role in this solvent. The CD studies sugest that even in the Aib or Acc^5 analogs of Leu-enkephalin there are solvent dependent structural changes. It is likely that well-defined conformational states like Type I' (III') or consecutive β-turn structures are populated for them peptides, in solution. However, equlllbria between them conformations must be considered.

The biological activity of all these analogs has not yet been determined. However, an in vivo behavioral assay is mice following intracerebral injection of peptide has been carried out on the three Aib analogs of Met-enkephalinamide. This study established the following order of activity: Aib-Aib Aib-Gly Gly-Gly Gly-Aib (Nagaraj et al. 1970; Sudha and Balaram 1983). while resistance to enzymatic degradation (Pert et al. 1976) likely to enhanoe the activity of the Aib analogs, relative to the native sequence in an in vivo assay, the observed activity does suggest that folded enkephalin conformations are in fact, recognized by appropriate receptors. This conclusion is based on the assumption that extended conformations are unlikely to be populated in these analogs seven at the receptor site, since favorable binding interactions are unlikely to offset unfavorable conformational energies.

This chapter has described an approach used in the authors' laboratory to restrict backbone conformational flexibility of the enkephalins. The use of alkylation at C^{α} as a means of limiting peptide backbone freedom (Spatola 1983) is a promising approach to the develoment of conformationally constrained analgos of biologically active peptides. The use of 1-aminocyoloalkane carboxlyic acids of varying ring size may permit altereations in the steric bulk and hydrophobicity of the residue being used to limit conformational flexibility. The potential of this approach is indicated by the observation that replacements of Leu(2) in the chemotactic peptide (Formyl-Met-Leu-Phe) by Aib, Acc^5, and Acc^6 leads to biologically

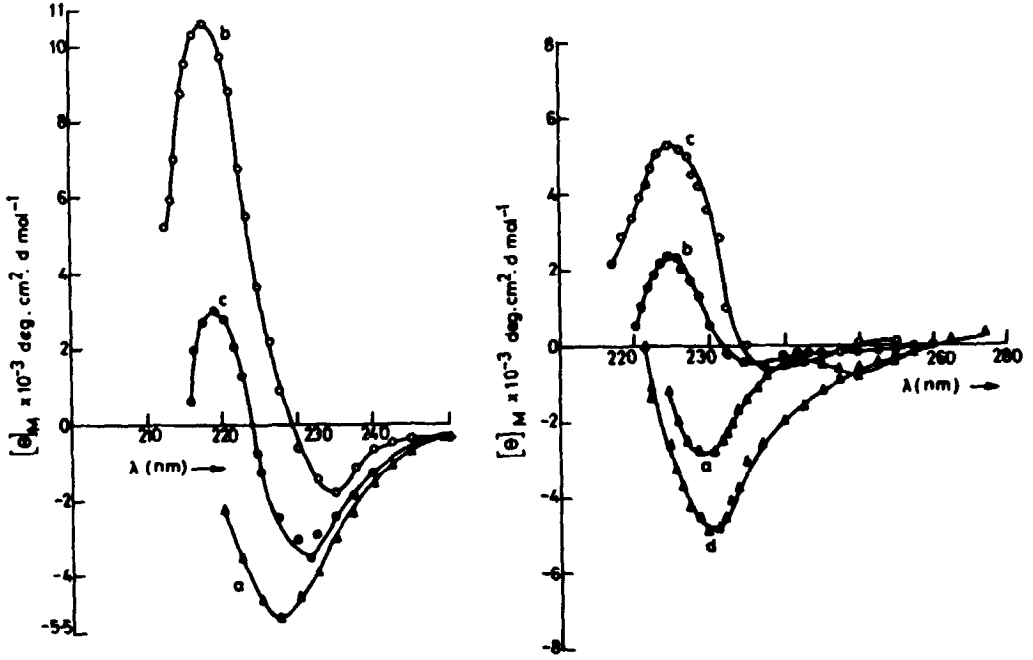


FIGURE 7

Left: CD spectra of Boc-Gly-Phe-Met-NH_2 (1.96) in a) TFE b) MeOH c) TFE MeOH, 1:1 (v/v). Right: CD spectra of Tyr-Gly-Gly-Phe-Met-NH_2 (1.74) in a) Me OH, b) TFE Boc-Tyr-Gly-Phe-Lue-NH_2 (1.53) in c) MeOH and d) TFE. (From Sudha and Balaram 1981, Copyright 1981, Elsevier/ North-Holland Biomedical Press).

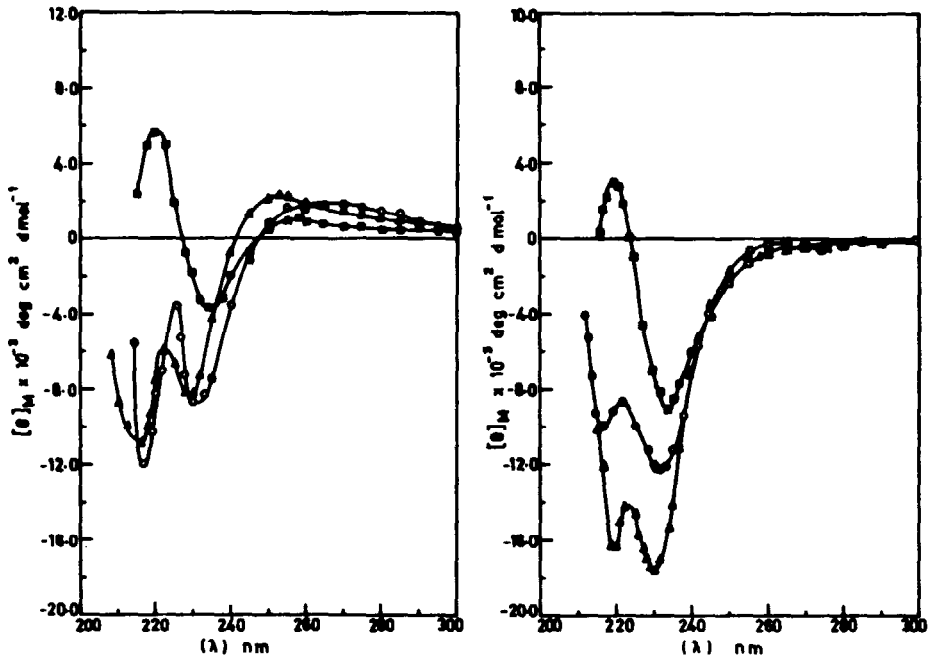


FIGURE 8

CD spectra of Tyr-X-Y-Phe-Leu-NH_2 in MeOH. Left: -Aib-Gly- □, -Gly-Aib- △, -Aib-Aib- O. Right: -Ace^5-Gly- □, -Gly-Acc^5- △, Acc^5-Acc^5- O.

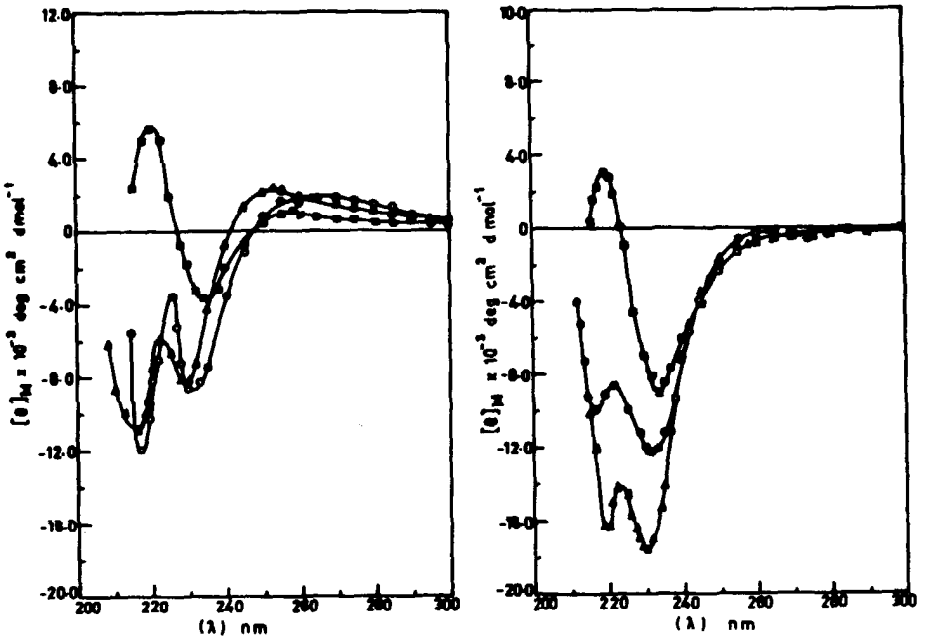


FIGURE 9

CD spectra of Tyr-X-Y-Phe-Leu-NH_2 in dioxane. Left: -Aib-Gly- □, -Gly-Aib- △, -Aib-Aib- O. Right: -Aoc[5]-Gly- □, -Gly-Acc[5]- △, -Acc[5]-Acc[5]- O.

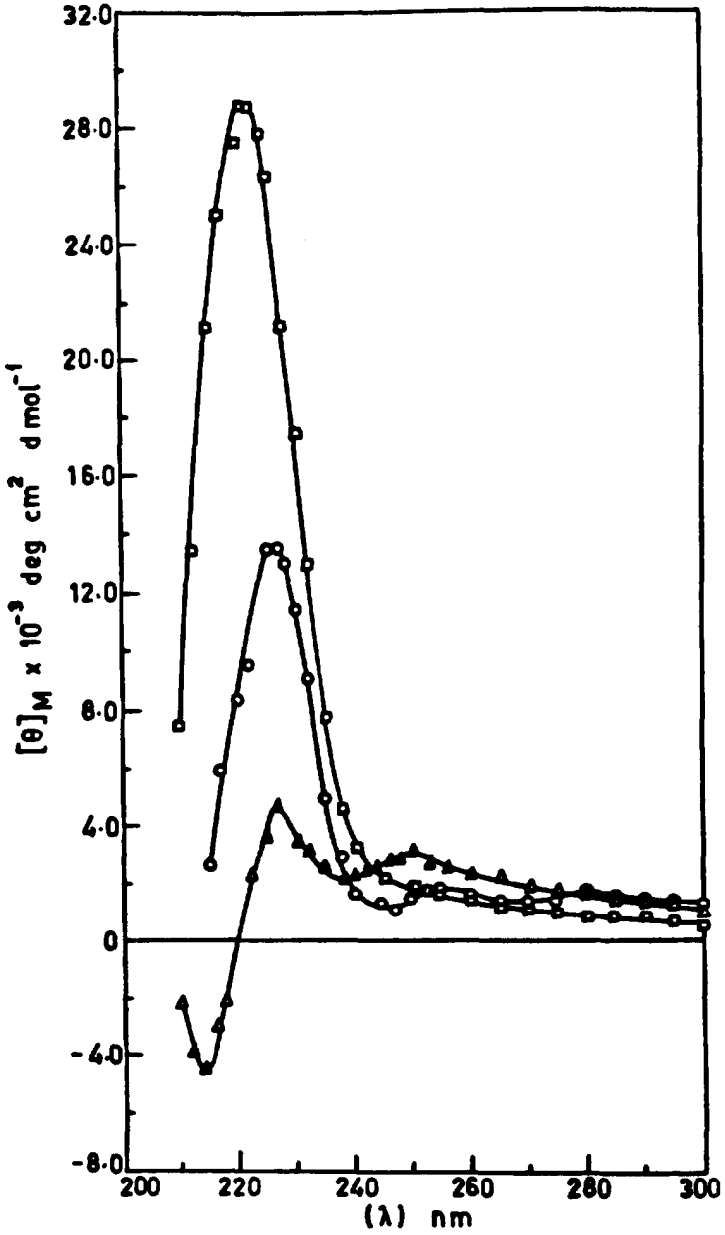


FIGURE 10

CD spectra of Tyr-X-YPhe-Leu-NH_2 in H_2O. -Aib-Gly-□, -Gly-Aib-△, -Aib-Aib- 0.

active analogs. The Acc^6 analog is a hundred fold more active than the parent peptide (Iqbal et al. 1984; Sukumer et al. 1985).

The studies described in this chapter outline a means of imparting specific folded conformations to analogs of enkephalins. Aditional restrictions improed on side-chain flexibility should complement these studies and lead to a better definition of the structra requirements for recognition by biological receptors.

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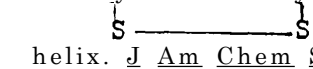

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Enkephalin Conformation in Solution: A Perspective From Vibrational Spectroscopic Studies

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The conformations of enkephalins have been assiduously explored since their discovery by Hughes et al. (1975). Numerous studies, often resulting in conflicting conclusions, have been reported in the literature (see Schfller 1984 for a review). A broad spectrum of physical methods has been utilized in derivin the solid and solution phase conformations of enkephalins. The central goal of the numerous studies has been to determine the bfoactfve conformation of enkephalins in their free state and, from the results obtained, derive the plausible conformation at the receptor site whose topography can at best be inferred. Many spectroscopic studies have been performed in organic solvents (Schiller 1984), which have provided much useful information but are of little direct relevance to the conditions likely to prevail at the receptor site. Of the numerous studies reported in the literature, only theoretical conformational analysis and molecular graphics have enabled researchers to postulate the receptor site conformations of enkephalins (Gorin et al. 1978).

In this study, FT-IR, Raman, and 2D-NMR spectroscopic methods were chosen to investigate the enkephalin conformations in solution, as these methods are suitable for obtaining fresh insight in the somewhat tedious and frustrating search for the elusive bfoactfve conformations of enkephalins. 2D-NMR studies and, in particular, 2D-NOE studies are still in progress; therefore, a perspective of enkephalin conformations from FT-IR and Raman spectroscopic studies (Renugopalakrishnan et al. 1985) is presented in this review. Previously, a Raman study of Leu^5-enkephalin was reported in the literature (Han et al. 1980). The present study differs in many respects from the previous Raman study of Leu^5-enkephalin. In the present study, the conformations of both Leu^5- and Met^5-enkephalins are investigated in aqueous and dimethyl sulfoxfde (DMSO) solutions. The latter solvent was used in order to compare the results of the present study with earlier studies (Schiller 1984). The FT-IR study of enkephalins in aqueous solutions

discussed here is the first of its kind and has utilized state-of-the-art advancements In this field. These studies were performed typically with millimolar concentrations of enkephalins in solution and, at this concentration level, the molecular aggregation of enkephalins can be expected to be minimal. The final answers to the bioactive conformations of enkephalins must await the isolation and purification of receptors in whose presence the conformations of enkephalins have to be determined using physical methods.

VIBRATIONAL SPECTROSCOPY AS A WINDOW ON SECONDARY STRUCTURE ON POLYPEPTIDES AND PROTEINS

Recent developments in vibrational spectroscopy have placed this technique in the forefront of physical methods for the elucidation of secondary structures of polypeptides and proteins. IR and Raman spectroscopic methods are based on the principles of vibrational spectroscopy, but differ on the fundamental mechanisms by which they arise: IR absorption occurs only when the dipole moment changes during the molecular vibration, whereas the Raman effect occurs through the induced dipole moment, namely the polarizability term when the incident light Interacts with the molecular system. Therefore, the two methods provide complementary information on a molecular system. For a general review of the application of FT-IR and Raman spectroscopy to biological molecules, see Parker (1983) and Tu (1982). Recently, the two methods and their variants have been applied to the elucidation of secondary structures of polypeptides and proteins (Renugopalakrishnan et al. 1984; Renugopalakrishnan and Bhatnagar 1984; Diem et al. 1984).

MID-IR AND RAMAN SPECTRA AS A BLUEPRINT OF THE PDLYPEPTIDE BACKBONE CONFORMATION

Most of the IR and Raman spectra of polypeptides and proteins reported in the literature contain much useful information in the mid-IR and Raman frequency region of 1000 to 2000 cm^{-1}, which can be broadly divided into the following three regions:

I. 1600 to 1700 cm^{-1}
II. 1500 to 1600 cm^{-1}
III. 1200 to 1300 cm^{-1}

These regions are usually designated as amide I, II, and III regions since they contain the three important vibrational modes of the peptide moiety, as shown in figure 1. The amide I vibrational mode consists of carbonyl bond stretching with a minor contribution from NH in-plane bending; the amide II mode consists of C'-N and N-C^{α} bond stretchings with a minor contribution from NH in-plane bending; whereas the amide III mode consists of C'-C^{α} and C'-N bond stretchings with a minor contribution from NH in-plane bending. The amide II mode

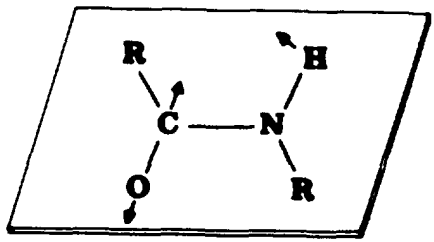
is usually either Raman inactive or weak in intensity. The three amide modes are generally sensitive to the secondary structures of the polypetide backbone, but it has been difficult to derive reliable secondary structural information from the observed frequencies. Part of the difficulty in deriving useful secondary structural information has been due to the absence of accurate IR and Raman data on polypeptides with well-characterized secondary structures. Much of the IR data reported in the literature before the introduction of the FT technique contained broad bands which lacked sufficient resolution to derive precise frequencies for secondary structural interpretation.

The introduction of lasers in the late sixties as the source of excitation in Raman spectroscopy (Lord and Yu 1970) has made it relatively easier to record Raman spectra of polypeptides and proteins, which was quite difficult with the older generation Raman spectrometers equipped with mercury arc lamps. Therefore, in recent years, a wealth of data on polypeptides and proteins has been reported in the literature setting the stage for a qualitative and quantitative interpretation of their IR and Raman spectra.

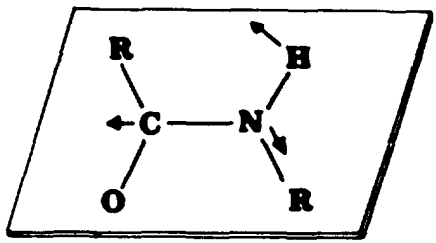
VIBRATIONAL FREQUENCIES OF SOME PROTEIN STRUCTURES RELEVANT TO ENKEPHALIN CONFORMATIONS: AMIDE I AND III FREQUENCIES OF β-SHEET AND β-TURN STRUCTURES

Synthetic polypeptfdes and proteins containing β-sheets, determined by other physical methods, manifest characteristic amide I frequencies ranging from 1669 cm^{-1} to almost 1690 cm^{-1}. For example, phosvitin (a phosphoglycoprotein from hen egg yolk containing more than 50% O-phosphoserines) assumes largely a β-sheet structure at pH 2 and manifests an R active band at 1690 cm^{-1} and a Raman active band at 1672 cm^{-1} (Renugopalakrishnan et al. 1985b). The amide I bands typical of β-sheets cluster at the higher frequency range, while those characteristic of α-helical structures cluster at the lower frequency range of the amide I region. The amide III frequencies characteristic of β-sheet and α-helical structures occur in the lower and higher frequency ranges of the amide III region respectively.

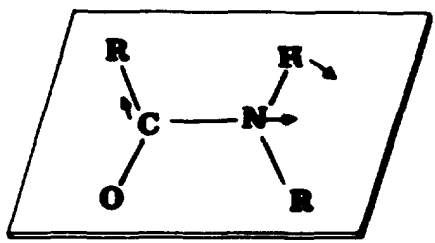
Amide I and III frequencies characteristic of β-turns have been calculated from normal vibration analysis of a tetrapeptfde, CH_3CO-(Ala-Ala-Ala-Ala)-NH-CH_3 by Bandekar and Krimm (1979). For effective transition dipole coupling perturbation, $\Delta\nu_{eff}$ between 0.35 and 0.45D, characteristic frequencies around 1640 to 1645 cm^{-1} and 1685 to 1690 cm^{-1} for type I β-turns and around 1660 to 1665 car and 1685 to 1690 cm^{-1} for type II β-turns were observed from these calculations. Antiparallel β-sheet structures manifest amide I frequencies near 1690 cm^{-1} (Miyazawa and Blout 1961) which partly overlap with the type I β-turn frequencies (Bandekar and Krimm 1979). Therefore, caution must be exercised in the interpretatlon of



Amide I



Amide II



Amide III

FIGURE 1

Amide I, II, and III vibrational modes of a peptide moiety. From Tu 1982. Copyright 1982, John Wiley and Sons.

the amide I frequencies. The amide II modes of the type I β-turn structures occur near 1550 to 1555 cm^{-1} and 1567 cm^{-1}, whereas type II e-turn structures exhibit amide II bands around 1545, 1555, and 1560 cm^{-1} respectively, corresponding to $\Delta\mu_{eff}$=0.27D (Bandekar and Krimm 1979). From the calculations, the amide III modes were found to occur in the 1290 to 1330 cm^{-1} region.

In general, a number of IR and Raman studies on β-turn structures have reaffirmed the theoretical calculations of Bandekar and Krimm for amide I and II frequencies. However, their observations on amide III frequencies, in general, are not corroborated by experimental observations (Tu 1982). Since the amide I frequencies of β-turn and β-sheet structures sometimes conflict with each other by occurring in the same region of IR and Raman spectra of polypeptides and proteins, caution must be exercised in drawing secondary structural interpretations.

From this wealth of information, the frequency range for different types of secondary structures commonly present in polypeptides and proteins is presented in figure 2, which was adapted from Tu (1982).

As may be observed from figure 2, the region designated as "random coil" is relatively smaller in area than the α-helical, β-sheet, and β-turn structures. In fact, this region contains the regions where β-turns, γ-turns, and collagenlike polyproline II structures manifest characteristic vibrations and, therefore, its definition remains quite arbitrary.

Interferences in the Amide I Region

The amide I region is usually obscured by bands arising from the solvents comnonly used in vibrational spectroscopic studies. Althouah diaital subtraction of solvent bands is possible, in reality it becomes difficult to completely subtract the solvent backaround. Therefore. sufficient caution must be exercised in assigning secondary structural significance to amide I bands. Fortunately, the introduction of the Fourier self-deconvolution technique (Kauppinen et al. 1981) has made possible the resolution of complex overlapping bands and has initiated a new era in the application of Raman and FT-IR spectroscopic methods to the determination of secondary structures. The interference of solvent in the amide II and III regions is less severe than in the amide I region, but the former vibrational modes are mixed modes involving the stretching of multiple bonds, making them less dependable as conformational probes.

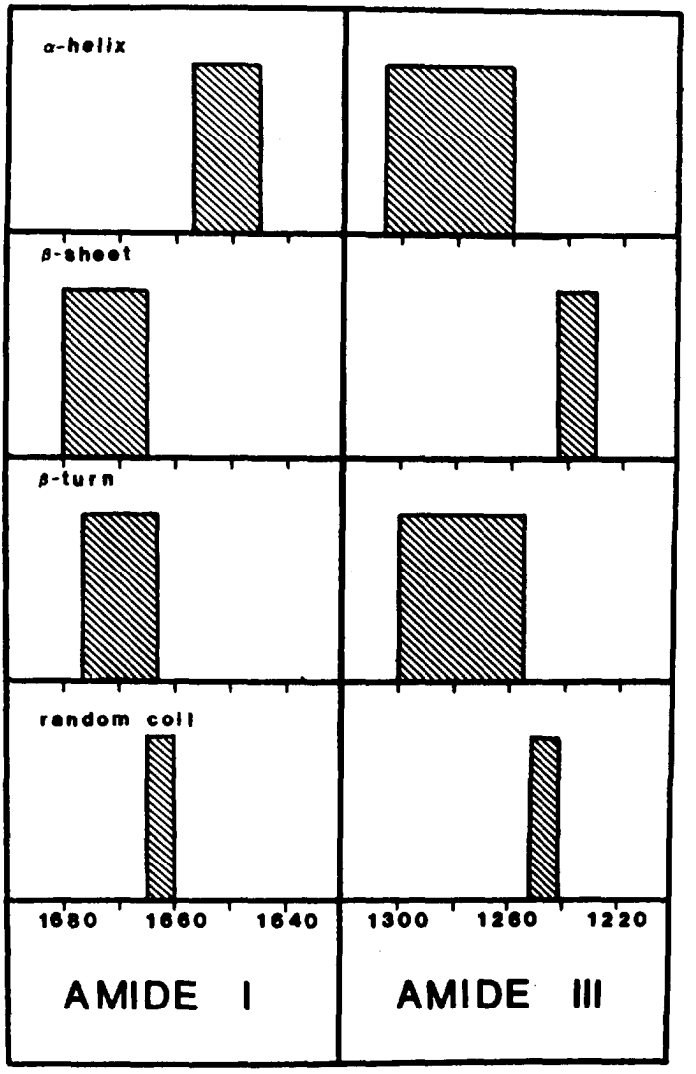


FIGURE 2

The range of observed amide I and III frequencies for α-helical, β-turn, β-sheet, and "random coil" structures. From Tu 1982. Copyright 1982, John Wiley and Sons.

RELATIONSHIP BETWEEN AMIDE I, II, AND III FREQUENCIES WITH THE SECONDARY STRUCTURE OF POLYPEPTIDE BACKBONE

The secondary structural dependence of amide I, II. and III vibrational modes are based on the concept that the vibrational frequencies of the peptide moiety are dampened to an extent dependent on their spatial orientation in the polypeptide backbone; peptide moieties with similar spatial constraints give rise to somewhat similar vibrations. For example, the amide I vibrational mode, figure 1, can be expected to depend on the orientation of the it peptide unit with respect to the preceding polypeptide backbone, figure 3, described by Ramachandran angle. ψ. The α-helical and β-sheet structures differ in, their ψ value, and if the amide I frequency for these two structures depend on ψ, then one would expect to see marked differences in their respective frequencies, as observed from figure 2. It is at present difficult to distinguish between α_L and α_R structures, as well as between e-sheet and β-turn structures, and β-sheet and collagenlike polyproline II structures based on amide I frequencies. It was recently suggested that the low-frequency vibrational modes where torsional interactions dominate are much more useful in providing a clearer distinction between various polypeptide structures (Renugopalakrishnan et al., in press [1985a]). It was demonstrated by Lord (1977) that the amide III frequencies of α-helical and β-sheet structures differ markedly and, hence, a correlation between amide III frequencies and, ψ 'was proposed. From numerous observations, this proposal has been further substantiated, although the amide III frequency is sensitive to the nature of side chain which makes Its use as a conformational probe quite difficult (Hsu et al. 1976). Raman and IR spectroscopic studies can provide a qualitative estimate of ψ, which at best can complement a time averaged value for ψ from NOE studies in solution phase (Leach et al. 1977; Urry et al. 1978).

With the introduction of FT technique in IR spectroscopy, FT-IR method is slowly gaining increasing popularity in protein structural studies. The previous disadvantage of utilizing aqueous solutions in IR studies has been largely overcome. The technical problem of fluorescence overshadowing the weak Raman lines has also been resolved by the introduction of the coherent anti-Stokes Raman technique (CARS), the use of pulsed lasers with electronic time gating to capture Raman photons, and the prolonged exposure of the sample to the laser beam which presumably bleaches the fluorescent moiety (Hudson 1977). The two methods, i.e., FT-IR and Raman spectroscopy, are well suited for structural investigations of the solid and solution phase of polypeptides and proteins and, therefore, offer a rapid method for the determination of their secondary structures.

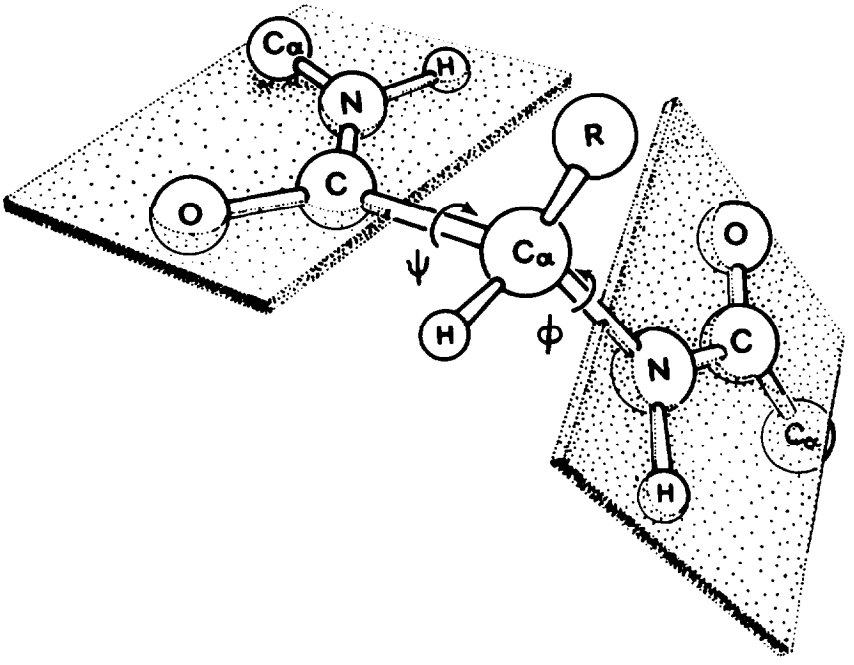


FIGURE 3

Ramachandran angles (ϕ, ψ) about $N-C^{\alpha}$ and $C^{\alpha}-C'$ bonds for two peptide units linked at the C^{α} atom. From Tu 1982. Copyright 1982, John Wiley and Sons.

A PERSPECTIVE OF ENKEPHALIN CONFORMATION FROM FT-IR AND RAMAN SPECTROSCOPIC STUDIES IN SOLUTION PHASE

Conformation of Leu^5-Enkephalin in Aqueous Solution

The FT-IR deconvoluted spectrum of Leu^5-enkephalin in aqueous solution is presented in figure 4. The amide I and II regions only are shown in figure 4 where the amide I bands occur at 1663 cm^{-1} with two shoulders at 1 58 cm^{-1} and 1675 cm^{-1} in addition to a peak at 1632 cm^{-1}. We have assigned the 1663 cm^{-1} band to a type II **β**-turn, whereas it is somewhat difficult to assign conformational significance to the shoulders at 1658 cm^{-1}. The shoulder at 1675 cm^{-1} and the peak at 1632 cm^{-1} can be assigned to the **β**-sheet structure. The amide I frequencies for the **β**-sheet structures also exhibit satellite bands in the 1630 to 1640 cm^{-1} region (Renugo-palakrishnan et a1. 1985b), which is not shown in figure 2. The IR active band at 1663 cm^{-1} for Leu^5-enkephalin is quite close to the 1665 cm^{-1} band observed in the Raman spectrum of Leu^5-enkephalin presented in figure 5, and its frequencies are tabulated in table 1. Raman spectrum of Leu^5-enkephalin in aqueous solution shows a multiplet structure in the amide I and III regions with the amide II mode being weak in this case. The most prominent amide I band occurs at 1665 cm^{-1}, whereas the amid III band occurs at 1263 cm^{-1} with a shoulder at 1255 cm^{-1} and a second peak at 1275 cm^{-1}. The amide III band at 1263 cm^{-1} is characteristic of **β**-turns, probably a type II **β**-turn (Bandekar and Krimm 1979). The shoulder at 1275 cm^{-1} is in the range observed for **α**-helical structures. Further support for the **β**-turn structure for Leu^5-enkephalin in aqueous solution is provided by the intense amide II band at 1547 cm^{-1} in the FT-IR spectrum of Leu^5-enkephalin (figure 4). The FT-IR spectrum of Leu^5-enkephalin (figure 4) shows somewhat less secondary structure than the Raman spectrum of the same (figure 5). but the frequencies of the observed bands in both are reasonably close to one another. The Raman bands at 1648 cm^{-1} and 1275 cm^{-1}, which are typical of **α**-helical structures, have no counterpart in the FT-IR spectrum.

Conformation of Leu^5-Enkephalin in Dimethyl Sulfoxide

The Raman spectrum of Leu^5-enkephalin in DMSO solution (figure 6) shows very few bands in the amide I and III regions, unlike the Raman spectrum of the same in aqueous solution, which was shown in figure 5. The amide I band occurs at 1616 cm^{-1} and 1686 cm^{-1}. While the origin of the band at 1616 cm^{-1} is not certain, the 1686 cm^{-1} is typical of the **β**-sheet structure (Renu opalakrishnan et al. 1985b). The conformation of Leu^{5}enkephalin in DMSO is quite different from that observed in aqueous solution.

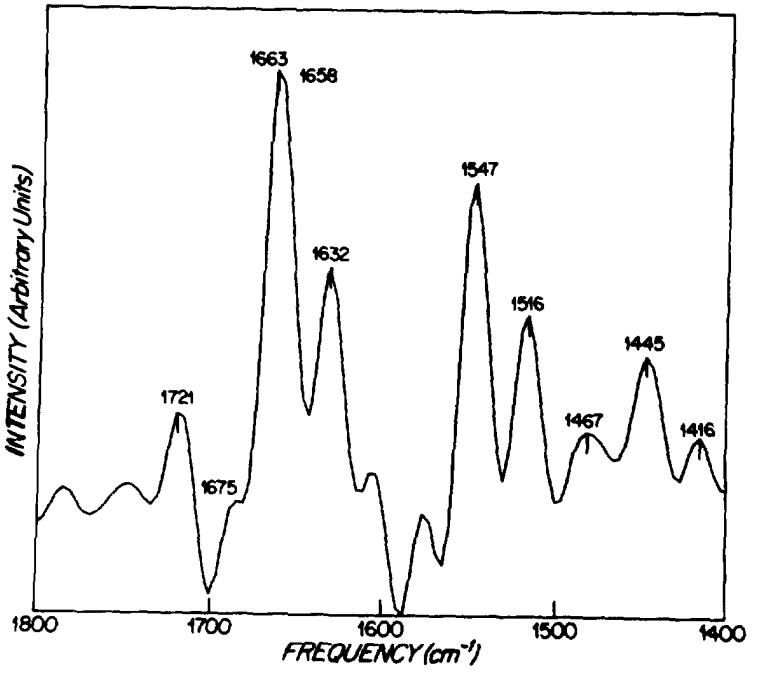


FIGURE 4

The FT-IR deconvoluted spectrum of Leu^5-enkephalin solution at a concentration of 0.37 mg/ml in water. The spectrum was recorded at room temperature. From Renugopalakrishnan et al. 1985. Copyright 1985, Academic Press.

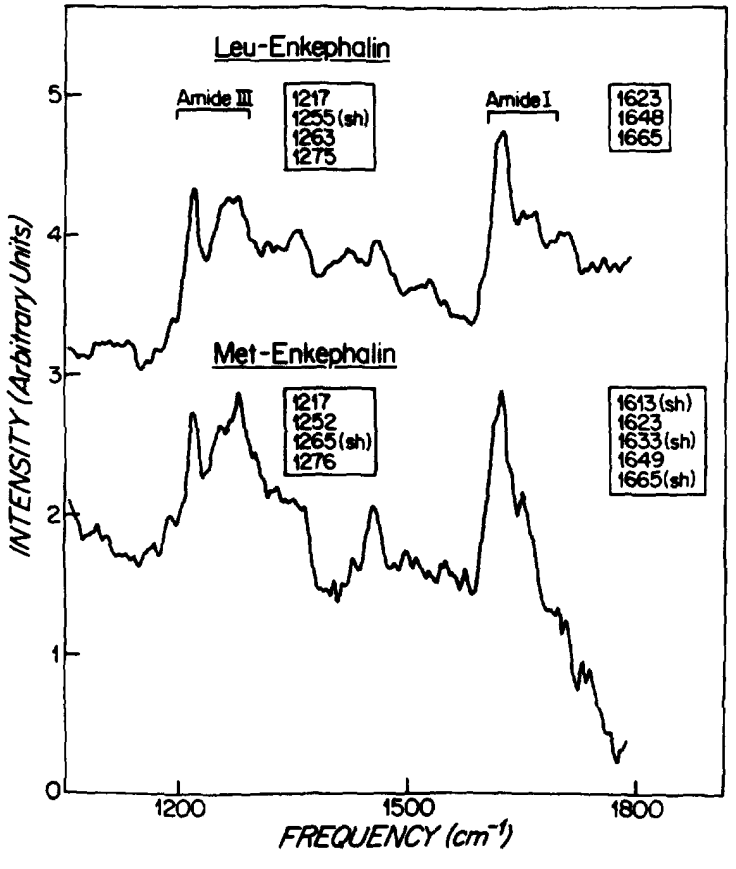


FIGURE 5

Raman spectrum of Leu^5-enkenphalin solution at a concentration of 0.95 mg/50 µl in water (top) and of Met^5-enkephalin solution at a concentration of 1.05 mg/50 µl in water (bottom). The spectra were recorded at room temperature using the 488 nm excitation line of an argon ion laser at a laser power of 600 mW. From Renugopalakrishnan et al. 1985. Copyright 1985, Academic Press.

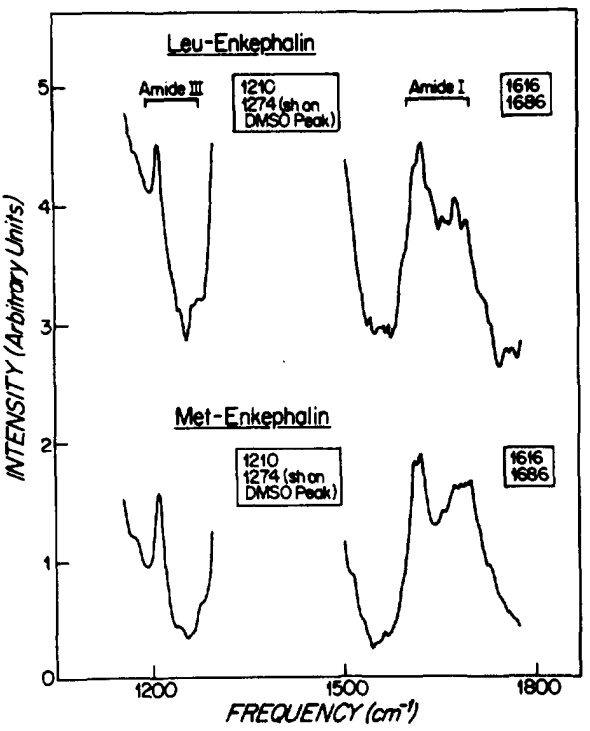


FIGURE 6

Raman spectrum of Leu^5-enkephalin solution at a concentration of 1.2 mg/50) µl of DMSO (top) and of Met^5-enkephalin solution at a concentration of 1.55 mg/50 µl of DMSO (bottom). The spectra were recorded at room temperature using the 488 nm excitation line of an argon ion laser at a laser power of 600 mW. The amide I and III regions only are shown. From Renugopalakrishnan et al. 1985. Copyright 1985, Academic Press.

The amide III region shows one intense peak at 1210 cm^{-1} with a shoulder (on DMSO peak) at 1274 cm^{-1}. The amide III peaks are hard to interpret in terms of the secondary structures and provide little conformational information.

Conformation of Met^5-Enkephalin in Aqueous Solution

The FT-IR spectrum of Met^5-enkephalin in aqueous solution is presented in figure 7 and the frequencies of amide I, II, and III mode are listed in table 1. The amide I mode occurs at 1636 cm^{-1} and 1678 cm^{-1}. The 1678 cm^{-1} is typical of **β**-sheet structure (Renugopalakrishnan et al. 1985b). In contrast, the amide III exhibits a triplet structure with bands occurring at 1246, 1273, and 1296 cm^{-1} respectively. The 1246 cm^{-1} band arises from the **β**-sheet structure whereas the 1273 cm^{-1} band is typical of **α**-helical structure. Bandekar and Krimm (1979) have predicted the 1290 to 1330 cm^{-1} region as characteristic of type I **β**-turns, but none have been reported to occur at this high frequency. Further work is necessary to assign the 1296 cm^{-1} band.

From the Raman studies, a somewhat different perspective of its conformation in aqueous solution emerges (figure 5) The amide I mode exhibits a multiplicity of bands: 1613 (sh) 1623, 1633 (sh), 1649, and 1665 (sh) cm^{-1}. An unequivocal assignment of the bands is certainly quite difficult. The 1633 (sh) cm^{-1} could well be a satellite band characteristic of **β**-sheet structure, but if this is true, its higher frequency component in the 1669 to 1690 cm^{-1} region is not observed in figure 5. The 1665 cm^{-1} band which is only a shoulder, unlike a peak at 1665 cm^{-1} in the case of Leu^5-enkephalin, is in the range typical of type II **β**-turn structures. The 1649 cm^{-1} band is against typical of **α**-helical structures. The shoulder at 1613 (sh) cm^{-1} and the 1623 cm^{-1} peak are hard to interpret. The amide III region shows three bands at 1252, 1265 (sh), and 1276 cm^{-1}; whereas the 1252 (sh) cm^{-1} is too high for **β**-μ-sheet structure, the other two can be assigned to the **β**-turn and **α**-helical structures. But, again, the **β**-turn frequency of 1265 cm^{-1} is only a shoulder in contrast to Leu^5-enkephalin.

Conformation of Met^5-Enkephalin in Dimethyl Sulfoxide

As mentioned earlier, the Raman spectrum of Met^5-enkephalin in DMSO solution is shown in figure 6. The spectrum manifests a doublet in the amide I region with bands occurring at 1616 cm^{-1} and 1686 cm^{-1}. The band at 1686 cm^{-1} is typical of **β**-sheet structure. The conformational flexibility of Met^5-enkephalin is greatly reduced in DMSO solution, which is similar to the observations on Leu^5-enkephalin in DMSO.

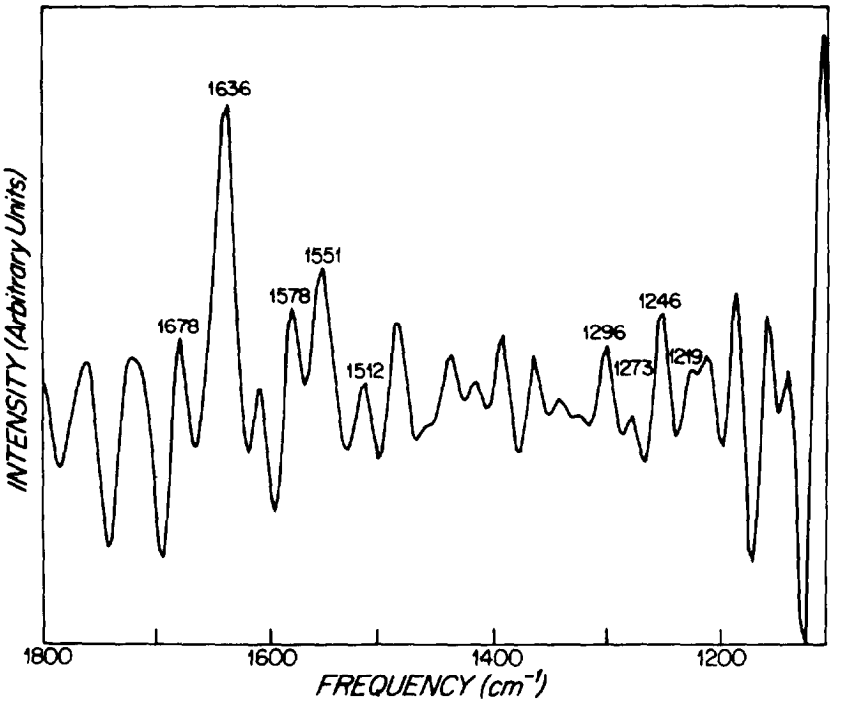


FIGURE 7

The FT-IR deconvoluted spectrum of Met^5-enkephalin solution at a concentration of 0.17 mg/ml in water. The spectrum was recorded at room temperature. From Renugopalakrishnan et al. 1985. Copyright 1985, Academic Press.

TABLE 1

The Amide I, II. and III Frequencies of Leu^5- and Met^5-enkephalins in Solution Phase From FT-IR and Raman Spectroscopic Studies

FT-IR	RAMAN		ASSIGNMENT
H_2O	H_2O	DMSO	
Leu^5 EnkEphalin			
	1217 cm^{-1}	1210 cm^{-1}	
	1255(sh)	1274(sh on DMSO peak)	AMIDE III
	1263		
	1275		
1516 cm^{-1}			AMIDE II
1547			
1632	1623	1616	
1658(sh)	1648	1686	
	1655		
1675(sh)			
			AMIDE I
Met^5-Enkephalin			
1246	1217	1210	
1373	1252	1274(sh on DMSO peak	
1296	1265(sh)		AMIDE III
	1276		
1512			AHIDE II
1551			
1578			
1636	1613(sh)	1616	
1678	1623	1686	AMIDE I
	1633(sh)		
	1649		
	1665(sh)		

CONCLUSIONS

Numerous studies on enkephalins using theoretical conformational analysis, X-ray diffraction studies on crystalline Leu^5- and Met^5-enkephalins, and spectroscopic studies of enkephalins in solution have led to widely divergent perspectives on the bioactive conformations of enkephalins. Unfortunately, none of the studies could be directed toward the bioactive conformations of enkephalins at the receptor site except for the theoretical conformational analysis of Gorin et al. (1978) and the 500 MHz ^1H NMR study of Beretta et al. (1984).

From the earlier studies, enkephalins have been postulated to be flexible molecules (Schiller 1984). This conclusion should not be surprising if one considers the absence of long-range stabilizing interactions in the pentapeptide. Hence, its conformation is likely to be determined by short-range interactions. It is plausible that the peptide exists in a conformational equilibrium modulated by the nature of the solvents, ionic strengths, etc. Among the structures commonly encountered for pentapeptide are **β-** and **γ-**turns; and, If molecular aggregation occurs, larger structures can be envisaged.

The FT-IR and Raman spectroscopic studies of enkephalins are Indicative of a conformational equilibrium prevailing in aqueous solution, whereas in DMSO, the conformational flexibility is greatly reduced. In DMSO, enkephalins form either **β-**turn or **β-**sheet structures or a combination of both; while, in aqueous solutions, both structures definitively coexist. While Leu^5-enkephalin in aqueous solution shows a **β-**turn structure, Met^5-enkephalin shows a less marked tendency to form **β-**turn structures. The occurrence of amide I bands at 1648 cm^{-1} in Leu^5-enkephalin and at 1649 cm^{-1} in Met^5 enkephalin (typical of **α-**helical structures) can probably be explained by the occurrence of (ϕ, ψ) values at the N- or C-terminal ends characteristic of **α-**helical structures. The peptides are too short to cause nucleation of **α-**helical structures (Chou and Fasman 1974). An equally valid explanation for the above bands is the likely presence of a type I **β-**turn structure which, according to the calculations of Bandekar and Krimm (1979). have low amide I frequencies in the region 1640 to 1645 cm^{-1}. At the present time, the results from this FT-IR study are more conclusive since it was possible to deconvolute the bands in the amide I and II regions. Han et al. (1980) concluded that Leu^5-enkephalin forms a **β-**turn structure in DMSO-d_6 with an amide I band occurring at 1691 cm^{-1}. This is slightly different from the frequency observed in our study, namely 1686 cm^{-1}. While an ambiguity probably exists in the interpretation of **β-**turn and **β-**sheet structures with frequencies of 1686 cm^{-1} and 1691 cm^{-1}, we would like to interpret this band as arising from s-sheet structure based on numerous observations (Renugopalakrishnan et al. 1985b).

Unfortunately. the earlier Raman study of Han et al. (1980) indicated a broad band centered around 1640 cm^{-1} for Leu^5-enkephalin in aqueous solution from which little conformational information could be derived. From their observations, Han et al. concluded that an ensemble of conformations existed in aqueous solution which is qualitatively similar to the conclusions reached in the present study. The IR spectrum of Leu^5-enkephalin in DMSO-d_6 (Han et al.) showed an intense band of 1666 cm^{-1} which was typical of a type II β-turn structure. This IR band at 1666 cm^{-1} is quite close to the 1663 cm^{-1} band observed in the present study of Leu^5-enkephalin in aqueous solution.

For Met^5-enkephalin, the propensity of the β-turn is reduced and evidence points to the presence of β-sheet structure as one of the prominent conformations. To date the observed difference in biological activity of Leu^5- and Met^5-enkephalins has not been explained based on conformational requirements. Even though the present study points toward differences in conformational populations in solutions of Leu^5- and Met^5-enkephalins, further studies are required to draw any definitive conclusions on the relationship between the conformational requirements and biological activity, such as μ/δ specificity.

It is tempting to speculate that each opioid receptor subtype obviously interacts with a single conformation from an ensemble of enkephalin conformations based on the topography and stereochemistry of both the receptor and the ligand. The rules which govern this selectivity are as yet unknown. However, Met^5-enkephalin, as compared to Leu^5-enkephalin, has higher affinity for binding at μ-receptor sites as compared to receptor sites, and, from our studies, it appears to preferentially exist as a β-sheet structure, over the β-turn. It might be that the β-sheet conformation interacts at the μ-receptor site, whereas as a β-turn structure (as in Leu^5-enkephalin), it interacts at the δ-receptor site. The present progress in the isolation and purification of opioid receptors (Demoliou and Barnard 1984), and the degree of sophistication in physical and theoretical studies attained, will pave the way in the future for understanding the rationale of enkephalin structure ⇄ dynamics ⇆ function relationships, in particular, and for polypeptides and proteins, in general.

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Conformational Features of the Opioid Peptides in the Solid State: A Review of X-Ray Crystallographic Research

Arthur Camerman, Ph.D., and Norman Camerman, Ph.D

INTRODUCTION

Isolation and characterization of the enkephalins led very quickly to attempts to elucidate their three-dimensional conformations. A variety of approaches, including model bullding experiments (Gradbury et al. 1976), high resolution nuclear magnetic resonance (NMR) (Roques et al. 1976; Jones et el. 1976), and empirical energy calculations (Isogal et al. 1977) were interpreted to suggest a folded conformation with a β-bend between either Gly^2-Gly^3 or Gly^3-Phe^4 as the most stable enkephalin structure. However, more comprehensive studies utilizing NMR, ultraviolet spectroscopy (UV) and circular dichroism (CD) data (Khaled et al. 1977), and sophisticated energy considerations (De Coen et al. 1977) demonstrated that fully extended enkephalin conformations are also consistent with spectroscopic data and with energy minima. From these studies, the existence of both folded and extended enkephal in molecules in solution was postulated. Seversl X-ray crystallographic investigations of leucine end methlonine enkephalins and enkephal in analogs in the solid state have recently been described. They have confirmed the occurrence of both types of conformation and have al loved us to observe directly the intra- and inter-molecular bondings which help to stablize them.

FOLDED CONFORMATIONS

Three crystal structure determinations--of an enkephalin, a substituted enkephalin, and an enkephalin analog--all have pentapepide molecules which exhibit folded conformations in the solid state. Despite the differences between the molecules themselves and in crystal environments (see table 1), the three molecular conformations are remarkably similar.

TABLE 1

Crystal Data for Enkephalins and Analogs whIch Have Been Crystallized

	Leucine						Methionine	
	1[a]		2[b]	3[c]	4[d]	5[e]	6[c,d]	7[d]
a	31.87	(31.93)	18.72	11.46	31.58	8.59	11.61	11.55
b	8.54	(17.08)	24.73	15.59	8.63	30.27	17.99	11.58
c	12.47	(24.86)	20.31	16.73	12.58	15.02	16.52	12.95
α	90.0	90.0	90.0	90.0	90.0	90.0	90.0	93.77
β	96.53	(95.54)	115.86	92.20	97.74	96.94	91.20	95.96
γ	90.0	90.0	90.0	90.0	90.0	90.0	90.0	86.96
z	4	(16)	8	4	4	4	4	2
Space Group	C_2	(A_2)	$P2_1$	$P2_1$	C_2	$P2_1$	$P2_1$	P_1

[a]Smith and Griffin 1978; (Blundell et al. 1979). Leucine enkephalin; folded conformation.
[b]Camerman et al. 1983 Leucine enkephalin; extended conformation.
[c]Blundell and Wood 1982 Leucine and methionine enkephalins; unsolved but likely both are extended.
[d]Ishida et al. 1984. [p-bromoPhe[4]] Leu-enkephalin) folded conformation. Methionine and [p-bromoPhe[4]] Met-entephalins unsolved but likely both are extended.
[e]Eckle et al. 1984. [D-Nle[2],L-NleS[5]] enkephalin; folded conformation.

Leucine Enkephalin

The first enkephalin X-ray diffraction structural information came from a crystal structure determination of Leu-enkephalin monohydrate in crystals grown from aqueous methanol (Smith and Griffin 1978). The molecular conformation is characterized by a Gly^2-Gly^3 β-bend stabilized by two intrmnolecular hydrogen bonds, Tyr^1(CO)-Phe^4(NH) and Tyr^1(NH)-Phe^4(CO). The tyrosine side chain is disordered and two positions of approximately equal occupancies are found for the hydroxyphenyl atoms. The Tyr and Phe side chains are located on the same side of the molecular backbone "plane" and are roughly coplanar with it, while the Leu side chain is on the opposite side and oriented perpendicular to the backbone.

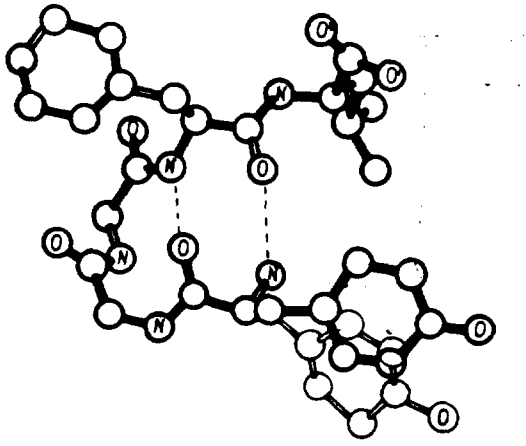


FIGURE 1

Molecular structure of leucine enkephalin in crystals grown from aqueous methanol Smith and Griffin 1978. Copyright 1978, American Association for the Advancement of Science

Subsequent to publication of this structure, Blundell et al. (1979) discovered that the unit cell of the Leu-enkephalin monohydrate crystals had been incorrectly characterized; the true unit cell (see table 1) contains four independent peptide molecules and the results of the structure analysis can be interpreted as dercribing a conformation averaged over four nearly identical molecules. The apparent disorder of the tyrosine side chains likely reflects two distinct tyrosine orientations in the four independent enkephalins.

[p-bromoPhe^4] Leucine Enkephalin

A leucine enkephalin with bromine substituted at position four of the phenylalanine phenyl ring was recently synthesized and crystallized from an ethanol/water mixture for an X-ray crystallographic structure investigation (Ishida et al. 1984). Reasons for bromine substitution were twofold: to add a "heavy" atom which makes the structure solution easier, and to see what effects slight modifications in chemical structure would have on enkephalin solid-state conformation. Results of the crystal structure determination show that even both the substituted bromine and crystalization from ethanol/water rather than methanol/cater, the conformation of [p-bromoPhe^4] Leu-enkephalin is almost identical to that of the previously reported Leu-enkephalin.

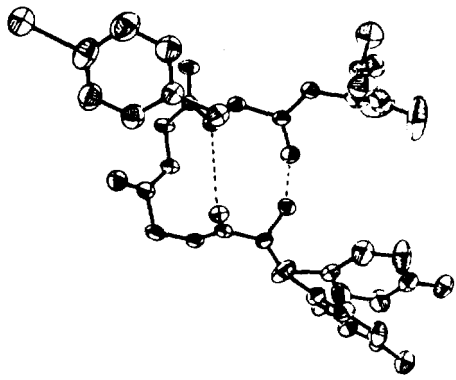


***FIGURE* 2**

Molecular conformation of [p-bromoPhe^4]leucine enkephalin. Ishida et al. 1984. Copyright 1984, The Biochemical Society, London.

The near identity of the the structures can be visualized by comparison of figures 1 and 2. In this crystal from, the [p-bromoPhe^4] Leu-enkephalin molecule exhibits a **β**-turn between Gly^2 and Gly^3 that is stabilized by two Tyr^1-Phe^4 hydrogen bonds exactly as found in Leu-enkephalin. Furthermore, just as initially described for Leu-enkephalin, the tyrosine side chain is disordered between two equally occupied positions. Torsion angles in the backbones and side chains of the two structures match very closely; the largest difference, 14°, is found in the leucine side chain and the average difference over 21 angles is less than 4° Since the

conformations of these two molecules are essentially identical, including disordered tyrosine positions, it is natural to question whether the space group reported for [p-bromoPhe^4] Leu-enkephalin may also be misinterpreted with, as found for the folded Leu-enkephalin, doubling of the b and c axes necessary to characterize the correct unit cell. As of this writing, Ishida and coworkers have not reported observing the weak diffraction intensities that could identify such a circumstance.

Tyr-D-Nle-Gly-Phe-NleS

X-ray crystallographic structure determination of a synthetic analog of leucine enkephalin in which glycine 2 is replaced by D-norleucine end leucine 5 by the sulfonic acid of L-norleucine has been reported very recently (Eckle et al. 1984). The compound was crystalized from an ethanol/water solution and the crystal asymmetric unit contains two enkephalin analog molecules, one ethanol molecule and five water molecules.

Despite the amino acid substitutions at positions 2 and 5, the conformations of both analog molecules are strikingly similar to that of folded leucine enkephalin. Again, each molecule exhibits a β-bend between amino acids at the 2 and 3 positions with intramolecular hydrogen bonds between Tyr^1(CO)-Phe^4(NH) and Tyr^1(NH)-Phe^4(CO).

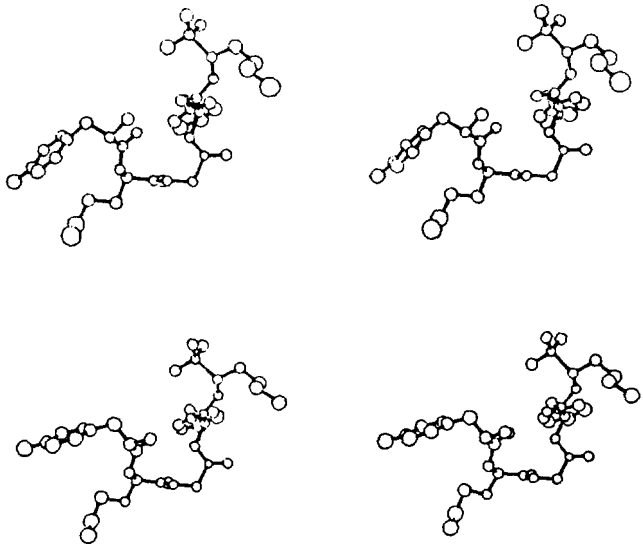


***FIGURE* 3**

Steroscopic projections of the two independent molecules of Tyr-D-NLe-Gly-Phe-NLeS. Eckle et al. 1984. Copyright 1984. International Union of Crystallography.

The major differences between the two molecules in the asymetric unit occur at the tyrosine, a situation analogous to the two different tyrosine orientations found in the previously discussed enkephalins. In contrast to those two enkephalins, however, the side chains in this enkephalin analog are oriented with the tyrosine and phenylalanine on opposite sides of the peptide backbone.

It is notewrthy, in light of the fact that [D-Ala^2] Leu-enkephalin is boiologically active, that insertion of a D-amino acid at position 2 does not alter the basic enkephalin folded backbone conformation.

EXTENDED CONFORMATIONS

Four enkephalin crystal forms have been reported in which the molecules either display or are strongly suggested to display extended β-pleated sheet conformations. Three of the four crystal structures have not yet been solved so that the molecular structures cannot be described with absolute certainty, but extremely strong indications in all three instances point to extended conformations. One completed crystal structure determination of leucine enkephal in with four independent pentapeptides in the asymmetric unit has found all four enkephalins to display extended backbone conformations.

Unsolved Structures

The first indications that both leucine and methionine enkephalins could have extended backbones in the solid state came from X-ray investigations of crystals of the two compounds obtained from ethanol/water solutions (Blundell and Woods 1982). Although these studies did not lead to successful structure elucidations, the X-ray data did allow determination of the pentapeptide backbone orientations. In both the leucine and methlonine crystals, the backbones appear to be extended and to form hydrogen-bonded β-pleated sheets.

A simllar situation involving X-ray data sets for methlonine enkephalin (the same crystal form as above) and [p-bromoPhe^4] Met-enkephalin was reported very recently (Ishida et al. 1904). These investigators also were unable to solve the crystal structures but were able to conclude that the methionine and bromo-substituted methionine enkephalins have extended conformations.

Leucine Enkephalin

A crystal form of leucine enkephal in grown from a water-DMF mixture was found from measurment of unit cell volume and density calculations to contain four independent enkephalin molecules plus a large amount of solvent in the asymmetric

unit. Elucidation of the crystal structure has shown that the four enkephalin pentapeptides exhibit slightly differing molecular conformations but all have extended linear backbones (Camerman et al. 1983; Karie et al. 1983).

The extended molecules form a slightly irregular antiparallel β-pleated sheet with the aminoacid side chains oriented above and below the backbone plane. The sheet is held together by hydrogen bonds largely between peptide backbone NH and CO groups.

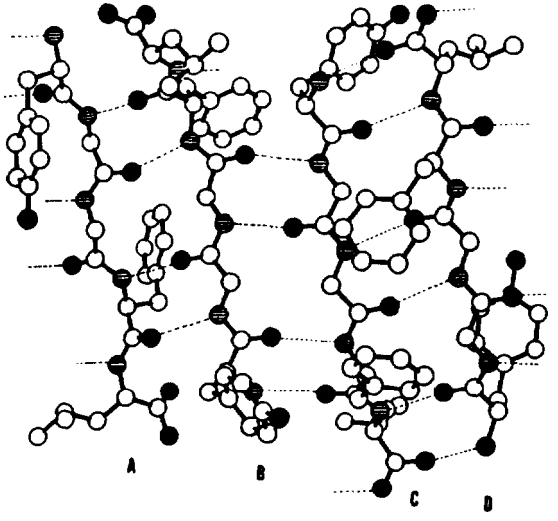


FIGURE 4

Conformations of the four indepandent peptides in Lmaiuo enkephalin crystals grown from water/DMF. Oxygen and nitrogen atom are darkened. Camerman et al. 1983. Copyright 1983. Macmillian Journals Ltd.

Comparison of me molecular conformations of me four independent enkephalins reveals that the greatest differences are the orientations of the tyrosine and leucine side chains; the phenylalanine side chain orientation, in contrast, differs only slightly from molecule to molecule.

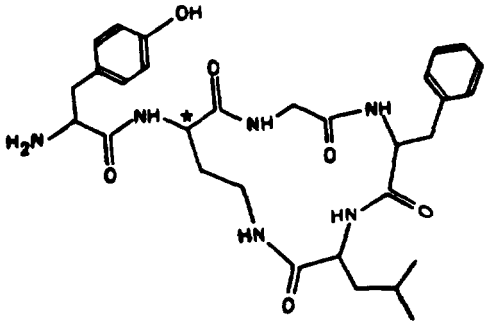
The peptide backbones of molacules A and D are similar and are more puckered than those of B and C, which also resemble each other. In all four molecules, the regular alternation of ride chain positions causes the Tyr and Phe side chains to be located on opposite sides of the backbone with the Leu side chain on the same side as the Tyr.

Conformational variations in the peptide backbones of leucine enkephalins are illustrated in table 2 which lists the backbone torslon angles in the four independent extended conformers and, for comparison, those in the folded molecule.

Analysis of the Φ and ψ angles for glycine residues 2 and 3 reveals that at these positions molecules A and D display conformations characteristic of L-amino acids, while in B and C the values are closer to those which would be favored by D-amino acids. The only other angles which differ appreciably between enkephalin molecules are those at leucine. These data are compatible with, and may provide an explanation for, the observations that substitution of D-alanine at position 2 and D-leucine at position 5 yield biologically active enkephalin analogs, while substitution of D-phenylalanine at residue 4 destroys activity.

Extended Conformation and Biological Activity

Determining which enkephalin conformations are responsible for binding to receptors and effecting the physiological actions of these compounds would greatly aid our understanding of the nature of opiate receptor binding and the molecular mechanisms of analgesia. A sinificant step in this direction has been the synthesis of cyclic Leu-enkephalin analogs with enhanced opiate μ receptor affinity (DiMaio and Schiller 1980; Schiller and DiMaio 1982; DiMaio et al. 1982). Substitution of D-α, γ-diaminobutyric acid for glycine in position 2 of the enkephalin sequence and cyclization of the γ-amino group to the leucine C-terminal carboxyl group has produced a compound that is twice as potent as its uncyclized analogue and 20 times as potent as Leu-enkephalin in inhibiting electrically evoked contractions of the guinea pig ileum.



Tyr-cyclo [-N^{γ}-DA$_2$bu-Gly-Phe-Leu]

A rationale for this enhanced activity is that cyclization produces a relatively rigid, fixed conformation in contrast to Leu-enkephelin which displays both extended and folded shapes in solution and in the solid state. Additionally, this fixed conformation is similar to the "active" conformation adopted by enkephalins when binding to μ receptors.

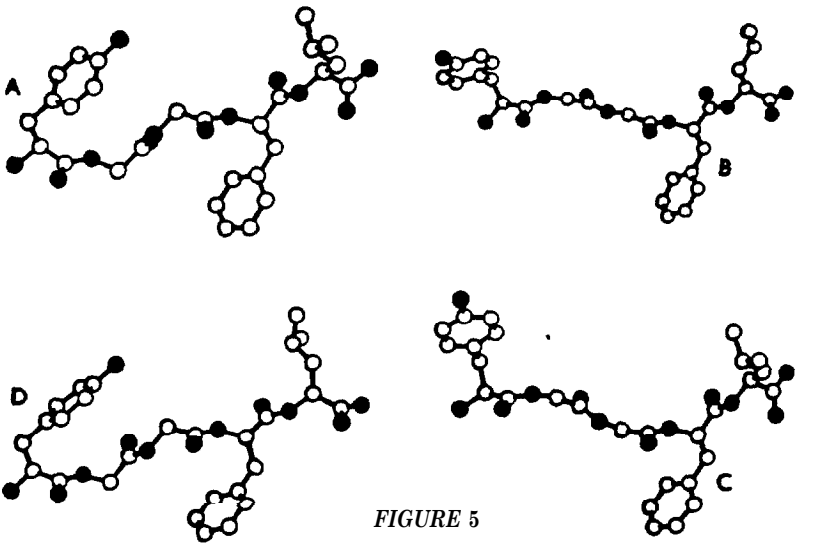


FIGURE 5

The four leucine enkephalin extended backbone conformations aligned in comparable orientations, Camerman et al. 1903. Copyright 1983. Nacmilian Journals Ltd.

TABLE 2

Torsion Angles (deg) in the Enkephalin Backbones[a]

Residue	Angle	A	B	C	D	F[b]
Tyr_1	ψ_1	135	154	155	137	126
	ω_1	172	177	177	173	173
Gly_2	ϕ_2	-144	151	141	-131	59
	ψ_2	114	-155	-157	142	25
	ω_2	-177	180	-178	179	179
Gly_3	ϕ_3	-122	154	174	-144	97
	ψ_3	132	-151	-170	131	-7
	ω_3	-179	-170	179	178	-174
Phe_4	ϕ_4	-122	-128	-119	-147	-136
	ψ_4	139	130	149	152	145
	ω_4	168	174	179	171	180
Leu_5	ϕ_5	-79	-72	-141	-141	-105
	ψ_5	176	167	137	151	-4

[a]Estimated standard deviations are 1.8-2.5°
[b]Folded leucine enkephalin.

It has been demonstrated with molecular models that the cyclized compound cannot retain the structural features of any of the folded enkephalin conformations; thus, the μ receptor active conformation almost certainly does not contain an intramolecular bend (DiMaio and Schiller 1980). We have investigated whether the crystal structure extended conformations are compatible with the cyclized enkephal in analog by constructing a model of the latter compound derived from an extended Leu-enkephalin molecule with the approprlate position 2 substituti and cycliatin added. The model is shown figure 6. Analysis of the model reveals that, in order to form the cyclic analog, the linear peptie backbone has to be made somewhat more pleated, with the largest changes in torsion angles occurring at Gly^3.

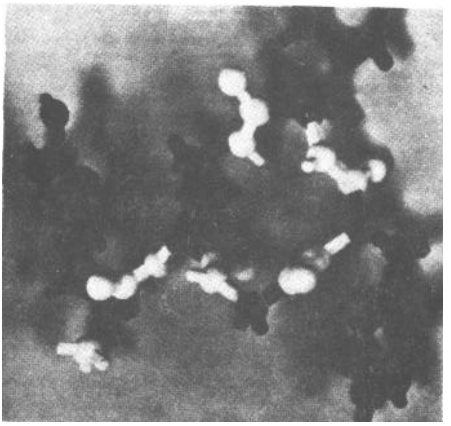


FIGURE 6

Molecular model of Tyr-cycol-[-γ-DA₂-bu-Gly-Phe-Leu-] from leucine enkephalin extended conformation coordinated. A₂bu: diaminobutyric acid. Peptide linkage are shown in white: α-carbons and side chains are dark. (Camerma et al. 1983. Copyright 1983, Macmillan Journals Ltd.

Comparison of figures 5 and 6 illustrates that although the molecular fit is not exact, the main structural features of the Leu-enkephalin extended conformation can be retained in the cyclized analogue: the peptide backbone is linears hydrogen bonding atoms are retained in similar positions and orientations: Tyr and Leu side chains are located on the same side of the backbone, while Phe is on the opposite side. Quantitatively, the Tyr and Phe rings are, respectively, 8.2, 13.3, 13.9, and 8.9 Å apart in the extended molecules A, B, C, and D and the same range of distances is permitted in the

cyclized model. In terms of arrangement of hydrogen bond acceptors and donors, the distance between Tyr^1 and Gly^3 carbonyl oxygen atoms ranges from 6.8 to 7.2 in the extended molecules, while Gly^2 and Phe^4 nitrogen atoms are 6.6 to 7.1 Å apart. These distances in the cyclized compound are 7.1 and 6.2 Å. It appears, therefore, that if the enhanced activity of the cyclized analog is due to forcing of the otherwise flexible multiconformational enkephalin molecule into a conformation similar to that adopted when bound to μ receptors, then conformations resembling the extended molecules are suitable models for μ receptor active enkephalin stereochemistry.

Apart from the extra atoms introduced by the substitution of diaminobutyric acid for glycine, the biggest change to the enkephal in structure on forming the cyclic analog is the movement of Leu due to its linkage to the side chain at position 2. It is of interest that for a series of cyclic compounds differing in the identity of the linkage substituent, inhibitory potencies in both the guinea pig ileum bioassay and the rat brain ^{3}H-naloxone binding assay increase with increasing length of the linking side chain (DiMaio et al. 1982). As the length of this side chain is increased, we would expect the change in position of the leucine from its location in the extended enkephal in conformation to be lessened in formation of the cyclic compounds. Thus, these biological data are also compatible with and support the concept of an extended enkephalin conformation as a primary candidate for binding to opiate μ receptors.

CONCLUSIONS

X-ray crystallographic inveqtigations of enkephalins and enkephalin analog have to date provided four complete three-dimensional structure determinations and three other data sets from which structural inferences can be draw. These investigations have enabled us to view directly enkephalinolecular stereochemistry in the solid state under a variety of envirormental conditions and to formulate conclusions about conformational preferences and stabilities

(1) The enkephalins are flexible molecules and when the whole pentapeptide is considered (side chains as well as backbone), several conformational arrangements are equally probable.

(2) There appeer to be two favored backbone conformations, the folded one characterized by a β-bend between residues 2 and 3 stabilized by two Tyr^1-Phe^4 hydrogen bonds, and the extended conformation stabilized by β-pleated sheet intermolecular hydrogen bonding.

(3) D-amino acids can be substituted for glycine 2 without changing either the folded or the extended backbone conformations. This correlates significantiy with the fact that enkephalins with D-amino acids substituted at position 2 retain biological activity.

(4) As it is possible for conformationally restricted cyclized enkephal in analogs with greatly enhanced receptor-mediated activity to maintain extended backbone stereochemistry but not folded conformations, It is likely that the enkephal in μ receptor active conformation is one with an extended peptide backbone.

It is not known at present what factors are responsible for the binding of enkephalins to each of the dlfferent classes of opiate receptors. One possibility, of course, is that dlfferent enkephalin conformations are required for interaction with the different receptors. If this were the case, then It would be reasonable to speculate from the structural data that extended enkephalin molecules might bind preferentially to the μ receptor and folded ones to the 8 receptor. Further structure analyses, particularly of conformationally restricted enkephalin analogs and other peptides that bind selectively to one class of receptors, will help to prove or disprove such speculations. They may also help to eventually identify the factors that govern all types of enkephal in-receptor interactions.

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The Search for Nonaddicting Strong Analgesics: Hopes and Frustrations

Sydney Archer, Ph.D.

INTRODUCTION

The pleasures of the poppy have been known to man for many centuries and have been glorified in literature by DeQuincy, among others. It was not until the middle of the last century that the use and abuse of opium derivatives became a serious cause for concern. Chemical examination of opium led to the isolation of morphine by Serturner in 1803. Continued research on Papaver somniferum resulted in the isolation and characterization of other alkaloids, and processes were developed to make pure morphine readily available. The unrestricted availability of this compound and the development of the hypodermic needle led to widespread abuse of this alkaloid. The recognition of the addiction potential of morphine stimulated attempts to find a substitute that would not be burdened by this undesirable property. The first example was heroin, the diacetyl derivative of morphine. This compound was indeed a satisfactory substitute in the sense that it is an excellent analgesic and, on administration to morphine-dependent subjects, prevented the appearance of the characteristic morphine withdrawal syndrome. It was soon discovered that heroin was just as insidious as its precursor. Two important lessons were learnt from this experience: first, analogues of morphine may be clinically effective analgesics and, second, there is the phenomenon of cross-dependence to be reckoned with.

There is very little relationship between the therapeutic dose of an opioid analgesic and its potential for abuse. Some factors which are important in determining overall abuse liability are: (1) the ability of a drug to induce tolerance and physical dependence; (2) whether abrupt withdrawal of the drug from a dependent individual will result in drug-seeking behavior; (3) whether administration of the drug or a congener will relieve withdrawal symptoms promptly; (4) whether the drug can be self-administered easily; and (5) whether high doses of the drug will produce unpleasant side effects.

INITIAL STUDIES

A systematic search for a nonaddicting, strong analgesic was begun in 1929 when the National Research Council and the National Academy of Sciences formed the Committee on Drug Addiction whose charge was to develop a program involving chemical synthesis, pharmacological evaluation, and clinical methodology to

determine analgesic potency and to assess addiction liability. The chemical group was headed by Dr. Lyndon Small, who at that time was a young assistant professor at the University of Virginia. Small investigated derivatives of the main phenathrene alkaloids in opium. He was joined by Dr. Erich Mosettig, a student of Professor Spaeth, from the University of Vienna, who concentrated on synthetic partial structures related to morphine. One of Mosettig's students, Dr. Everette L. May, joined the group in 1935, and continued this work which led to many significant advances.

The pharmacology program was started in 1930 under the leadership of Dr. Nathan B. Eddy, who later became Secretary of the Drug Addiction Committee. In order for this fruitful collaboration to be even more effective, the two groups were transferred to the National Institutes of Health (NIH) in 1938. About this time, a primate facility was established at the University of Michigan under the leadership of Professor Maurice Seevers, who developed techniques for measuring dependence liability in monkeys.

During the first decade of this program, over 200 morphine analogues were synthesized and evaluated. During the course of the next three decades, this NIH facility tested over 9,000 compounds, most of which were submitted by laboratories engaged in analgesic research with the same goals in mind. About 1,000 of these compounds were examined in Dr. Seevers' laboratory, of which approximately 10% were tested in the Addiction Research Center in Lexington, Kentucky.

The Addiction Research Center was established in 1935 to develop methods to quantitatively assay the addiction potential of new analgesics. The first Director was Dr. Clifton K. Himmelsbach. One of the first methods perfected under Himmelsbach's direction was the direct substitution procedure. Prisoner addicts were stabilized on a given dose of morphine for about 1 week. Then a putative analgesic synthesized in either Dr. Small's laboratory or elsewhere was substituted for the morphine, using doses suggested by Dr. Eddy's assessment of analgesic potency in mice. When suitable doses were used, in every instance the addicts were not aware that a drug had been substituted for morphine. It was judged that drugs which could substitute successfully had a high addiction potential.

It was well known that when morphine is withdrawn from dependent individuals, a syndrome develops which is characterized by a number of signs and symptoms. Himmelsbach developed a method to quantitate the morphine abstinence syndrome as shown in table 1. By plotting these Abstinence Syndrome Intensity scores against time, the entire clinical process can be seen graphically as in figure 1.

By the time World War II started, methodologies were available to determine the potency of strong analgesics in rodents and their addiction potential in primates and man. A major gap remained: methodology for the clinical evaluation of analgesic potency. Henry Beecher was a pioneer who led the way to close this gap. Two of his students, Louis Lasagna and Arthur Keats, as well as Raymond Houde, made major contributions to solving the problem of determining analgesic potency in man (Houde et al. 1965). A time-effect curve measuring the analgesic potency of oral and intramuscular morphine is shown in figure 2.

TABLE 1

Point System for Measuring Abstinence Syndrome Intensity by the Day (D) or Hour (H)

Signs	(D) By day		(H) By hour	
	Points	Limit	Points	Limit
Yawning	1	1	1	1
Lacrimation	1	1	1	1
Rhinorrhea	1	1	1	1
Perspiration	1	1	1	1
Mydriasis	3	3	3	3
Tremor	3	3	3	3
Gooseflesh	3	3	3	3
Anorexia (40% decrease in caloric intake)	3	3		
Restlessness	5	5	5	5
Emesis (each spell)	5		5	5
Fever (for each 0.1°C rise over mean addiction level)	1		1	10
Hyperpnea (for each resp./min. rise over mean addiction level)	1		1	10
Rise in a.m. systolic B.P. (for each 2mm Hg over mean addiction level)	1	15	1	10
Weight loss (a.m.) (for each lb. from last day of addiction)	1			

Total abstinence syndrome intensity per day or per hour is the sum of the points scored in the (D) or (H) columns respectively, with due attention to the limits.

From Himmelabach, C.K., and Andrews, H.L. 1943. Copyright 1943 by The American Society for Pharmacology and Experimantal Therapeutics.

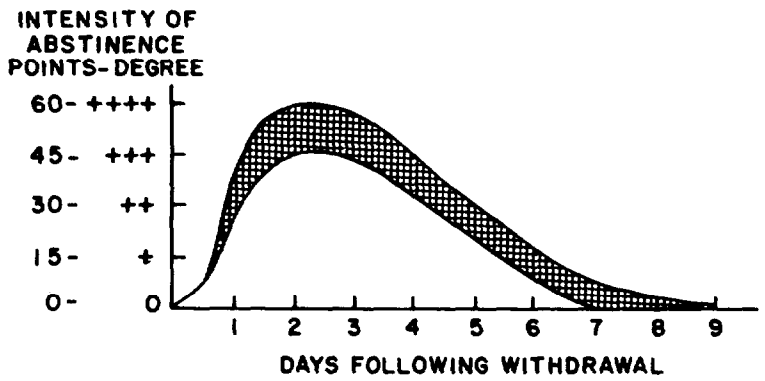


FIGURE 1

Morphine Abstinence Syndrome

Perhaps the only clinically useful compound from the research efforts of Small and his group during the 1929 to 1939 decade was metopon,1, prepared in several steps from the enol acetate of dihydrocodone (Small et al. 1939).

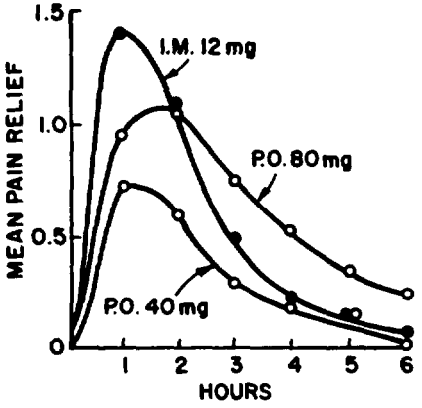
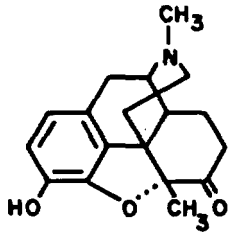


FIGURE 2

Time-Effect Curves For Oral (P.O.) And Intramuscular (I.M.) Morphine From Houde, Wallenstein and Beaver 1965. Copyright 1965 by Academic Press.

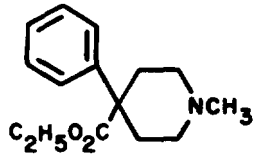


1, Metopon

This drug is about 3 times as potent as morphine and is reported to be more effective orally than morphine. It readily substitutes for morphine in stabilized addicts and is no longer available in the United States.

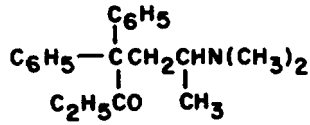
SYNTHETIC OPIOIDS

In the late 1930s. Schaumann discovered that meperidine (Demerol), 2, prepared by Eisleb (Eisleb and Schaumann 1939). as an anticholinergic agent was in fact a strong analgesic, a laboratory result that was confirmed in man. At first, it was thought that meperidine produced less physical dependence than morphine, but this was before it was appreciated that meperidine has a shorter duration of action than morphine and tolerance develops more slowly. Continued administration of high doses of the drug does produce physical dependence. Abrupt withdrawal from dependent subjects results in a withdrawal syndrome resembling that of morphine with somewhat less autonomic effects. Drug-seeking behavior does occur. The finding that a totally synthetic agent could mimic the effects of morphine led to the beginning of many research programs devoted to the synthesis of analgesics.



2. Meperidine (Demerol)

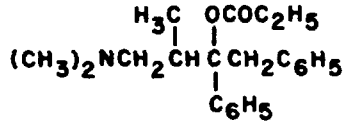
One of the more successful efforts was that of Bockmuhl and Ehrhart (1948), who synthesized methadone. 3, and many of its congeners. Methadone is an effective strong analgesic in man when administered either parenterally or orally.



3. Methadone

The drug substitutes for morphine in addicts and this property is the basis of the methadone maintenance program. The abuse potential of methadone is comparable to that of morphine.

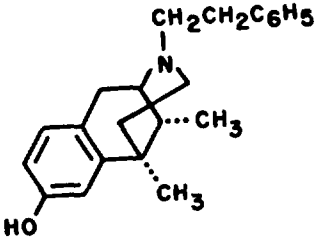
d-Propoxyphene (Darvon), 4, an analogue of methadone, in which a methylene group is inserted between the phenyl ring and the quaternary carbon, was prepared in 1953 (Pohland and Sullivan 1953). In clinical trials, it was difficult to show that the 32 mg oral dose was superior to a placebo. It was estimated that 90 to 120 mg of d-propoxyphene was equivalent to 60 mg of codeine (Beaver 1966). Combinations with aspirin give greater pain relief than either agent alone (Lasagna 1964). Because d-propoxyphene does not substitute for morphine as well as codeine, the drug was not classified as a narcotic, despite the fact that when administered the subjects recognized it as an opioid.



4. d-Propoxyphene (Darvon)

However, the drug was so irritating when administered parenterally. that its parenteral abuse was limited. Extensive experience with the drug led to the

conclusion that at equianalgesic doses the abuse potential is the same as codeine. Huge doses of d-propoxyphene resulted in deaths of some individuals. For these reasons, the drug has been placed in Schedule IV of the Controlled Substances Act.



5, Phenazocine

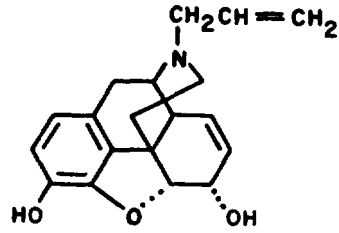
Phenazocine, 5, was synthesized in NIH laboratories by Everette May (May and Eddy 1959). It is about 10 times as potent as morphine in rodent tests and does not substitute well for morphine in dependent monkeys. This encouraging result prompted extensive clinical studies which showed that the drug was about 3 to 4 times as potent as morphine in man. Human studies at the Addiction Research Center showed that. in contrast to the findings in monkeys, 1 mg of phenazocine substituted adequately for 8.5 mg of morphine, and direct addiction studies showed that phenazocine produced a morphinelike physical dependence.

These are just a few examples which illustrate how the hopes of divesting strong analgesia from addiction liability were dashed on the rocks of experience. All the synthetic opioids which emerged from the laboratory as strong analgesics in rodent tests are, in modern terminology, mu agonists and as such were found to have morphinelike properties. The situation apppeared to be so bleak that Schaumann was prompted to write:

> It is therefore not correct to say that the depression of respiration and the constipating effect of the analgesics are side effects. They are inseparable from their analgesic action. This unfortunately is also true for the liability to cause addiction. It will, therefore, not be possible to find morphine-like analgesics without this undesirable addiction and, in fact, all efforts in this direction have been unsuccessful. (Schaumann 1956)

ANTAGONISTS AND AGONIST-ANTAGONISTS

A major development in opioid pharmacology was the finding by Unna (1943) that N-allylnormorphine, nalorphine, 6, was a morphine antagonist. Nalorphine precipitated a withdrawal syndrome in morphine-dependent subjects. Early studies in postaddicts (Isbell 1956) led to the conclusion that the drug had no dependence potential. In later investigations. it was found that after chronic administration of high doses of nalorphine, abrupt withdrawal resulted in a syndrome characterized by “electric shocks to the head,” and light headedness. as well as some other signs found in the morphine withdrawal syndrome, but the intensity of these effects were far less. There was no drug craving or drug-seeking behavior.



6, Nalorphine

Lasagna and Beecher (1954) reported that nalorphine was an analgesic in man. This finding was confirmed by Keats (Keats and Telford 1956). who found that 6 was equal in milligram potency to morphine. Psychotomimetic effects occurred in about 20% of the patients. The high incidence of unpleasant CNS side effects precluded the use of nalorphine as a clinically acceptable analgesic. The Baylor group (Telford et al. 1961) investigated a short series of narcotic antagonists as analgesics. None of these showed agonist activity in the usual rodent tests, in fact, they were antagonists. Some of the drugs proved to be potent analgesics in man, but those that were also produced undesirable CNS side effects.

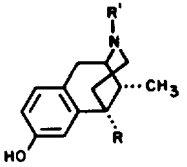
Some of us who were experiencing the frustrations in dealing with the problem of separating analgesia from addiction were following these new developments with keen interest. A critical analysis of all the pharmacology and addiction studies carried out in my laboratory in 1959 led to the conclusion that the rodent analgesic assays in use at that time were better predictors of addiction liability than of analgesic potency. At the time we initiated our studies, there were no laboratory tests which could evaluate the analgesic action of antagonists. A series of benzomorphans of varying antagonist potency were prepared and several of them were evaluated as analgesics in man by Keats (Keats and Telford 1964) and Lasagna (Lasagna and deKornfeld 1963). The results are summarized in table 2.

Cyclazocine and pentazocine were chosen for further investigation because in these early studies they produced less CNS side effects at analgesic doses than any of the other antagonists. Further studies revealed that the morphine equivalent dose of pentazocine was nearer to 30 mg than 20 mg, and that at doses of 60 to 90 mg naloxone-reversible dysphoric effects occurred. Early studies at the Addiction Research Center with pentazocine suggested that there was little, if any, abuse potential for this drug, but more extensive investigations showed that this was too optimistic a conclusion (Jasinski et al. 1970).

After chronic administration of 60 to 90 mg of pentazocine every 4 hours, postaddicts develop a withdrawal syndrome possessing some features of both the nalorphine and morphine withdrawal syndromes. The effects are milder in intensity, but drug-seeking behavior is present.

TABLE 2

Benzomorphan Antagonists In Man

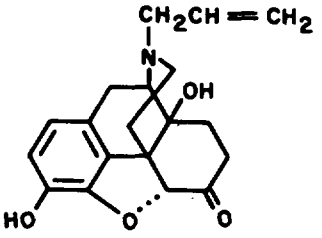


No.	Compound R	R'	Analgesic Dose(mg/70kg)	Psychoto-Mimetic Effects	Potency as Narcotic Antagonists
	CH_3	$-CH_2CH=CH_2$ (SKF-10047)	> 15	++	+++
	C_2H_5	$-CH_2CH=CH_2$	5	++	+++
	CH_3	$-CH_2CH=CHCl$ (cis)	> 2	++	+++
7	CH_3	$-CH_2C_3H_5$(c) (Cyclazocine)	0.25	+	+++
8	CH_3	$-CH_2CH=(CH_3)_2$ (Pentazocine)	20	0	+
	C_2H_5	$-CH_2CH=(CH_3)_2$	30	0	+
5	CH_3	$-CH_2CH_2CH_2C_6H_5$ (Phenazocine)	3.5	0	0
6	Nalorphine		10	++	++
	Morphine		10	0	0

Instances of abuse of the parenteral form were reported soon after the drug was made available commercially, but for many years the drug was not seriously abused by heroin addicts. About 1977, a combination of pentazocine (extracted from crushed tablets) and the antihistamine tripelennamine taken intravenously became a popular form of drug abuse in a few urban centers. In order to counteract this abuse, the manufacturers introduced an oral formulation of pentazocine and naloxone. Since the latter is a very poor antagonist when given orally but a powerful one when administered parenterally, there would be no interference with the oral efficacy of the drug but the parenteral actions would be antagonized by the naloxone. As a result, there has been a substantial reduction in the abuse of oral pentazocine.

Cyclazocine is a potent opioid antagonist which, as an analgesic, is about 40 times as potent as morphine and is highly effective when given orally. Abrupt withdrawal after prolonged administration resulted in a nalorphinelike mild withdrawal syndrome without drug-seeking behavior. Extensive clinical studies showed that although the incidence of dysphoric effects at clinically useful doses was not as high as with nalorphine, they occurred too frequently to allow the introduction of cyclazocine into clinical medicine.

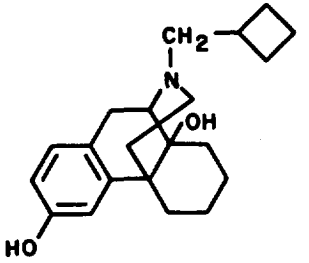
The realization that agonist-antagonist opioids can be clinically effective, strong analgesics with reduced dependence liability and limited dysphoric effects, prompted other investigators to enter the field. Naloxone, and naltrexone, the cyclopropylmethyl analog, were developed in the Endo Laboratories (Lowenstein 1964; Lowenstein and Fishman 1967; Blumberg et al. 1967).



7. Naloxone

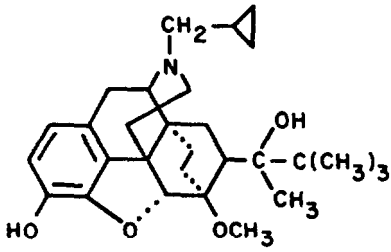
Both naloxone and naltrexone are potent opioid antagonists essentially devoid of agonist properties. Naloxone is used clinically and in the laboratory as a "pure" opioid antagonist. Nalbuphine, an analog of naltrexone differing only in having an N-cyclobutylmethyl group instead of the N-cyclopropylmethyl and an alcohol rather than a keto function at C-6, is a mixed agonist-antagonist. It has about the same milligram potency as morphine. At these clinical doses, dysphoric effects are minimal or absent and it is only when doses of 70 mg are used that unpleasant CNS symptoms appear. Chronic administration of nalbuphine followed by naloxone precipitated a mild abstinence syndrome with drug-seeking behavior. It is estimated that its abuse potential is similar to that of pentazocine (Jasinski and Mansky 1972).

Butorphanol, 8, is a synthetic analog of nalbuphine (Monkovic et al. 1972, 1975) and resembles the latter pharmacologically as well as clinically. As in the case of nalbuphine, the presence of the 14-beta hydroxyl group lowered the incidence of CNS side effects. Direct addiction studies at the Addiction Research Center revealed that the drug produced a withdrawal syndrome of mild intensity similar to other mixed agonist-antagonists.



8. Butorphanol

Buprenorphine, 9 (Lewis et al. 1971) is a derivative of thebaine. It is an agonist-antagonist whose parenteral analgesic potency is about 25 to 50 times thatof morphine. Doses of 0.4 to 0.8 mg given sublingually produce satisfactory analgesia in postoperative patients.



9. Buprenorphine

When naloxone was given to subjects who were receiving doses of 8 mg of the drug daily, no withdrawal syndrome was precipitated as in the case of other mixed agonist-antagonists. Nevertheless, abrupt withdrawal did elicit a syndrome which included drug-seeking behavior (Jasinski et al. 1978).

The original hope that mixed agonist-antagonists could be clinically acceptable strong analgesics with no abuse potential remains unfulfilled. What has been accomplished is more modest. Drugs in the potency range equal to or greater than morphine with few dysphoric effects and reduced abuse potential are now available for clinical use. From a pharmacological standpoint, this class of drugs was of immense value in leading to a far better understanding of how opioids behave at a cellular and receptor level. Their diverse actions led to the formulation of the concept of multiple opiate receptors (Martin 1967). He postulated the presence of mu, kappa, and sigma receptors to which at least one more, the delta receptor, has now been added (Kosterlitz et al. 1980). There are no highly specific ligands for each of these receptors, but compounds of varying degrees of selectivity are known. It is almost certain that a mu-specific ligand would resemble morphine so closely that the chances are virtually nil for finding a compound of this type with no addiction liability. The clinically available agonist-antagonists are mixed mu-kappa agonists. It would be of great interest to have specific kappa and delta agonists available for study.

FUTURE OUTLOOK

The seminal discovery of Hughes and Kosterlitz and their associates (Hughes et al. 1975) that the brain produces its own opioids ushered in a new area of research. Soon thereafter several new polypeptides from brain and pituitary were isolated, and a host of synthetic peptides having opioid properties were prepared in many laboratories throughout the world (Morley 1982). Much of the efforts, particularly in industrial laboratories, were directed toward finding a drug with no addiction liability, based on a tenuous hope that the human body would not synthesize compounds which would be addictive. Unfortunately, this proved to be another unfulfilled hope. Many of the opioid peptides are ineffective

analgesics when administered parenterally. In order to demonstrate opioid effects in mammals, they must be administered intraventricularly. When this was done in morphine-dependent rodents, the analgesic peptides prevented the appearance of a withdrawal syndrome.

Addiction liability is not the only problem to be solved in the development of a peptide analgesic. Intraventricular administration is not a clinically acceptable method of giving drugs. The putative peptide drug must be effective when given parenterally, preferably by the subcutaneous or intramuscular routes. If the drug were not effective when given orally or sublingually, it would be limited in value. Effectiveness is only one part of the story. Side effects, if present, must be acceptable to the patient. Finally. there is a cost factor which must be considered. In order to avoid diastereomeric mixtures, optically active amino acids must be used. In order for the cost of manufacture to be acceptable, the drug must be very potent so that the price of a dose would be economically competitive. For all these reasons, it is difficult to be optimistic that the solution to the opioid addiction problem will be found in an opioid peptide.

It is not generally appreciated that the structure and conformation of ligands are extremely important for recognition by an opioid receptor, but postreceptor activation events are independent of these factors. A great deal is known about the pharmacological events following the activation of the mu receptor because, at pharmacologically effective concentrations in vivo, morphine and some of its congeners are essentially pure mu agonists. On the other hand, very little is known about the pharmacological sequelae of activating the delta or kappa receptors because of the lack of pure kappa or delta agonists. It is essential to have such compounds because postreceptor events following activation of kappa or delta receptors probably would be different from those following mu-receptor activation. Although the optimism of my younger days has been eroded, it is for the considerations just discussed that I do not share Schaumaoo's extreme pessimism that addiction liability and analgesic action are not separable.

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