Developing Fluorogenic Reagents for Detecting and Enhancing Bloody Fingerprints

Award 2007-DN-BX-K171

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Abstract

Fingerprints are the most common and useful physical evidence for the apprehension and conviction of crime perpetrators. Fluorogenic reagents for detecting and enhancing fingerprints in blood, however, have several associated challenges. For instance, they are generally unsuitable for dark and multi-colored substrates. Luminol and fluorescin and other chemilumigens and fluorigens can be used with dark and often multi-colored substrates, but are not compatible with fixatives and their oxidation products are not insoluble. They therefore diffuse away, degrading detail. The oxidation of luminol is a fast and irreversible reaction, so the user must act quickly to observe and capture an image or it will be lost. The chemiluminescence of luminol and the fluorescence of fluorescein and other standard fluorophores are quenched in acidic media.

The main goal of the proposed Phase 1 research was to develop fluorogenic compounds for detecting fingerprints in blood that have suitable sensitivity and stability, enhance and preserve print details, and will work on dark and multi-colored substrates. Reagents and protocols were developed that preserved detail for substantially longer periods than fluorescein. Solubility and fluorescence under acidic conditions and peroxidase-specific chemistry were all successfully achieved. The proposed materials thus improved upon the current fluorescin/fluorescein and luminol systems in many respects.

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Executive Summary

1. Problem and Purpose. Fingerprints are the most common and useful physical evidence for the apprehension and conviction of crime perpetrators. Fingerprints, foot and shoe prints in blood are especially informative for crime scene reconstruction and prosecution of defendants because they are made at or about the time of the crime's being committed.

Although numerous reagents have been developed for detecting and enhancing latent prints on a variety of substrates, limitations remain:

(**i**) most are unsuitable for dark and multi-colored substrates,

(**ii**) the visualizable oxidation products of reagents such as luminol and fluorescein are not insoluble enough to prevent unwanted diffusion resulting in degradation of detail,

(**iii**) the oxidation of luminol is a fast and irreversible reaction, so the user must act quickly to observe and capture an image or it will be lost and

(**iv**) the chemiluminescence of luminol and the fluorescence of fluorescein is quenched in acidic fixing media.

Beyond the Phase 1 goals of the project described herein are the longer term issues involving the creation fluorogenic compounds and solutions for prints in blood that:

(i) exhibit high sensitivity and stability,

(ii) are practical for use with large, variable surface types,

(iii) will enhance and preserve print details,

(iv) will work on dark and multi-colored substrates,

(v) would allow for subsequent DNA typing, and

(vi) are relatively safe for users.

The Table on the following page summarizes each of the most commonly used fingerprint reagents and the issues encountered with their usage:

Table 1. Common print reagents

Compound	Use	Type of stain	Color after development	Formula	Documentation	Shelf life
Amido black Alcohol or water base Naphthol blue black	Latent prints and to enhance visible blood prints on painted surfaces	Protein	Dark Blue	HOAc/MeOH or Citric Acid/Kodak Photo Flo 600 solution	---	Indef.
Diaminobenzidine (DAB)	Latent prints and to enhance visible blood prints	Catalytic Oxid.	Brown	Sulfosalicylic acid/H_2O_2	In the presence of light background development occurs due to dye photoionization	24 h at rt, 48 h if refrig.
ABTS	Latent prints and to enhance visible blood prints	Catalytic Oxid.	Bright green	Water/H_2O_2	Fading of detail may occur within 2 weeks.	48h refrig.
DFO (1,8-diazafluoren-9-one)	Latent prints on porous surfaces	Protein	Yellow fluorescence and red-pink ridge detail	MeOH/EtOAc/HOAc	Treated porous items may become stained w/ a yellow discoloration after some time	6 months
Ninhydrin	Latent prints on porous surfaces	Protein	Purple	Acetone MeOH/IsoprOH Petroleum ether	Spraying, dipping or painting 75-80% relative humidity is needed	1 year
Comassie brilliant blue R (acid blue 83)	Latent prints and to enhance visible blood prints	Protein	Blue.	HOAc/MeOH	---	Indef.
Crowle's double stain	Latent prints and to enhance visible blood prints	Protein	Blue	HOAc/TCAA	----	Indef.
Leucocrystal violet (LCV)	Latent prints, deposited blood on non-porous surfaces, visible blood prints	Catalytic Oxid.	Purple	Sulfosaliccylic acid H_2O_2	In the presence of light background development occurs due to dye photoionization	30 days
Fluorescein	Reagent for latent bloodstains	Catalytic Oxid.	Green fluorescence	EtOH/HOAc/ H_2O_2 Water/ H_2O_2	Photographs should be taken within a few hours	Up to 24h, recomm. to prep and use fresh
Luminol	Reagent for latent bloodstains	Catalytic Oxid.	Blue glow	Water/ H_2O_2, KOH	Photographs should be taken immediately	Solution has to be prepared and used asap

Summary:

1. **Amido black and ninhydrin** form dark-colored dye complexes and have been used successfully on light colored transparent surfaces. They are not typically used on dark colored surfaces.

Ninhydrin has a low background color but is unsuitable for porous surfaces since it runs off, and either distorts the print or fails to react before detail can be photographed.

Amido black is very sensitive and works well on non-porous surfaces but its high background color (light to medium blue) compromises contrast on porous surfaces from which the stain can not be removed by rinsing.

2. **DFO (1,8-diazafluoren-9-one)** is a very sensitive dye that gives yellow fluorescence, but its use is limited, since for development the specimen has to be heated at 100 ^{o}C. Treated porous items may become tainted with a yellow discoloration after time.

3. **Luminol** has had limited success since there is only briefly appearing luminescence. It is thus hard to photograph and fails to resolve fine ridge detail.

4. **Leucocrystal violet (LCV)** is used to develop prints mainly on light-colored backgrounds. Background development however can occur under intense light due to photoionization products of the dye which may react with other substances not specific to blood. Porous materials can also strongly absorb the dye.

5. **ABTS** is a non-carcinogenic alternative to DAB. One must photograph the developed impressions within two weeks of development, as fading of the detail may occur. Some reports state a low recovery for DNA typing.

6. **Diaminobenzidine (DAB)** is highly toxic. Photographs must be taken shortly after development, due to light-induced photoionization.

Essentially each reagent exhibits advantages and disadvantages. The goal of this Phase 1 one year project was to achieve proof-of-concept in addressing common, fundamental significant challenges such as

(i) utility on dark or multicolored surfaces,

(ii) fluorescence under acidic fixative media conditions,

(iii) utility on variable porous and non-porous surfaces and

(iv) improved print detail preservation.

2. Research Design. Specific Aims:

Specific Aim 1. *The synthesis of benzofluorone fluorophores.*

Specific Aim 2. *Characterization, sensitivity, and stability studies of the benzofluorones.*

We have synthesized and evaluated a new class of xanthene dyes, the benzofluorones (Figure 1). These new dyes afforded us a successful avenue by which to address current issues in crime scene print detection.

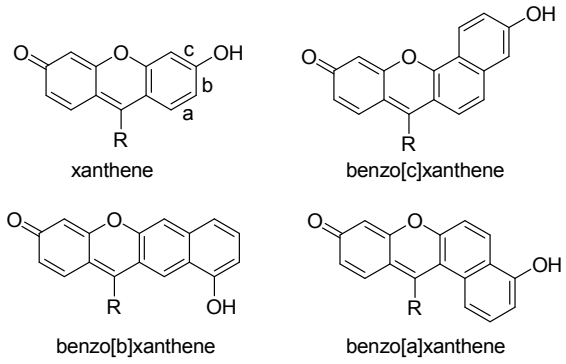


Figure 1. The xanthene dye framework (upper left, typified by fluorescein) and three types of benzoxanthenes. Benzoxanthene types are each distinguished via the orientation of their naphthyl moieties. We have synthesized the first known benzo[b] and [c]xanthenes and have prepared the benzofluorones corresponding to types a, b, and c. The benzofluorones, unlike xanthenes such as fluorescein, do not contain carboxylic acid/lactone moieties and thus are not quenched below pH = 5 (see Figure 2).

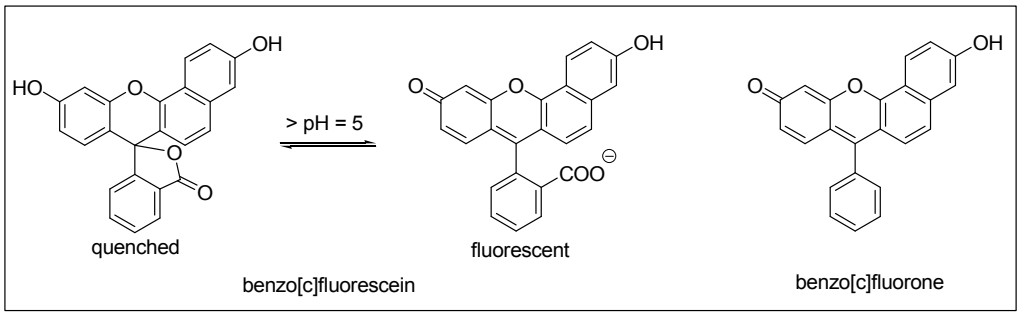


Figure 2. The difference between fluoresceins (LEFT) and the proposed fluorones (RIGHT) is the absence of the carboxylate moiety which promotes quenching of fluorescence via lactone formation in acidic media.

We have previously reported a novel synthesis of xanthene dyes (Yang, 2005). It involves the initial formation of methylated carbinol intermediates, followed by demethylation and concomitant condensation. This simple new method is inexpensive, efficient and rapid, and has led to the very first syntheses of benzo[a]- and [b]xanthene dye frameworks (Yang, 2006). The benzofluorones are structurally related to fluorescein, a material already used in bloody fingerprint detection; however, they exhibit several advantages. They are able to absorb and emit at relatively longer wavelengths, thereby reducing sample auto-fluorescence signals. Specific dyes in this new series exhibit multiple emission bands. They are pH sensitive and can absorb and emit over a very broad > 400 nm range, spanning most of the visible (and part of the UV) region.

The compatibility of a prototypical dye in the benzofluorone series with commercial UV, blue and red imaging filter sets, and several dye laser excitation wavelengths, has already been demonstrated. This latter new compound was much more photostable compared to fluorescein and exhibited no cytotoxicity in imaging studies performed to date. Additionally, the benzofluorone fluorophores are less water soluble than fluorescein. We thus expected these materials to potentially preserve fingerprint detail better than fluorescein. The benzoxanthenes synthesized in our labs were also designed to be fluorescent over a broad solution pH range whereas fluorescein is essentially colorless and poorly fluorescent below pH 5 due to its carboxylic acid/lactone pH-dependent equilibrium.

Specific Aim 1. The synthesis of benzofluorone fluorophores.

Hypothesis: Multicolor-emitting benzofluorones can be designed to be soluble and stable in acidic media and colorless until exposed to the peroxidase activity of heme at which time they become both insoluble and visible.

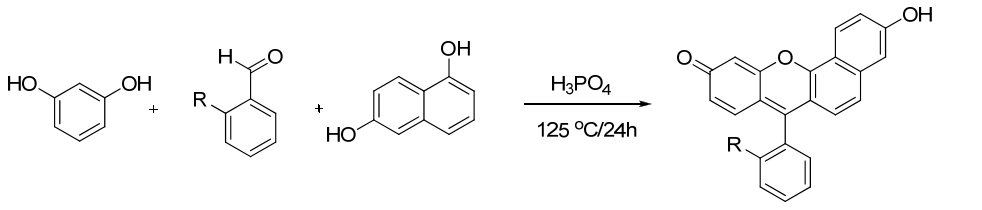
Rationale: These features would overcome the current limitations of the fluorescin (colorless dehydrofluorescein)/fluorescein system while preserving its advantages. Preliminary results from the PI's lab show that this new class of compounds is (**i**) easily synthesized from inexpensive materials in good yield, (**ii**) fluoresces in acidic media and (**iii**) is amenable to ready transformation to the corresponding pro-oxidant class of analogs.

Approach: The goal was to create and study new pro-oxidant leuco benzofluorones.

Results: Multicolor-emitting benzofluorones were attained which were soluble and stable in acidic media and colorless until exposed to the peroxidase activity of heme at which time the oxidized dyes became less soluble and visible. New leuco dye compounds were successfully and easily synthesized using zinc as the reducing agent. This was a very convenient process, analogous to that used in the common fluorescin systems. We also created new benzorhodafluor **33**.

Schemes 3 and 4 summarize the new fluorophores and their syntheses achieved during the award period:

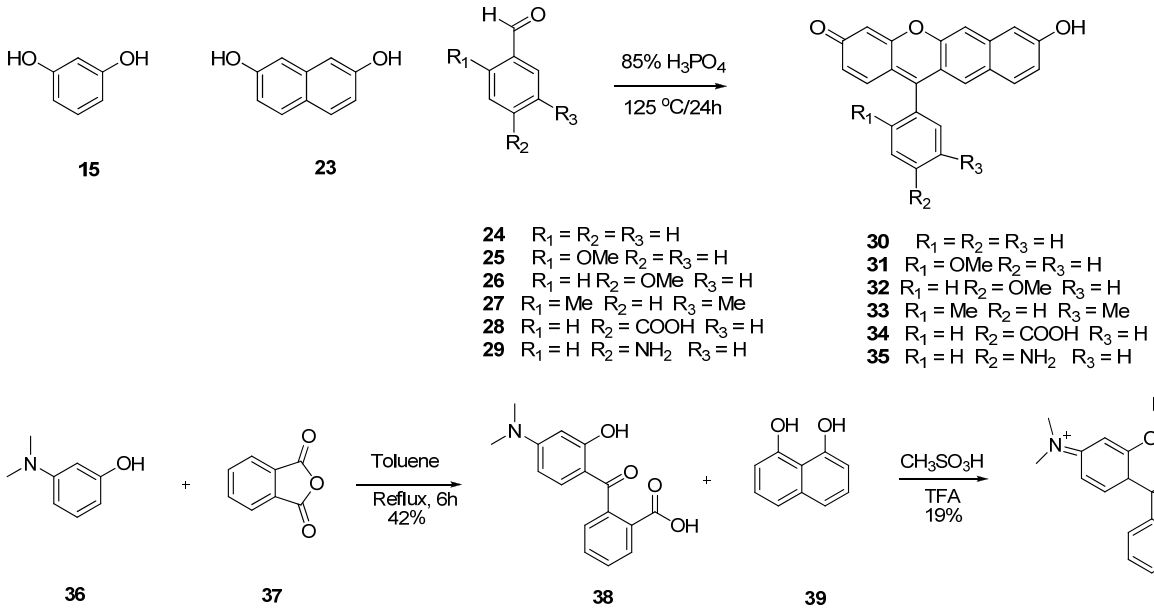
Scheme 3



15 **16** R = H, **17** R = OMe, **18**

19 R = H, 13 % yield
20 R = OMe, 15 % yield

Scheme 4



15 **23**

24 $R_1 = R_2 = R_3 = H$
25 $R_1 = OMe$ $R_2 = R_3 = H$
26 $R_1 = H$ $R_2 = OMe$ $R_3 = H$
27 $R_1 = Me$ $R_2 = H$ $R_3 = Me$
28 $R_1 = H$ $R_2 = COOH$ $R_3 = H$
29 $R_1 = H$ $R_2 = NH_2$ $R_3 = H$

30 $R_1 = R_2 = R_3 = H$
31 $R_1 = OMe$ $R_2 = R_3 = H$
32 $R_1 = H$ $R_2 = OMe$ $R_3 = H$
33 $R_1 = Me$ $R_2 = H$ $R_3 = Me$
34 $R_1 = H$ $R_2 = COOH$ $R_3 = H$
35 $R_1 = H$ $R_2 = NH_2$ $R_3 = H$

36 **37** Toluene, Reflux, 6h, 42% → **38** **39** CH_3SO_3H, TFA, 19% → **40**

Specific Aim 2. Characterization, sensitivity, and stability studies of the benzofluorones. *Hypothesis:* The initial lead candidate dyes shoild exhibit superior properties to fluorescein and luminol for the proposed applications.

Rationale: Our published data shows that specific benzofluorones created in our lab have requisite solubility and optical activity in acidic media.

Approach: The most promising lead compounds attained during Aim 1 studies would be more extensively evaluated for the following desired properties:

(i) requisite solubility and optical activity in acidic media,

(ii) solubility and stability in strong acid, and

(iii) preservation of print detail.

Although numerous fluorescent substrates have been reported to detect peroxidase activity, they are often non-specific. Hence, we evaluated our novel dyes that were indeed found responsive to peroxidase activity, according to procedures described by Laberge (Laberge, 2001). Each of the proposed Specific Aim 2 property studies entailed direct comparison of results using the benzofluorones against benchmark data obtained with fluorescein and typical fingerprint dyes. Quantitative measures of peroxidase specificity were also evaluated against the literature values for other materials (Castle, 1995; Egerton, 1957; Laberge, 2001; Sugane, 2001). Detail preservation and function in acidic print fixative media was also evaluated.

Results and Summary of Key Findings

From the screening of a series of nine new SNAFR synthetic dyes, three compounds were selected for fingerprint development based on the performance of their corresponding leuco bases when subjected to the oxidation mediated by HP (horseradich peroxidase)/H_2O_2 or commercial pig blood/H_2O_2 in solutions under varying pH conditions.

The main issues to overcome as stated in the original application included the facts that:

(**i**) most common fingerprint reagents are unsuitable for dark and multi-colored substrates,

(**ii**) the visualizable oxidation products of luminol and fluorescein are not insoluble enough to prevent unwanted diffusion resulting in degradation of detail,

(**iii**) the oxidation of luminol is a fast and irreversible reaction, so the user must act quickly to observe and capture an image or it will be lost and

(**iv**) the chemiluminescence of luminol and the fluorescence of fluorescein is quenched in acidic fixing media.

Each of these issues has been addressed during the one year study. Outstanding progress has been achieved. Highlights include:

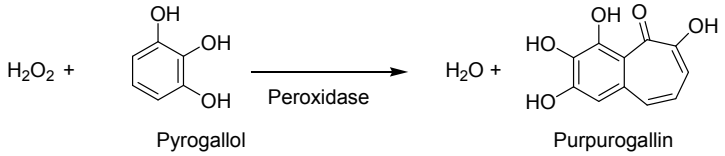
1. The oxidation reaction mediated by HP/H_2O_2 or pig blood/H_2O_2 indeed afforded fluorescent products in acidic media. This addresses issue **(iv)** above.
2. The use of NaOAc and PEG MW 35 000 as additives to the reaction mixture accelerated the oxidation reaction. It was not a goal to speed the development process. This is an observation that may be of value for future work.
3. The reduced form of the dyes is soluble at acidic pH (2-5) and is also compatible with strong protein denaturing solutions (i.e. sulfosalycilic acid, 20g/L). This addresses an important aspect of solubility properties issue **(ii**) above in that the non-visualizable leuco dye forms should be soluble during application but turn into insoluble products when oxidized and visualized to enhance preservation and detail.
4. The oxidized forms have diminished solubility [issue **(ii**)]. Formation of colloidal type precipitates were observed for oxidations carried out in solution at acidic pH. Thus improvement in detail preservation may be possible.
5. Because of their broad absorption range, fingerprints developed using these dyes were visualized over the range of 400 – 570 nm [issue **(ii**)], using alternative light sources equivalent to several dye laser excitation wavelengths. Thus the use of UV-light for visualization which can damage DNA can be avoided-a promising area for further development.
6. The oxidation products are stable, allowing one to capture detailed images after long periods of time (at least 10 months) that do not differ from those taken immediately [(issue (**iii**)].
7. Improvement in the fluorescence emission is obtained by spraying the treated fingerprint with a solution of higher pH. Promising results were obtained on pig blood fingerprint trials on glass and colored paper board using the newly-created dyes.

In summary, the new fluorophores are useful on multicolor and dark substrates in a variety of fixative media, initial detail is not degraded even after many months. The dyes found initial utility on both porous and non-porous surfaces.

I. Introduction

Statement of the problem: Fingerprints are the most common and useful physical evidence for the apprehension and conviction of crime perpetrators. Fluorogenic reagents for detecting and enhancing fingerprints in blood, however, have several associated challenges. For instance, they are generally unsuitable for dark and multi-colored substrates. Luminol and fluorescin and other chemi- lumigens and fluorigens can be used with dark and often multi-colored substrates, but are not compatible with fixatives and their oxidation products are not insoluble. They therefore diffuse away, degrading detail. The oxidation of luminol is a fast and irreversible reaction, so the user must act quickly to observe and capture an image or it will be lost. The chemiluminescence of luminol and the fluorescence of fluorescein and other standard fluorophores are quenched in acidic media. The main goal of the proposed Phase 1 research was to develop fluorogenic compounds for detecting fingerprints in blood that have suitable sensitivity and stability, enhance and preserve print details, and will work on dark and multi-colored substrates. Reagents and protocols were developed that preserved detail for substantially longer periods than fluorescein. Requisite solubility, fluorescence under acidic conditions and peroxidase-specific chemistry were all successfully achieved. The proposed materials to date thus improved upon the current fluorescin/fluorescein and luminol systems in many ways and thus are amenable for further development.

Literature citations and review: Reagents are often employed for the detection and/or enhancement of blood and bloody fingerprints. The most common methodology to visualize bloody finger prints relies on the perioxidase-like activity inherent to red blood cells. The heme group of hemoglobin catalyzes the breakdown of H_2O_2 into H_2O while a second redox active species (often dyes or stains) are concurrently oxidized. Consequently, alterations in the redox states of the dyes is observed via an optical response whereby the fingerprints can be visualized from any desired matrix (Caldwell, 2000). Benzidine and leuco dyes (e.g., such as phenolphthalin, leucocrystal violet (aka Gentian violet), and leucomalachite green (Adler, 1904), o-tolidine (Ruttan, 1912), diamino and tetramethyl-benzidine (TMB; Holland, 1974), leucorhodamine 6G (Yapping, 2004), leucoeosin Y (Wang, 2007), diaminobenzidine, and others (Gaensslen, 1983; Caldwell, 2002) have long been used with hydrogen peroxide or sodium perborate to detect blood and bloody prints via the psudo-peroxidase activity of the heme moiety of hemoglobin.



Scheme 1. Peroxidase-mediated oxidation of pyrogallol to purpurogallin (http://www.sigmaaldrich.com/img/assets/17380/second-reaction.gif).

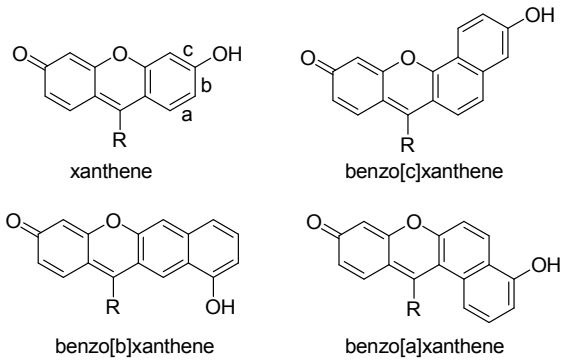


Figure 1. The xanthene dye framework (upper left, typified by fluorescein) and three types of benzoxanthenes. Benzoxanthene types are each distinguished via the orientation of their naphthyl moieties. We have synthesized the first known benzo[b] and [c]xanthenes and have prepared the benzofluorones corresponding to types a, b, and c. The benzofluorones, unlike xanthenes such as fluorescein, do not contain carboxylic acid/lactone moieties and thus are not quenched below pH = 5 (see Figure 2).

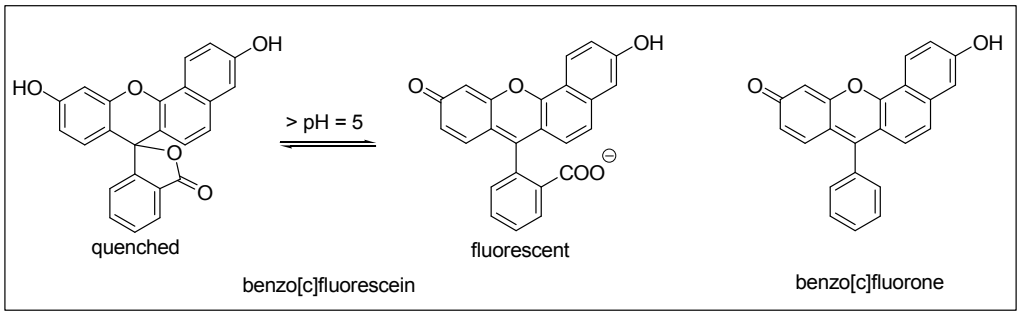


Figure 2. The difference between fluoresceins (LEFT) and the proposed fluorones (RIGHT) is the absence of the carboxylate moiety which promotes quenching of fluorescence via lactone formation in acidic media.

Chemiluminogens and fluorogens also have a long history. Luminol yields a photon during its oxidation and was first used in 1937 (Specht). Its chemiluminescence swiftly decays unless the reaction is slowed by one of several techniques. Young (2006) compared BlueStar, a proprietary formulation based on luminol and/or its homologs and reported to have significantly greater sensitivity and longer lasting chemiluminescence, with luminol (and fluorescin) for blood detection, but not for bloody fingerprint enhancement.

Lumigens are a family of acridan-based chemicals that exhibit both chemiluminescence and fluorescence in the presence of peroxide and horseradish peroxidase. They have not been assessed for detection of blood and bloody fingerprints. Generally speaking, chemiluminescence is excellent for blood detection in certain cases. However, it is not ideal for bloody fingerprint enhancement because it is difficult to estimate how much reagent to apply and difficult to ascertain appropriate image capture parameter values, including the length of time available for capture and exposure.

In contrast, chemifluorescence affords fairly precise control of parameters by excitation intensity and wavelength and emission wavelength. Time is not a significant problem and, in fact, can be exploited in time-delay techniques whereby the finite lifetimes of excited species are used for subtraction of background fluorescence (Ong, 2004; Menzel, 2004). Fluorescin (reduced fluorescein; Cheeseman, 1995) and 2,2'-azino-di-[3-ethylbenzthiazolinesulfonate (ABTS, Caldwell, 2000) have been reported for the chemifluorescent detection of bloody fingerprints. Several other fluorogenic peroxidase substrates are have been synthesized for use in biochemical and cytological studies. Quantiblue (Pierce Chemical, Rockford, IL) and Amplex Red and Oregon Green (Invitrogen, Carlsbad, CA) are commercially available. However, none of these are compatible with strong protein denaturing (fixing) solutions. Many fluorophores are also subject to photo-bleaching by intense excitation light sources and susceptible to quenching by low pH.

As mentioned above, these latter compounds enhance bloody fingerprints through their oxidation by reactive oxygen released from a peroxide (most often hydrogen peroxide or a perborate salt) by the pseudoperoxidase activity of the hemoglobin heme moiety. Additionally, the high protein content of blood lends itself to non-specific protein staining. Many researchers (Jones, 1982), coomassie brilliant blue R (Norkus, 1986), and Crowle's double stain (Becraft, 1987). Sears (2000) compared a number of general protein stains for efficacy. Two fluorescent general stains for bloody fingerprints have also been reported: acid fuchsin (aka Hungarian Red, Theeuwen, 1998), and acid yellow 7 (Sears, 2005). Amino acid detection reagentshave also been employed, the most common being ninhydrin and 1,8-diazafluoren-9-one (DFO),a fluorescent analog). A number of other ninhydrin analogs have been synthesized but not screened for bloody fingerprint enhancement, including the 1-2-indandione family (Hauze, 1998).

There are several challenges associated with the available blood print reagents and stains. In particular, non-fluorescent reagents are useful for detection and are compatible with protein fixative agents in acidic media such as sulfosalicylic acid to preserve detail, but they are not well suited to dark and multi-colored substrates. In contrast, luminol and fluorescin can be used with dark and often multi-colored substrates, but cannot be used in conjunction with fixatives for detail preservation. In fact, they are not only insoluble or stable in strong acids, but their chemiluminescence or fluorescence is quenched. Furthermore, their oxidation products are not insoluble, and so tend to diffuse away, degrading detail. Benzidine and its homolog, TMB, benefited in this regard by the insolubility of their oxidized products. Krieg (2004) has interestingly demonstrated that fluorogenic substrates can be designed to self-anchor for histological staining by covalent bonding. However, the majority of fluorogenic reagents require treatment with a separate fixing agent.

The oxidation of luminol is a fast and irreversible reaction, so the user must act quickly to observe and capture an image or it will be lost. Because of the inability to predict substrate color and the possibility of multi-colored substrates, a large range of emission wavelengths by a series of fluorphores would be useful, but a

single fluorophore capable of wavelength shifts via a simple method such as pH change, or the addition or concentration change of some cation or anion, would be especially useful.

Although not studied in detail, the effectiveness of the previously reported dyes and stains depends very much on surface type (Sears, 2005). For instance, while Acid black 1 and acid violet 17 are effective on all surfaces, acid yellow 7 is the choice of reagent for nonporous surfaces. Similarly, DFO and Ninhydrin can be employed only on porous surfaces. Hence, the study of dyes and stains on variable surfaces is of particular interest in developing more user-friendly and relatively simpler methods for bloody print detection.

In addition to seeking to detect, enhance and preserve bloody print detail, a new concern has developed about the possible interference of print detection and enhancement processes with the ability to successfully DNA type the residual blood content of the print (Lee, 1989; Laux, 1991; Fregeau, 2000; Budowle, 2000; Martin, 2004). UV light and peroxides cause of DNA damage. However, in general, most fingerprint enhancement treatments do not significantly degrade DNA. Nonetheless, any proposed new methods for detecting, fixing, or enhancing bloody prints should be validated for DNA testing.

Statement of hypothesis or rationale for the research: We have recently reported a novel synthesis of xanthene dyes (Yang, 2005). It involves the initial formation of methylated carbinol intermediates, followed by demethylation and concomitant condensation. This simple new method is inexpensive, efficient and rapid, and has led to the very first syntheses of benzo[a]- and [b]xanthene dye frameworks (Yang, 2006). The benzofluorones are structurally related to fluorescein, a material already used in bloody fingerprint detection; however, they exhibit several advantages. They are able to absorb and emit at relatively longer wavelengths, thereby reducing sample auto-fluorescence signals. Specific dyes in this new series exhibit multiple emission bands. They are pH sensitive and can absorb and emit over a very broad > 400 nm range, spanning most of the visible (and part of the UV) region.

The compatibility of a prototypical dye in the benzofluorone series with commercial UV, blue and red imaging filter sets, and several dye laser excitation wavelengths, has already been demonstrated. This latter new compound was much more photostable compared to fluorescein and exhibited no cytotoxicity in imaging studies performed to date. Additionally, the benzofluorone fluorophores are less water soluble than fluorescein. We thus expected these materials to potentially preserve fingerprint detail better than fluorescein and to function on dark and multicolored surfaces. The benzoxanthenes synthesized in our labs are also designed to be fluorescent over a broad solution pH range whereas fluorescein is essentially colorless and poorly fluorescent below pH 5 due to its carboxylic acid/lactone pH-dependent equilibrium (Figure 2). Therefore our fluorophores should function in acidic fixative media.

II. Methods

Screening of NBD- and Benzidine-Basic Dyes-HP reaction. Stock 0.05 – 0.1 % basic dye solutions were prepared in PBS pH 8.0. NBD (Naphthol Basic Dye, **13**) and benzidine **14** solutions were prepared as described in the literature (Mauro, 1985). The reaction was performed at room temperature under basic conditions using 50 mM TRIS buffer pH 8.5. Controls included the basic dye solution, the basic dye + H_2O_2, and the basic dye + HP. Reactions were monitored 15 min after addition of reagents or HP by UV-Vis and fluorescence.

Chemistry. Unless otherwise indicated, all commercially available starting materials were used directly without further purification. 1,6-Dihydroxynaphthalene was recrystallized from toluene. 1,8-dihydroxynaphthalene was synthesized as described in the literature (Yang, 2008). Silica gel Sorbent Technologies 32-63 μm was used for flash column chromatography. ^{1}H NMR was obtained on a ARX-400 Advance Bruker spectrometer. Chemical shifts (δ) are given in ppm relative to *d_6*-DMSO (2.50 ppm, ^{1}H, 39.00 ppm ^{13}C) or $CDCl_3$ (7.26 ppm, ^{1}H, 77.00 ppm ^{13}C). MS (LRMS) and ESI spectra were obtained at Mass Spectrometry Facility of Georgia State University, Atlanta, GA.

Synthesis of compound 19. Compound **19**, was prepared as described in the literature (Sibrian-Vazquez, 2009). Briefly, 1,3-dihydroxybenzene (0.5g, 4.54 mmol) and 1,6-dihydroxy naphthalene (0.727g, 4.54 mmol) are ground to a fine powder. The powder is transferred to a 100 mL round bottom flask and 25 mL of 85% phosphoric acid is added, the suspension is stirred and benzaldehyde (0.482g, 4.54 mmol) is added in one portion. The reaction mixture is heated at 125 ^{o}C for 24 h. The mixture is poured into 200 mL DI water, the precipitate is filtered and washed with water until neutral pH is attained. The solid residue is dissolved in MeOH, dried over Na_2SO_4, filtered and the solvent evaporated under vacuum, to afford a red precipitate. The target compound is isolated by flash chromatography on silica gel using EtOAc:MeOH 95:5 for elution. Yield 0.205g, 13 %. ^{1}H-NMR, ^{13}C NMR, and MS are consistent with published data.

Synthesis of compound 20: 1,3-Dihydroxybenzene (0.5g, 4.54 mmol) and 1,6-dihydroxy naphthalene (0.727g, 4.54 mmol) are ground to a fine powder. The powder is transferred to a 100 mL round bottom flask and 25 mL of 85% phosphoric acid is added, the suspension stirred and 2-methoxy-benzaldehyde (0.482g, 4.54 mmol) is added in one portion. The reaction mixture is heated at 125 ^{o}C for 24 h. The mixture is poured into 200 mL of DI water, the precipitate is filtered and washed with water until neutral pH is attained. The solid residue is dissolved in MeOH, dried over Na_2SO_4, filtered and the solvent evaporated under vacuum, to furnish a red precipitate. The target compound is isolated by flash chromatography on silica gel using EtOAc:MeOH

95:5. Yield 0.25g, 15%. ^{1}H NMR (*d*-DMSO, 400 MHz): δ (ppm) 10.57 (1H, s), 8.49-8.51 (1H, d, J = 9.08 Hz), 7.63-7.64 (1H, t, J = 8.46 Hz, J = 7.41 Hz), 7.53-7.65 (1H, d, J = 8.78 Hz), 7.30-7.34 (2H, m), 7.21-7.23 (2H, m), 7.02-7.04 (1H, d, J = 9.58 Hz), 6.93-6.95 (1H, d, J = 8.87 Hz), 6.48-6.52 (2H, m), 3.70 (3H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 183.478, 159.215, 158.168, 156.369, 149.575, 147.829, 137.537, 131.357, 130.440, 130.252, 129.848, 124.423, 123.375, 123.083, 120.944, 120.710, 119.643, 118.360, 115.993, 113.523, 111.863, 109.946, 104.454, 55.598. LR ESI (M - H^+) found 367.0, calculated for $C_{24}H_{16}O_4$ 367.1.

Synthesis of Compound 38: 3-Dimethyl amino phenol (5g, 36.44 mmol) and phthalic anhydride (5.39g, 36.44 mmol) are dissolved in 30 mL of toluene and refluxed 6 h. The solvent is evaporated under vacuum to leave a purple residue. The residue is dissolved in ethyl acetate and the mixture passed trough a plug of silica gel using EtOAc:Hexanes 1:1, EtOAc:Hexanes 3:1, and EtOAc for elution. Yield 4.32g, 42%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 12.50 (1H, s), 8.08-8.1 1H, d, J = 7.89 Hz), 7.61-7.63 (1H, t, J = 1.30, Hz, J = 7.52 Hz), 7.52-7.55 (1H, t, J = 1.31 Hz, J = 7.72 Hz), 7.34-7.36 (1H, dd, J = 1.01 Hz, J = 7.56 Hz), 6.87-6.89 (1H, d, J =9.07 Hz), 6.15 (1H, d, J = 2.48 H), 6.05-6.08 (1H, dd, J = 2.51 Hz, J = 9.07 Hz), 3.02 (6H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 198.61, 170.51, 165.12, 155.93, 141.18, 134.28, 132.79, 131.06, 129.17, 127.98, 110.28, 103.96, 97.73, 39.93.

Synthesis of Compound 40: Compound **38** (0.57g, 2 mmol), 1,8-dihydroxynaphthalene (0.48g, 3 mmol) were dissolved in 5 mL methanesulfonic acid and TFA (5 mL) is added. The mixture is heated at 80^{o}C for 2 h, and allowed to cool to rt. The mixture is poured into 200 mL water, the red solid is filtered and washed with water (3 x 100 mL) and dried under vacuum. The target compound is separated by flash chromatography on silica gel using $CHCl_3$:MeOH 95:5. Yield 0.153g, 19%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 9.12 (1H, s), 8.04-8.07 (1H, d, J = 6.75 Hz), 7.6-7.68 (2H, m), 7.44-7.48 (1H, t, J = 7.93 Hz), 7.39-7.41 (1H, d, J =8.82 Hz), 7.31-7.33 (1H, d, J = 7.25 Hz), 7.15-7.18 (1H, d, J = 6.64 Hz), 7.06-7.08 (1H, d, J = 6.69 Hz), 6.67-6.70 (1H, m), 6.49-6.54 (2H, m), 3.02 (6H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 169.529, 154.155, 153.356, 152.060, 150.784, 148.425, 136.465, 135.011, 129.713, 128.991, 128.839, 126.809, 125.064, 124.269, 123.944, 123.900, 119.522, 113.216, 112.557, 112.399, 110.062, 105.677, 99.984, 97.768, 83.231, 40.280. LR ESI (M + H^+) found 410.1, calculated for $C_{26}H_{20}NO_4$ 410.13.

Preparation of the Leuco Materials: Reduction of Dyes. Compounds **19** and **20** were reduced in both basic and acid conditions using literature (Cheeseman 1999) protocols as follows:

Dye Reduction under basic conditions: 2.5 mg of the dye was dissolved in 1.5 mL water. 25 Mg. NaOH is added, upon which an intense pink color developed. Zn (50 mg) is added and the mixture stirred at rt.

After 10 min the solution turns pale yellow, and stirring is continued 20 min. The solution is decanted and used to prepare the working solution.

Dye Reduction under acidic conditions: 2.5 mg. of the dye is dissolved in 1.5 mL EtOH. Glacial acetic acid (0.125 mL) is added, upon which an intense orange color develops. Zn (50 mg) is added and the mixture stirred at rt. After 15 min the solution turns pale yellow, and stirring is continued 15 min. The solution is decanted and used to prepare the working solution.

Fluorescein Acid Reduction: 5 mg fluorescein is dissolved in 3 mL EtOH followed by the addition of 250 µL g of glacial acetic acid, then Zn (100 mg) is added in one portion and the mixture stirred 40 min at rt. A colorless solution is obtained. The solution is decanted and used to prepare the working solution.

Fluorescein Basic Reduction: 5 mg fluorescein is dissolved in 3 mL DI water. 50 mg of NaOH is added followed by the addition of Zn (100 mg) in one portion. The mixture is stirred 30 min at rt. A colorless solution is obtained. The solution is decanted and used to prepare the working solution.

Reaction of Reduced SNAFR with Peroxidase. Peroxidase reactions of the reduced dyes were investigated using Peroxidase Type II from Horseradish EC 1.11.1.7 (HP). Working solutions of HP were prepared by dissolving 5 mg HP (181 units/mg) in 1 mL of 50 mM TRIS buffer pH 8.5 or 1 mL of 50 mM acetate buffer pH 4.0.

The oxidation reaction was carried out as follows:

1. Dye working solution: 100 µL of the reduced dye solution is diluted to 10 mL using water or EtOH.
2. 2 mL reduced dye working solution is transferred to a vial and 10 µL 0.03% H_2O_2 is added followed by the addition of 10 µL of HP working solution. The reaction is monitored by UV-vis and fluorescence.
3. The reactions of reduced dyes with H_2O_2 and HP are monitored as control reactions.
4. As a control, the reduced fluorescein (fluorescin) reaction with HP under the same conditions is also included in this study.

*Fluorescein working solution***:** 2.5 µL of reduced fluorescein-containing solution was diluted with 9.975 mL of DI water or EtOH.

Screening of the oxidation reaction of compound 22 mediated by HP/H_2O_2 or Pig Blood/H_2O_2 at acidic pH to determine conditions for leuco dye oxidation and to visualize fluorescence properties

Solutions and reagents:

Pig blood: From Lampire Biological Laboratories Cat. # 7204909

Isotonic solution, 0.9% NaCl w/v in DI water

Sulfosalycilic acid 20g/L: 2 g of sulfosalycilic acid are dissolved in 100 mL of DI water.

1:500 blood dilution: 500 µL of pig blood are transferred to a 250 mL volumetric flask and diluted to the mark with isotonic solution.

0.5% MBTH solution. 50 mg of MBTH (3-methyl-2-benzothiazolidine hydrazone hydrochloride) is dissolved in 10 mL of DI water.

10 μM PEG solution. 35 mg of PEG MW 35 000 is dissolved in 100 mL of 50 mM acetate buffer pH 4.0.

For testing: 1 mL reduced dye, 10 μL of 1:500 pig blood dilution and 10 μL of 0.03% H_2O_2 are mixed.

Blood Control: 1 mL of water, and 10 μL od 1:500 pig blood dilution

Dye control: 1 mL of reduced dye

Dye-HP-H_2O_2: 2 mL of reduced dye, 10 μL of HP solution, 10 μL of 0.03% H_2O_2.

Influence of the amount of blood on the oxidation reaction at acidic pH. The reduced working solution was prepared by dilution 100 μL of reduced dye to 10 mL using water, 50 mM acetate buffer pH 4, or sulfosalycilic acid. For the test: 1 mL of dye working solution and 10 μL of 0.03% H_2O_2 are mixed followed by the addition of different amounts (10 – 100 μL) of 1:500 pig blood dilution. Controls for blood, reduced dye and reduced dye in the presence of HP-H_2O_2 are included for comparison.

Reaction of reduced dye and MBTH with H_2O_2 at pH 7.00 to determine the effect of additives to enhance dye precipitation. Influence of the H_2O_2 concentration. (a) 1 mL of reduce dye in water, 50 μL of 0.03% H_2O_2 10 μL of HP solution, 50 μL of MBTH solution. (b) 1 mL of reduce dye in water, 50 μL of 3% H_2O_2 10 μL of HP solution, 50 μL of MBTH solution. Controls: Reduced dye, reduced dye and HP, 0.03% H_2O_2, and MBTH in HP, 0.03% H_2O_2 were included for comparison.

Reaction of reduced dye in the presence of PEG at acidic pH to determine the effect of additives to enhance dye precipitation. The reduced dye working solution was prepared by dilution of 100 μL of reduced dye to 10 mL with 10 μM PEG solution. (a) 1 mL of the reduced dye in PEG solution, 50 μL of HP solution and 50 μL of 0.03% H_2O_2 are mixed. (b) 1 mL of the reduced dye in PEG solution, 50 μL of 1:500 pig blood dilution and 50 μL of 0.03% H_2O_2 are mixed. Controls including the reduced dye and pig blood in PEG solution are included as comparison.

Reaction of reduced compound 22 with HP and Blood Pig monitored by UV-vis and Fluorescence. Reduced dye and working solution were prepared as described above. The oxidation of the reduced dye mediated by HP or pig blood in the presence of H_2O_2 were monitored by UV-vis and fluorescence. The oxidation reaction was monitored at pH 4, pH 7 and pH 9.0. 0.1M Acetate buffer pH 4.0, 0.1 M phosphate buffer pH 7.0, and 0.1 M TRIS buffer pH 9.0 were used. Mixtures were prepared as follows: 20 μL of the reduced working solution, 130 μL of 1:500 pig blood dilution, 5 μL of H_2O_2, were diluted to 2.0 mL using buffer of the desired pH. Controls including pig blood and reduced dye were included as comparison. The reaction was monitored by both UV-vis and fluorescence every 10 min, until no change was observed.

Screening of reduced SNAFR analogues reaction with pig blood/H_2O_2 in sulfosalicylic acid solution to determine optimal fluorescence properties.

Solutions:

Leuco crystal violet solution: Dissolve 2 g of sulfosalycilic acid, 0.74g of NaOAc, and 0.2g of leuco crystal violet (make sure crystals are white, if they are yellow, do not use).

1:500 Pig blood dilution: Prepare this solution as described above.

20g/L Sulfosalicylic acid: Dissolve 2g in 100 mL of DI water.

3% H_2O_2 in sulfosalicylic acid: 2 g of sulfosalicylic acid are dissolved in 100 mL of 35 H_2O_2. For testing the dyes: To 1 mL of the corresponding sulfosalycilic acid solution are added 50 µL of the reduced dye working solution and 50 µL of the 1:500 pif blood dilution. LCV concentration on the actual test corresponds to 2 mg/mL, while for reduced dye it corresponds to 0.08 mg/mL. The time for the color change and/or precipitate formation are monitored.

Fluorescence Spectroscopy. Fluorescence spectra were collected on a Cary Eclipse Fluorescence Spectrophotometer using a 1 cm path length quartz cell. Emission spectra were collected following excitation with a Xenon pulse lamp, pulsed at 80 Hz. Emission wavelengths were scanned with 2 nm step size, emission bandwidth 5 nm, excitation bandwidth 2.5 nm, the averaging time per point was 0.1 s, and 600 V was applied to a R928 PMT.

UV-vis Spectroscopy. UV-vis spectra were collected on a Cary 50 Spectrophotometer, using a 1 cm path length quartz cell, on the dual beam mode. Spectra were scanned with a 1 nm step size, the average time per point was 0.0125 seconds.

Optimization of pH conditions. pH Profiles were determined for each dye from pH 4 -9. UV-vis and fluorescence spectra were collected for each pH tested. Solutions and controls were prepared as follows: *a)* 1:500 Pig blood solution was prepared before analysis: 200 µL of pig blood [Lampire Biological Laboratories, Cat. # 7204909] were diluted in a 100 mL volumetric flask using isotonic solution (0.9% NaCl). *b)* HP solution: 5 mg of HP EC 1.11.17 was dissolved in 1 mL of 50 mM TRIS buffer solution pH 8.5. *c)* Reduced Dye-blood-H_2O_2 mixture. 20 µL of reduced dye solution, 130 µL of pig blood dilutionm 10 µL of 3% H_2O_2 solution and 1845 µL of the corresponding buffer. *d)* Reduced Dye-HP-H_2O_2 mixture: 20 µL of reduced dye solution, 10 µL of 3% H_2O_2 solution 10 µL of HP solution and 1845 µL of the corresponding buffer. *e)* Blood-H_2O_2 control: 20 µL of water, 10 µL of 3% H_2O_2 solution, 130 µL of 1:500 pig blood dilution and 1845 µL of the corresponding buffer. *f)* Reduced dye control: 20 µL reduced dye solution, 10 µL of 3% H_2O_2 solution, 130 µL of isotonic solution (0.9% NaCl), and 1845 µL of the corresponding buffer.

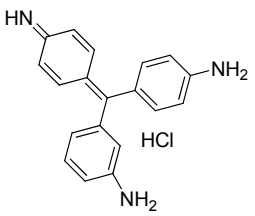
Bloody fingerprints. Commerical pig blood (10 µL) [Lampire Biological Laboratories, Cat. # 7204909] was pipetted onto the finger with a Eppendorf pipette using 250 µL tips. The blood was spread immediately with the pipette tip across the surface of the finger while the finger was held in horizontal position.

Langenburg (2008) has recently reported that for optimal detail in bloody finger-marks, approximately 10 µL of blood should be used to produce high quality bloody finger-marks when deposition is immediate (within 45 s).

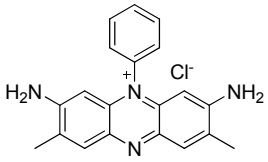
III. Results

III.1 Screening of NBD- and Benzidine-Basic Dyes-HP reaction

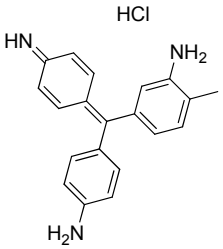
The purpose of this preliminary experiment was to screen the peroxidase mediated reaction of NBD, and DAB with the basic dyes series (compounds **1-12,** Figure 1) in solution (Scheme 2). These dyes have been used in histological work. However, the protocols for histological and fingerprint work are very different. The aim was to investigate the formation of solid/polymeric products as well as changes in the spectroscopic properties of these dyes as a result of this reaction and their stability towards H_2O_2 and HP. The formation of solid/polymeric products can be desirable in order to preserve/enhance fingerprint details. Thus, the possibility of inducing this polymerization/solid formation via either covalent or non-covalent interactions with dyes or fluorophores sensitive to peroxidase is being sought using NBD as a model system.



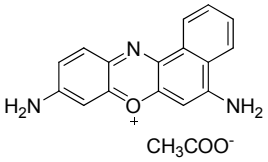
Pararosaniline, **1**



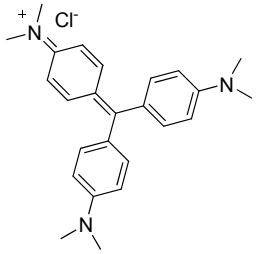
Safranine O, **2**



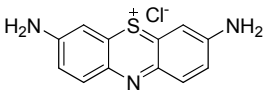
Basic Fuchsin, **3**



Cresyl violet Acetate, **4**



Crystal violet, **5**



Thionin, **6**

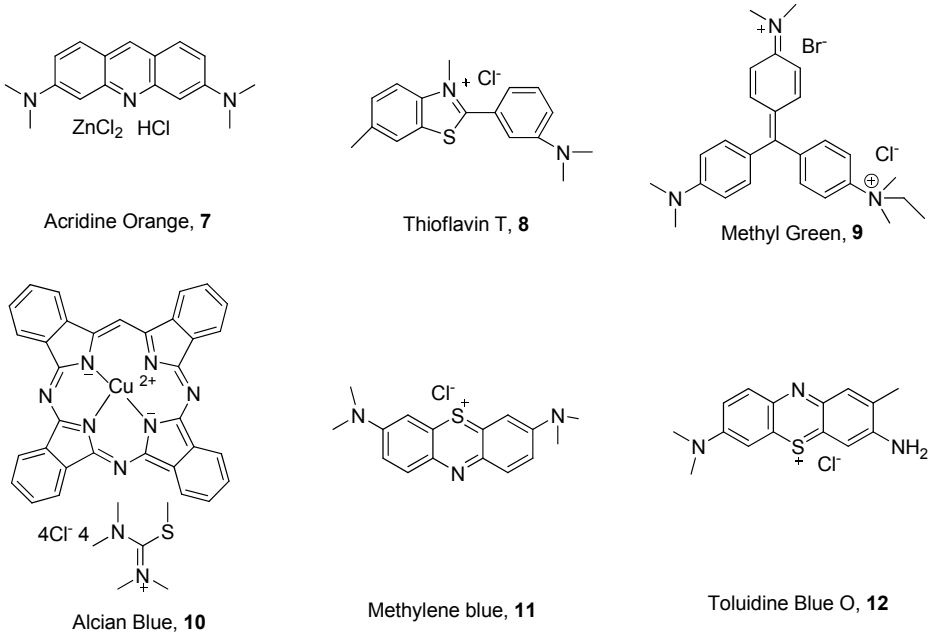
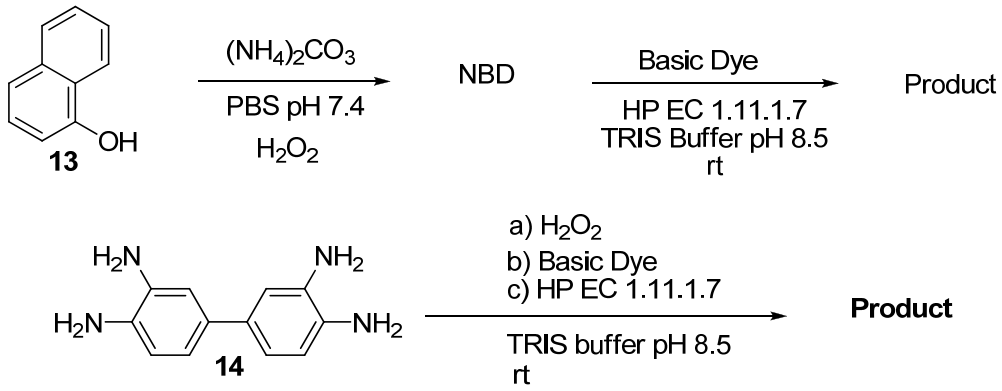


Figure 3. Chemical structures of basic dyes used for screening.

Scheme 2



Changes observed in fluorescence after adding benzidine and NBD to each of the dye solution are due to the emission of benzidine and the oxidation product, when an excitation wavelength at 280 nm is used. After addition of HP, the fluorescence of NBD and benzidine solutions is quenched. The mixtures changed color and a precipitate was formed, (entries 1 and 2, Table 3). Although a precipitate was formed for the HP reaction with Safranine O and Toluidine blue, no changes in absorption or fluorescence spectra were observed. No solid formation or changes in absorption or fluorescence were observed for Azure A (entry 12, Table 3). After addition of HP, solid formation, changes in the absorption spectra and reduction or quenching of dye fluorescence was observed for dyes in entries 3, 5, 8, 9, 10, 13, 14, 15, and 16 when NBD was present. These changes may be attributed to the formation of precipitate that reduces the dye concentration in the mixture. When benzidine was present, in general, solid formation was not observed within 15 min; however, after 24 h at rt small amount of solid precipitated from each mixture. In the

presence of NBD after addition of HP, solid formation and a slight increase in the fluorescence of Pyronine Y was observed. No major changes were observed when benzidine was used. In the presence of NBD or benzidine and after addition of HP, Methyl Green showed an increase in absorbance. Fluorescence emission is observed at 412 nm, (ex. 280 nm). A purple solid is formed in the presence of NBD and no precipitate is observed for the benzidine-methyl green mixture.

These preliminary results show that under the conditions tested, combinations of NBD-basic dyes that produce a precipitate, and enhance either absorbance or fluorescence, represent potential candidates for further studies, for ex. Pyronine Y and Methyl Green. UV-vis and fluorescence spectra for all conditions tested are given in Appendix I.

Table 2. UV-Vis and Fluorescence characteristics of basic dyes.

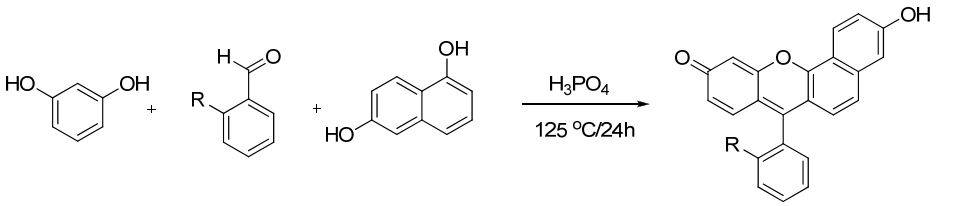
Entry	**Compound**	**Absorption Max. (nm)**	**Emision (nm) (exc. 280 nm)**
1	NBD	291	465
2	Benzidine		410
3	Basic Fuchsin Flagella	540	---
4	Safranine O	519, 275, 251	---
5	Pararosaniline	540, 286, 243	---
6	Pyronin Y	546	558
7	Toluidine Blue	630, 288	648
8	Methyl Green	632, 255, 216	652
9	Crystal Violet	590, 303, 250	590
10	Thionine	600, 283	619
11	Methylene Blue	665, 290, 246	679
12	Azure A	635, 287	647
13	Giemsa Stain		410, 540, 678
14	Cresyl Violet	585, 269	321, 623
15	Acridine Orange		530
16	Thioflavine		530

Table 3.

Entry	Compound	NBD + HP		Benzidine + HP	
		Solid	Solution	Solid	Solution
1	NBD	Purple	Purple		
2	Benzidine	---------	--------	Brown	Brown
3	Basic Fuchsin Flagella	Purple	Colorless	--------	yellow
4	Safranine O	Dark red	Pink	Red	Red
5	Pararosaniline	Purple	Colorless	--------	Light brown
6	Pyronin Y	Dark red	Red	--------	Red
7	Toluidine Blue	Dark blue	Navy blue	--------	Navy blue
8	Methyl Green	Purple	Colorless	--------	Brown
9	Crystal Violet	Purple	Purple	--------	Purple
10	Thionine	------	Purple	--------	Purple
11	Methylene Blue	Dark blue	Dark blue	--------	Green
12	Azure A	------	Dark blue	--------	Dark blue
13	Giemsa Stain	Purple	Purple	--------	Brown
14	Cresyl Violet	Purple	Purple	--------	Brown
15	Acridine Orange	Brown	Brown	Brown	Brown
16	Thioflavine	Brown	Olive-green	--------	Orange

III.2. Synthesis of SNAFR2 analogues. SNAFR-2 compound **19** and the new methoxy analog, compound **20**, were synthesized by condensation of the corresponding aldehydes with resorcinol and 1,6-dihydroxynaphthalene in 85 % H_3PO_4 at 125 ^oC/24 h. Isolation of the title compounds was achieved by flash chromatography on silica gel using EtOAc:MeOH 95:5.

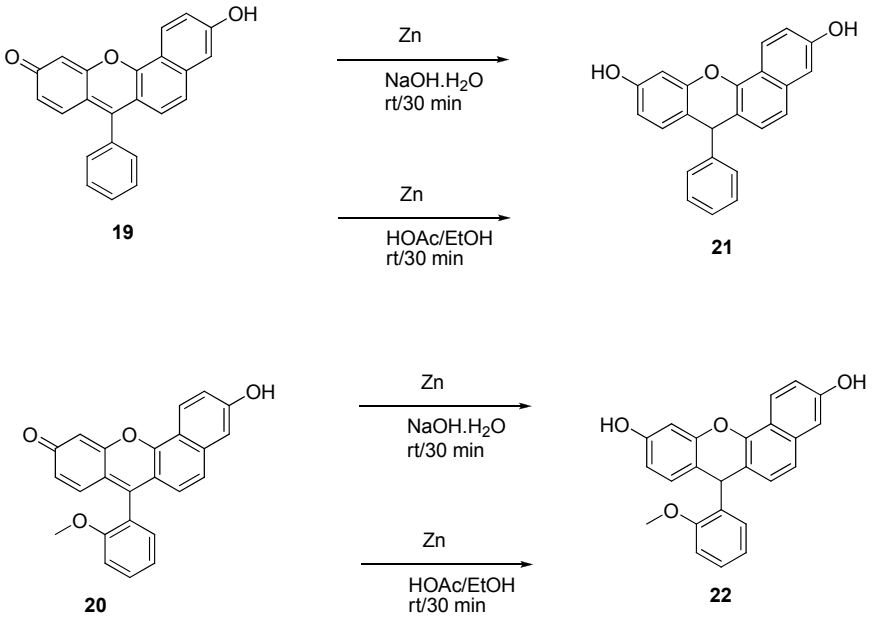
Scheme 3



15 **16** R = H, **17** R = OMe **18** **19** R = H, 13 % yield, **20** R = OMe, 15 % yield

III.3 Reduction of SNAFR2 analogs. Compounds **19** and **20** (Scheme 4) were reduced to the corresponding dihydro derivatives (leuco forms) under both basic and acidic conditions. Deep colored solutions changed to colorless or pale solutions once they are reduced.

Scheme 4



III.4 Reaction of Reduced SNAFR with Peroxidase. The peroxidase reaction with **19** and **20** and their corresponding products (**21** and **22**) from the reduction with Zn in basic and acidic conditions were investigated. Fluorescein was included in this study as a control and the reaction was evaluated using HP and H_2O_2/HP systems. Reduced **21** and **22** give pale yellow solutions in either aqueous or ethanolic media. Absorbance or fluorescence emission is observed only after reaction of the reduced dyes with HP or H_2O_2/HP. HP or H_2O_2/HP mediated oxidation of reduced dyes give fluorescence emission spectra when common Ar laser lines (488, 514, 529, 543, and 578 nm) were used for excitation. Fluorescein gives fluorescence emission only at a 488 excitation wavelength.

The kinetics of HP or H_2O_2/HP mediated oxidation of dyes reduced under basic conditions are faster as compared to the reduced products obtained in acidic media. The reaction is relatively fast, and after 20 min maximum absorbance and fluorescence emission is obtained.

While differences in the oxidation by HP or H_2O_2/HP were found for **21**, its reduced analog **22** reacts at to same extent with either HP or H_2O_2/HP. In general low absorbance and no fluorescence emission for oxidized products was observed for excitation wavelengths at 543 and 578 when the HP or H_2O_2/HP reaction was performed with the reduced products obtained in acidic conditions. Reduced compound **22** showed higher fluorescence emission as compared to reduced compound **21**. Quenching of the fluorescence emission was

observed to a small extent for both dyes; however, it was lower as compared to that observed for fluorescein. Reaction of unreduced **5** and **6** with HP or H_2O_2/HP produced quenching of the fluorescence emission after 40 min.

III.4.1 Reaction of Reduced SNAFRs with Peroxidase, UV-Vis.

The reaction of Zn-reduced dyes **21** and **22** was monitored by UV-vis. Similar protocols for evaluating other fluorophores have been reported (Setti, 1998). Results show that while compound **22** and fluorescein reduced under basic conditions react to the same extent with either HP or H_2O_2/HP compound **21** shows different kinetics when reacted with HP or H_2O_2/HP. The reaction with H_2O_2/HP is almost twice as fast as compared to HP alone. No major changes in absorbance were observed after 40 min.

III.4.2 Reaction of Reduced SNAFRs with Peroxidase, Fluorescence

The HP reaction with reduced dyes was monitored by fluorescence. Excitation wavelengths used correspond to the common Ar laser lines at 488, 514, 529, 543, and 578 nm. Fluorescence emission was monitored over a period of 40 min in all cases. Results show that after reaction of HP or H_2O_2/HP with SNAFR dyes reduced under basic conditions (compounds **21** and **22**), emission of the oxidized products is obtained for all the excitation wavelengths used (Appendix II) Higher fluorescence emission is observed for the oxidation product of compound **22**.

In contrast, the emission fluorescence of fluorescein is only observed using 488 nm excitation wavelength. Also for fluorescein, quenching of fluorescence was observed, as the emission decreases over time. This quenching is also observed for the oxidized products compounds **19** and **20**, but to a much lower extent as compared to fluorescein. Fluorescence emission for the reaction of peroxidase with the reduced dyes obtained under acidic condition (Appendix II) was in general lower than that observed for those obtained using basic conditions. HP oxidation kinetics was slower as compared to that of H_2O_2/HP. Emission intensity for the SNAFR dyes (compounds **19** and **20**) was higher as compared to the emission of fluorescein.

III.5 Screening of the oxidation reaction of reduced compound 22 mediated by HP/H_2O_2 or Pig Blood/H_2O_2 at acidic pH. The purpose of these experiments was to compare the oxidation reaction of the reduced compound **22** mediated by pig blood/H_2O_2 using the conditions previously established for the reaction with HP/H_2O_2, where a concentration of 0.03% was found to work well. The use of pig blood in similar tests has been reported (Bratkovskaja, 2004). Oxidation of reduced compound **22** at neutral pH with pig blood (dil 1:500)/ H_2O_2, proceeds slower as compared to the oxidation mediated by HP/H_2O_2. Color development appears after 15-20 min. The reduced compound **22** is not completely oxidized by pig blood (dil 1:500)/ H_2O_2 even after 12h and no formation of precipitate is observed. At pH 4 and in sulfosalicylic acid solution (SSA) with pig blood (dil 1:500)/ H_2O_2 the oxidation of compound **22** proceeds slower as compared to the oxidation mediated by HP/H_2O_2. Color development appears after 15-20 min. The reduced compound **22** is not completely oxidized by pig blood (dil 1:500)/ H_2O_2 even after 12 h; however, the formation of an orange precipitate is observed after

2-3 h. The precipitate formed is a very fine solid with colloidal appearance, once it is re-suspended in the solution it takes more the 1 h to completely settle out again.

Experiments using additives that may promote precipitate formation were carried out. For this purpose MBTH and PEG were used. It has been reported in the literature that HP catalyses the oxidative coupling of MBTH with methoxy-phenols to form red compounds. It has been reported that the introduction of PEG and other globular polymer may act as nucleation centers for the polymers formed during the oxidation of 1-naphtol mediated by HP (Caldwell, 2000). Oxidation of reduced compound **22** in the presence of MBTH by HP/H_2O_2 in water, produces the formation of an orange-brown suspension, after more than 12 h, a brown precipitate settles out. On the other hand, addition of 10 μM PEG (MW 35 000) in acetate buffer pH 4 improves the oxidation of reduced compound **22** mediated by pig blood/ H_2O_2 as shown by color development after 2 min. However, oxidation is still diminished as compared to the oxidation with HP/H_2O_2. Precipitate formation is observed for the reaction with HP/H_2O_2 after 6 h.

Overall, the reaction with blood/H_2O_2 was slower than with HP/H_2O_2. Slight visual color changes appear after addition of different amounts of blood and color development is time dependent on the amount of blood added, so it is hard to get a concentration correlation based on these subtle visual changes.

The most important finding from these experiments is the formation of a fine orange precipitate that forms upon oxidation and precipitates in acidic media. This should be desirable in order to enhance and preserve fingerprint detail. These precipitates correspond to oxidized dye, not to denatured protein from the blood, since no orange precipitate was observed on controls containing only blood. Moreover, aqueous solutions of this dye are orange colored in acidic media. Solubility tests have shown that the dye is partially soluble in aqueous solutions at pH 4. Fluorescence spectra of these solutions show significant signal loss as a result of precipitate formation.

Moreover, it is known that PEG is an amphiphilic molecule that associates with water. Previous studies have shown that each -O-CH_2CH_2- moiety in PEG is associated with 2-3 molecules of water. However, its concentration is relatively low, 10 μM, and it was incorporated in the solution to act as nucleating agent as has been reported for the oxidation of 1-naphthol mediated by HP. No apparent gel formation was observed in the solutions containing PEG.

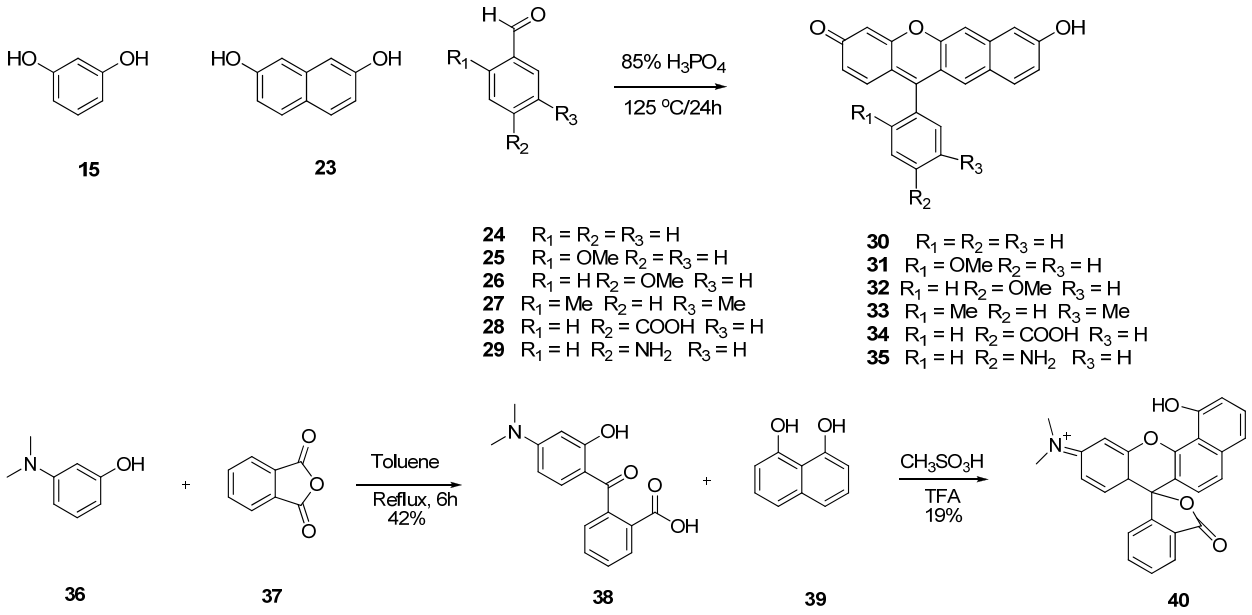
III.6 Reaction of reduced compound 22 with HP and blood pig monitored by fluorescence. The HP reaction with reduced compound **22** at pH 7.0 and in the presence of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) was monitored by fluorescence. Excitation wavelengths used correspond to the common Ar laser lines at 488, 514, 529, 543, and 578 nm. Fluorescence emission was monitored over a period of 40 min in all cases. Results show that after reaction of HP/H_2O_2 with reduced compound **22** under basic conditions, emission is obtained for excitation wavelengths at 488, 514, 529, and 543. However, when MBTH is

present, no fluorescence emission is observed at all excitation wavelengths used. This may indicate that no free dye is in solution due to the precipitate formed after the reaction occurs.

The oxidation reaction of reduced compound **22**, was also monitored by fluorescence in acetate buffer pH 4, phosphate buffer pH 7.0 and in sulfosalicylic acid (20g/L). Fluorescence emission was pH dependent, the higher emission was observed at pH 7.0, followed for the one at pH 4.0. Emission in SSA was rather low, but that may be the result of the reduced amount of free dye in solution due to precipitate formation. No significant contribution of the hemin present in the blood was observed and emission of the reduced dye was always lower as compared to those conditions when pig blood was present.

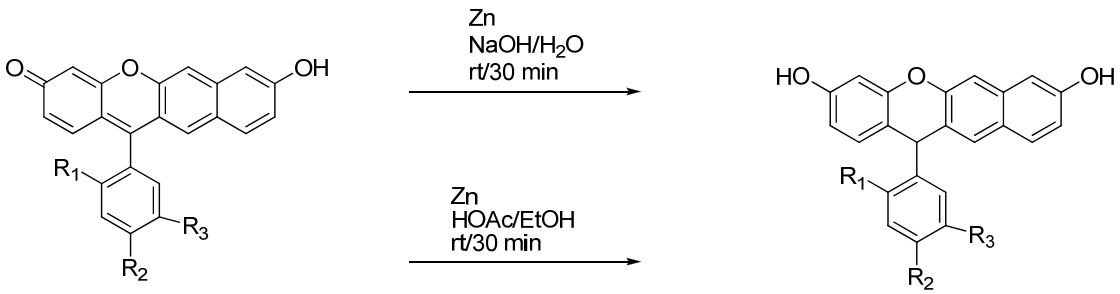
III.7 Synthesis of SNAFR-6 analogues. New SNAFR-6 analogues (Scheme 5) were synthesized by condensation of the corresponding aldehydes with resorcinol and 2,7-di-hydroxy naphthalene in 85 % H_3PO_4 at 125 ^oC/24 h. Isolation of the title compounds was achieved by flash chromatography on silica gel using EtOAc:MeOH mixtures. Yields obtained were in the range of 4 – 22%. Compounds **30** – **35** were characterized by ^1H NMR, ^{13}C NMR, and MS. A new semi-naphtorhodafluor **40** was synthesized by condensation of the carboxy-amino ketone **38** with 1,8-dihydroxynaphthalene using a mixture of methanesulfonic acid:TFA 1:1 at 80 ^oC, (Scheme 5).

Scheme 5



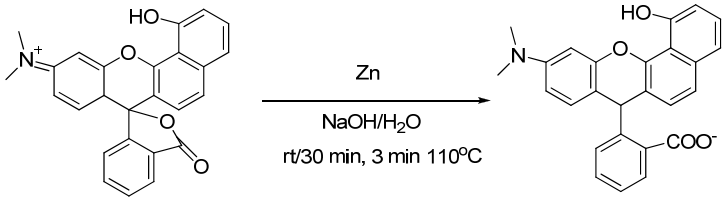
III.8 Reduction of SNAFR analogues. As shown in Scheme 6, SNAFR-6 analogues **30** – **35** and **40** were reduced under basic conditions as described in the literature.[4] The initial deep colored solutions of the corresponding dyes changed to pale yellow after reduction occurred.

Scheme 6



30 $R_1 = R_2 = R_3 = H$
31 $R_1 = OMe\ R_2 = R_3 = H$
32 $R_1 = H\ R_2 = OMe\ R_3 = H$
33 $R_1 = Me\ R_2 = H\ R_3 = Me$
34 $R_1 = H\ R_2 = COOH\ R_3 = H$
35 $R_1 = H\ R_2 = NH_2\ R_3 = H$

41 $R_1 = R_2 = R_3 = H$
42 $R_1 = OMe\ R_2 = R_3 = H$
43 $R_1 = H\ R_2 = OMe\ R_3 = H$
44 $R_1 = Me\ R_2 = H\ R_3 = Me$
45 $R_1 = H\ R_2 = COOH\ R_3 = H$
46 $R_1 = H\ R_2 = NH_2\ R_3 = H$



40 **47**

III.9 Screening of reduced SNAFR analogues reaction with Pig Blood/H_2O_2 in sulfosalicylic acid solution. Reaction of reduced SNAFR-2 and SNAFR-6 analogs (compounds **21**, **22**, and **41** – **46,** 0.08 mg/mL) with pig blood was compared to the standard reaction of Leuco Crystal Violet (LCV) at a 2 mg/mL concentration in solution. The results are summarized as follows (a) Reaction with Leuco Crystal Violet (LCV) is fast, color change is observed within 1-2 min. (b) Reaction with reduced compound **22** is faster as compared to other reduced compounds tested in this series, color change is observed after 4 min, but then a precipitate starts forming after 10 min. The reactivity order based on visual observation of color development was **22** > **21** > **41, 42** > **43, 44** > **45, 46**. (c) The reaction in the presence of NaOAc in the sulfosalicylic solution is faster.

III.10 Optimization of pH. In order to determine optimal pH for the oxidation reaction mediated by blood in solution, the reaction was monitored for each of the selected dyes by UV-Vis and fluorescence at a pH range from 4-9 (Figure 4). Compounds tested correspond to reduced dyes **21**, **22**, and **47** obtained under basic reduction conditions of **19**, **20** and **40**. While UV-Vis spectra show that reaction proceeds a higher rate at acidic pH with maximums between 4.5 and 5.6 for all compounds tested, fluorescence spectra shows that optimal conditions for fluorescence emission from the oxidized products are slightly shifted to higher pH. This can be attributed to the different equilibria involved in solution for these dyes as have been reported. These results also

show that a small contribution of the reduced dyes is observed and that the signal from the blood at the excitation wavelengths is also minimum or negligible. It is interesting to note that while the oxidation products of compounds **21** and **22**, show an increased fluorescence emission at higher pH, the fluorescence emission for the oxidation product from compound **40** decreases or is minimal. The oxidation reaction mediated by H_2O_2-HP was included as a control. UV-vis spectra show that this reaction occurs to a higher extent as observed by the higher absorbance obtained; however, fluorescence emission is only observed at pH 9 for the oxidation products of compounds **21** and **22**.

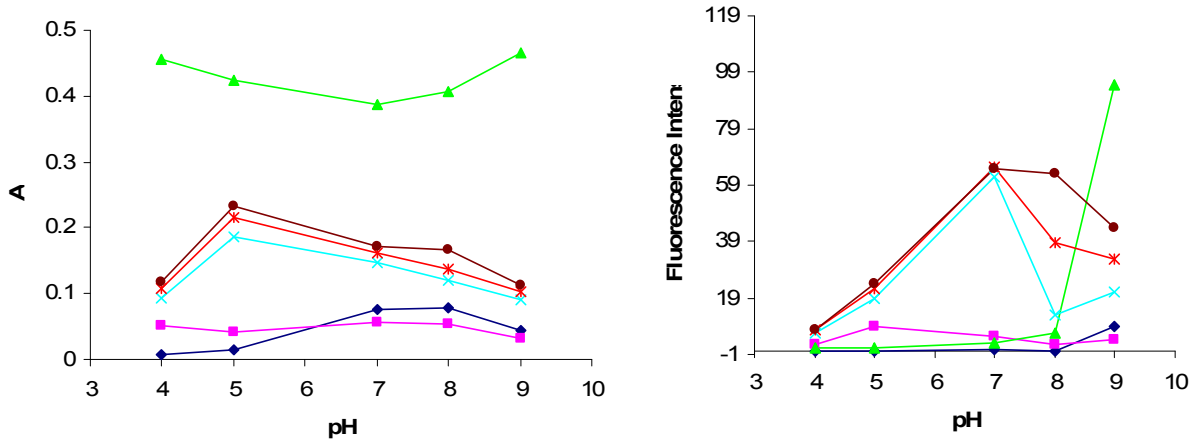


Figure 4. pH profile for reduced compound **21**. Left: determined by UV-vis. Right (fluorescence). Black; blood-H_2O_2, magenta; reduced dye- H_2O_2, green; reduced dye- H_2O_2-HP, blue, reduced dye-pig blood- H_2O_2, 10 min, red; reduced dye-pig blood- H_2O_2, 20 min, brown; reduced dye-pig blood- H_2O_2, 30 min

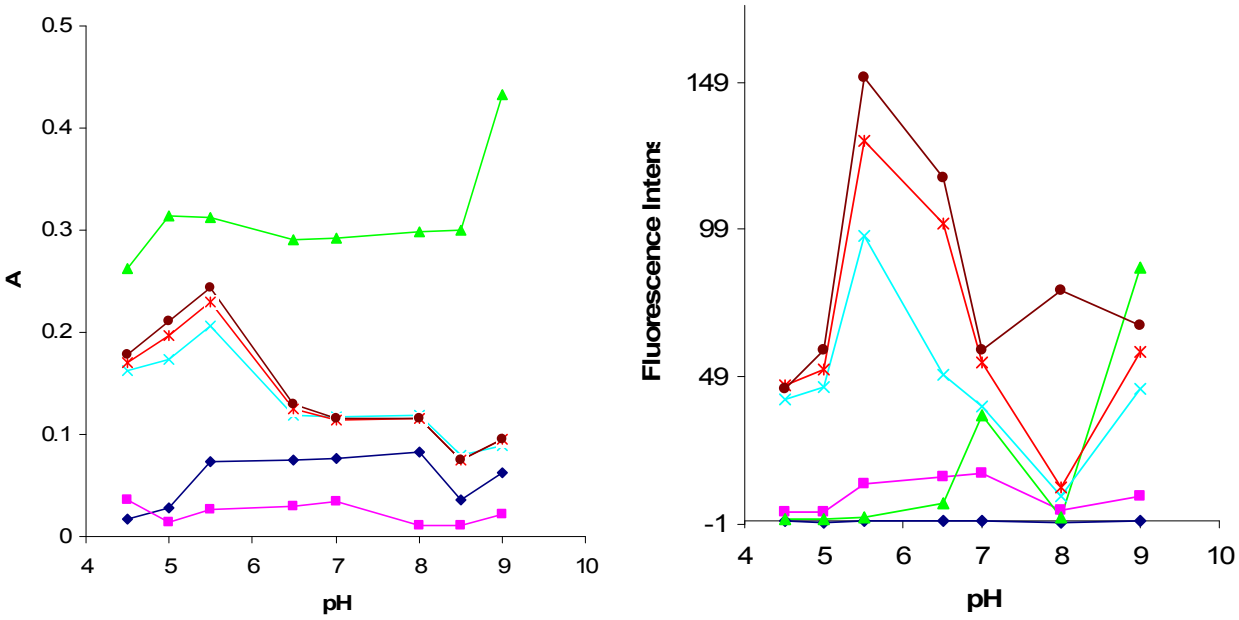


Figure 5. pH profile for reduced compound **22**. Left: determined by UV-vis. Right (fluorescence). Black; blood-H_2O_2, magenta; reduced dye- H_2O_2, green; reduced dye- H_2O_2-HP, blue, reduced dye-pig blood- H_2O_2, 10 min, red; reduced dye-pig blood- H_2O_2, 20 min, brown; reduced dye-pig blood- H_2O_2, 30 min

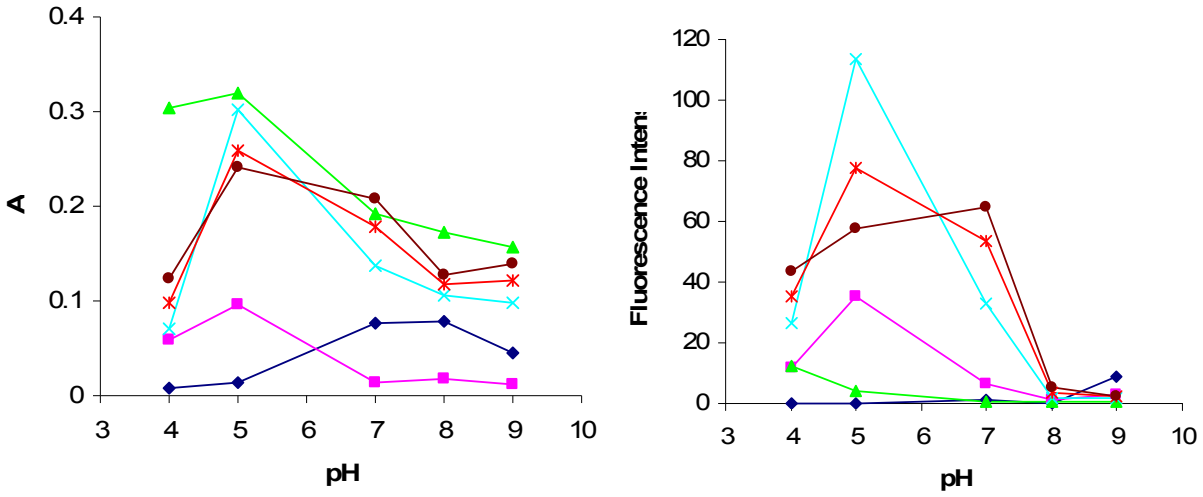


Figure 6. pH profile for reduced compound **47**. Left: determined by UV-vis. Right (fluorescence). Black; blood-H_2O_2, magenta; reduced dye- H_2O_2, green; reduced dye- H_2O_2-HP, blue, reduced dye-pig blood- H_2O_2, 10 min, red; reduced dye-pig blood- H_2O_2, 20 min, brown; reduced dye-pig blood- H_2O_2, 30 min

III.11 Screening of SNAFR analogues fluorescence in surfaces (Filter paper)

Control experiments. 10 µL of a 60 µM dye solution in MeOH (compounds **19**, **20**, **30-35**, and **47)** was spotted on filter paper dried. Fluorescence emission was screened using an ALS (alternative light source) at different wavelengths: 395, 455, 470, 505, 530, 590, and 625 nm. SNAFR analogues show yellow, orange, and pink fluorescence under these conditions. Better wavelengths for excitation were 455 nm, 470 nm, 505, and 530 nm. The use of yellow, orange, and red filters enhanced fluorescence visualization.

III.12 Sensitivity of SNAFRs on Filter Paper

Based on the fluorescence emission observed for each dye in this series and their behavior in the redox reaction mediated by pig blood in solution, compounds **19**, **20** and **40** were selected for further testing for blood fingerprint development. Oxidation of reduced **21**, **22**, and **47** on filter paper was tested using different pig blood dilutions (1:100, 1:200, 1:300, 1:400, and 1:500). 10 µL of the blood dilution were deposited on filter paper and air dried. The test was performed after 2h. The reduced dye solution was sprayed and reated 3 min, then 3% H_2O_2/EtOH was sprayed. Colored spots developed a few seconds after the H_2O_2 spray was applied. While reduced compounds **21** and **22** develop a peach spot, reduced compound **47** develops a dark pink spot. On the other hand, while background starts developing after 25 min for compound **47** (purple color on the filter paper surface), background development is observed for compounds **21** and **22** after 8 h. The blood-dye redox reaction is visualized using an ALS at 455 nm and with the aid of yellow, orange and red filter barriers. ALS at 395 and 470 nm showed similar patterns, ALS at 505 and 530 nm gave visible results with the aid of a red filter.

III.13 Blood fingerprint development on surfaces (glass and paper)

Blood fingerprints were deposited on glass microscope slides, gray and black cardboard using pig blood. 10 μL of pig blood was loaded on the finger and the fingerprint impression was made after 20-30s on each surface. The blood fingerprint was allowed to dry 2-4 h before any test was performed.

III.13.1 Blood fingerprint development on glass surfaces

From the several conditions tested, it was found that better fingerprint development was obtained when the reduced dye in a sulfosalicylic acid/sodium acetate/PEG solution is sprayed and reacted for 3-5 min, after which a spray of 3% H_2O_2/EtOH is applied and allowed to dry. Enhancement of the color/fluorescence for compounds **21** and **22** is obtained by applying a final spray with a 50 mM phosphate buffer solution pH 7.00. Fingerprint detail is observed when an ALS at 455 nm is used and visualized using different filter barriers and remains practically unchanged after 24h. Fluorescence emission for the oxidation product of compound **21** is also observed using an ALS at 505 nm and a red filter. For compound **47**, fingerprint development occurs at acidic pH, no improvement was observed after spraying with buffer solution of pH ranging from 5-9; however, it was observed that delaying the 3% H_2O_2/EtOH spray by 20 min after the reduced dye is applied, enhancement of the developed fingerprint is obtained. As observed with compounds **21** and **22**, the fluorescence emission of the developed fingerprint remains also unchanged after 24h.

III.13.2 Blood fingerprint development on paper surfaces

Enhancement of blood fingerprints deposited on gray and black cardboard was also evaluated under the same conditions as described above using the reduced compounds **21**, **22**, and **47**. On gray paper only the reduced compound **22** gives good results, fluorescence emission is observed using an ALS at 455 nm and with the aid of an orange and red filters; although reduced compound **21** also shows some fluorescence, no detail is observed. On black paper only partial areas of the fingerprints were developed for each of the compounds tested, this may be the result of the slow diffusion of the reagent into the deposited blood. However, detail is observed with all of them, and the fluorescence emission remains unchanged after 24 h, (not shown).

III.14 Procedure for blood fingerprint development using SNAFR analogues

III.14.1 Chemicals

1. Sodium Acetate anhydrous Fluka, Cat. 71185
2. Zinc mossy, Fisher, Cat, Z11-500
3. Polyethylene glycol 35 000, Fluka, Cat. 94646
4. 5-Sulfosalicylic acid di-hydrate, Fisher Cat. A297-100
5. Zinc powder, Riedel-deHaen, Cat 14409
6. Sodium Hydroxide, pellets Fisher S320
7. Sodium phosphate monobasic, NaH_2PO_4, Fisher, Cat BP 329500
8. Ethanol denatured, histological grade, Fisher Cat. CDA19

9. 30% Hydrogen Peroxide, Aldrich
10. DI water
11. Compound **19**, $C_{23}H_{14}O_3$ MW 338.36
12. Compound **20**, $C_{24}H_{16}O_4$, MW 368.38
13. Compound **40**, $C_{26}H_{20}NO_4$ MW 410.44

III.14.2 Equipment

1. Vials (8 and 20 mL)
2. 250, 1000 mL beakers
3. 100 and 1000 mL Volumetric flasks
4. Scale
5. Magnetic stirrer and stir bars
6. TLC Chromist type spryers; PREVAL spray unit, Aldrich Cat. Z365556
7. Oil bath at 110^{o}C (optional)
8. pH meter
9. AlS kit, **megaMAXX™** from Arrowhead Forensics Cat. A-6986 containing the following: 395nm megaMAXX™ UV Light w/5 LEDs, 455nm Light, 470nm Light, 505nm Light, 530nm Light, 590nm Light, 625nm Light, White Light, Light Diffuser, Tripod, FAL208 Yellow Goggles, FAL207 Red Goggles, BMS300 Orange Goggles, 797GV UV Protective Spectacles, TIFFEN 52 mm orange 16 Barrier Filter, TIFFEN 52 mm red 25 Barrier Filter, TIFFEN 52 mm 8 yellow 2 Barrier Filter.
10. Canon, PowerShot S515 Digital Camera.

III.14.3 Solutions

III.13.4.1 3% H_2O_2/EtOH

10 mL of 30% H_2O_2 are diluted to 100 mL using Ethanol.

III.13.4.2 Sulfosalicylic acid:sodium acetate:PEG solution.

2.0 g	sulfosalicylic acid di-hydrate
0.74 g	sodium acetate anhydrous
0.035 g	Polyethylene glycol MW 35 000
100 mL	DI water

Reagents are weighed, transferred to a 250 mL beaker, and dissolved in 100 mL of DI water.

III.13.4.3 50 mM Phosphate Buffer pH 7.0

1. Dissolve 7.1g of NaH_2PO_4 in approx. 900 mL of distilled water.

2. Titrate to pH 6.99 at the lab temperature of 25^{o}C with 0.1N NaOH.

3. Transfer to a 1000 mL volumetric flask and dilute to the mark with distilled water. The buffer will be pH 7.00 when used at 25 ^{o}C

III.13.4.4 Dye Stock Solution

5 mg	Dye
50 mg	Sodium Hydroxide
100 mg	Zinc powder
2.5 mL	DI water

5 mg Dye is transferred to an 8 mL vial and suspended in 2.5 mL of DI water. 50 mg NAOH is added to the dye suspension. The mixture is stirred until the dye dissolves completely (1-2 min), then 100 mg of Zn are added and the vial is capped. The solution turns from a dark pink or dark green to a pale yellow color after 5 minutes of stirring. Stirring is continued for additional 30-40 min. Reduction can be speeded up by bringing the mixture to boil for 3 min by placing the uncapped vial in a oil bath at 110^{o}C, it is removed from the oil bath and stirred until it cools down to room temperature. The stock solution is kept over Zn dust, if a darker color develops, the mixture is stirred 1-2 min (it will turn pale yellow again) before preparing the working solution.

III.13.4.5 Dye working solution

The reduced spraying dye solution is prepared by pipetting 0.4 mL of the stock solution and diluting to 10 mL using the sulfosalicylic acid:sodium acetate:PEG solution. The final dye concentration is 0.08 mg/mL. A cloudy solution may form. The working solution is best when used within 30 min and should be discarded after the spraying is done.

III.13.4.6 Reagent sensitivity

Prepare a series of blood dilutions (1:100, 1:200, 1:300, 1:400, 1:500, 1:1000). Transfer 10 µL of each dilution to a filter paper and let it dry (1-2h). Spray with the working reduced dye solution, wait 1-2 min, then overspray with 3% H_2O_2/EtOH solution. Peach or dark pink colored spots depending on the dye used, develop after a few seconds. Fluorescence and/or background development is visualized using an alternate light source ALS (455 nm, 470 nm, 505, or 530 nm) with the aid of yellow, orange, or red filter barriers.

III.13.4.7 Spraying Procedures

III.13.4.7.1 Procedure A

1. The target is slightly mist with the reduced dye solution and it is let to react 3 min.
2. Spray the target with 3% H_2O_2/EtOH.
3. Overspray with 50 mM Phosphate buffer pH 7.0 solution.

III.13.4.7.2 Procedure B

1. The target is slightly mist with the reduced dye solution and it is let to react 3 min.
2. Spray the target with 3% H_2O_2/EtOH.

III.13.4.7.3 Procedure C

1. The target is slightly mist with the reduced dye solution and it is let to react 20 min.
2. Spray the target with 3% H_2O_2/EtOH.

III.13.4.8 Visualization of developed target

Developed target is visualized using an alternate light source ALS (455 nm, 470 nm, 505, or 530 nm) with the aid of yellow, orange, or red filter barriers.

III.13.4.9 Documentation

Photographs are taken with a digital camera Canon, PowerShot S515 Digital Camera: supermacro mode, manual focus, flash off, ISO 400 or 800 depending on the substrate and using yellow, orange or red filters in front of the lens.

IV. Conclusions

From the screening of a series of nine SNAFR analogues, three potential compounds were selected for fingerprint development based on the performance of their corresponding leuco bases when subjected to the oxidation mediated by HP/H_2O_2 or pig blood/H_2O_2 in solution at different pH.

1. The fluorescence emission observed at acidic pH made them suitable for further testing.
2. The use of NaOAc and PEG MW 35 000 as additives on the reaction mixture accelerated the oxidation reaction.
3. While the reduced form of these dyes is soluble at acidic pH (2-5) and compatible with strong protein denaturing solutions (i.e. sulfosalycilic acid, 20g/L), the corresponding oxidized form is partially soluble, exactly as targeted. Formation of colloidal type precipitates were observed for

oxidations carried out in solution at acidic pH. Thus it was observed that improvement of detail was achieved.

4. Because of their long wavelength absorption range, fingerprints developed using these dyes was visualized in the range of 400 – 570 nm, using alternative light sources equivalent to several dye laser excitation wavelengths. Thus the use of UV-light for visualization which can damage DNA is avoided.
5. The oxidation products are stable, allowing one to capture images after long periods of time (at least 8 months).
6. Improvement of fluorescence emission is obtained by spraying the treated fingerprint with a solution at higher pH.
7. Promising results were obtained on pig blood fingerprint trials on glass and colored paper board using these new SNAFR analogues. A protocol using the practical spraying technique was developed.

Future work

a) Validation of results using human blood.

b) Minimization of background. Dye structure can be modified in order to achieve this goal.

c) Effects of other additives to improve diffusion of reagent within blood fingerprints.

d) Evaluate the influence of potentially interfering species (metals, myoglobin, plant materials, etc).

e) Evaluate the effect/compatibility of other amino acid detection agents (ninhydrin, DFO, 1,2-indanedione) currently used for latent fingerprint.

f) Evaluate the effect of these dyes on subsequent DNA analysis.

g) Improvement of detail and fluorescence intensity

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Appendix I

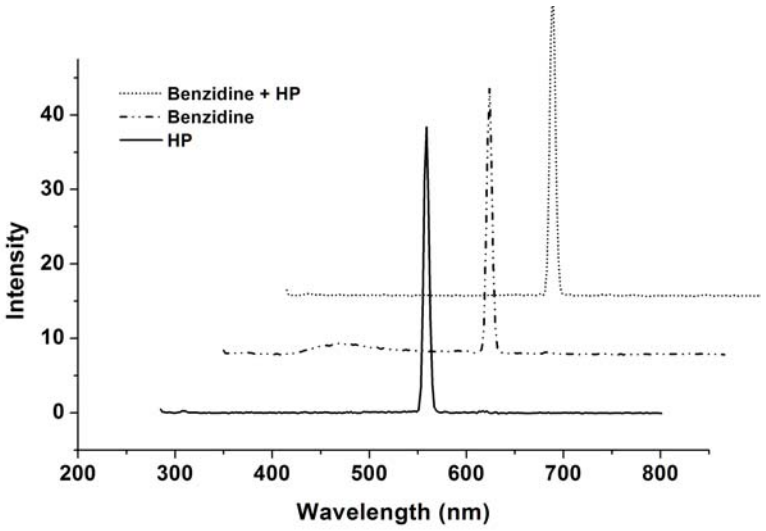


Figure 1. Fluorescence spectra for reaction of DAB, **14** with HP EC 1.11.1.7, exc 280 nm.

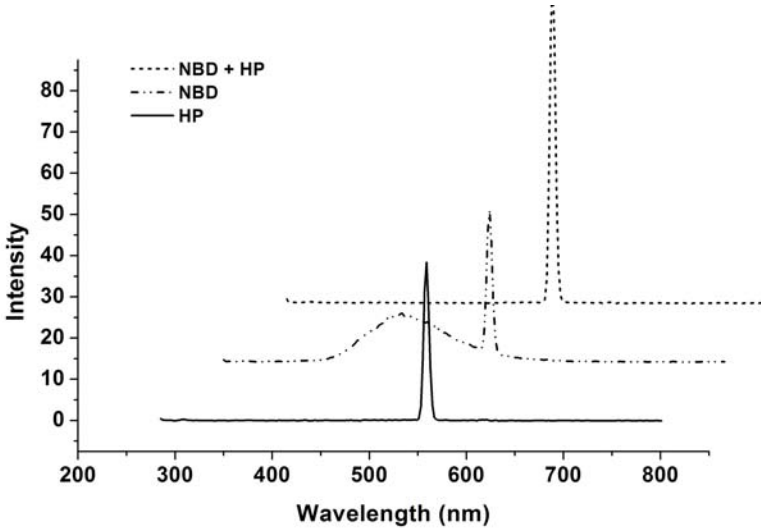


Figure 2. Fluorescence spectra for reaction of NBD with HP EC 1.11.1.7, exc 280 nm

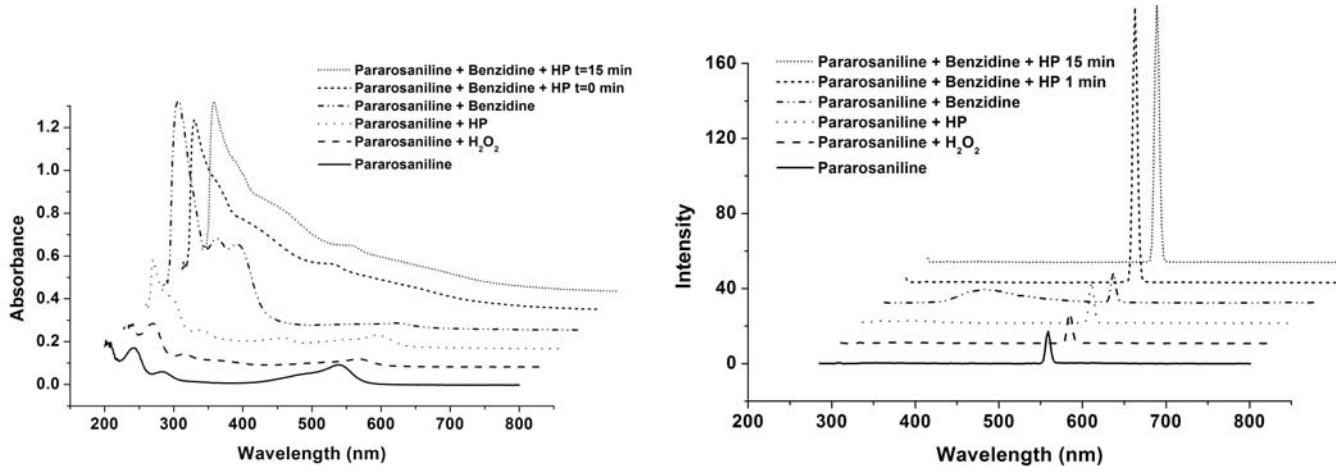


Figure 3. UV-Vis and Fluorescence spectra for reaction of Pararosaniline **1** with DAB, exc 280 nm.

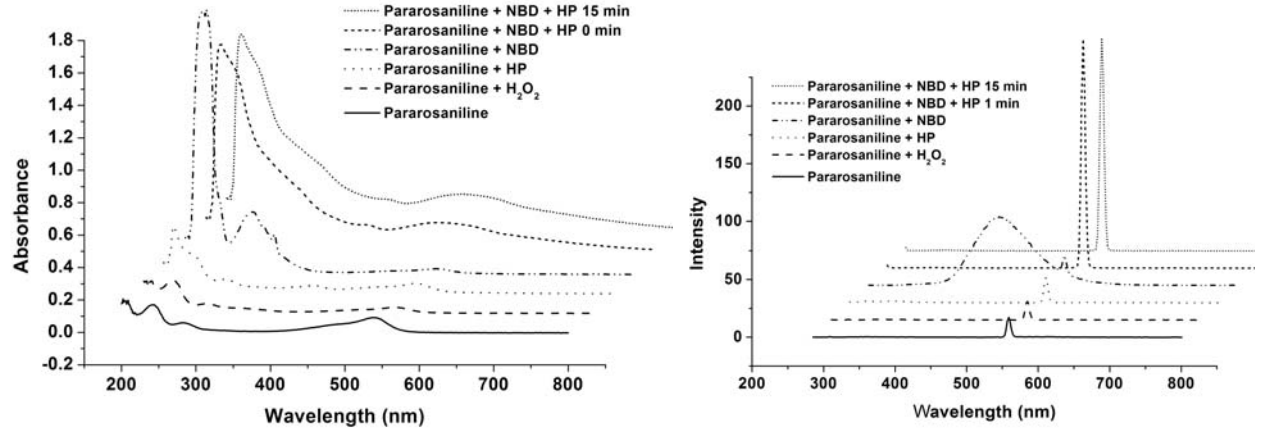


Figure 4. UV-Vis and Fluorescence spectra for reaction of Pararosaniline **1** with NBD, exc 280 nm.

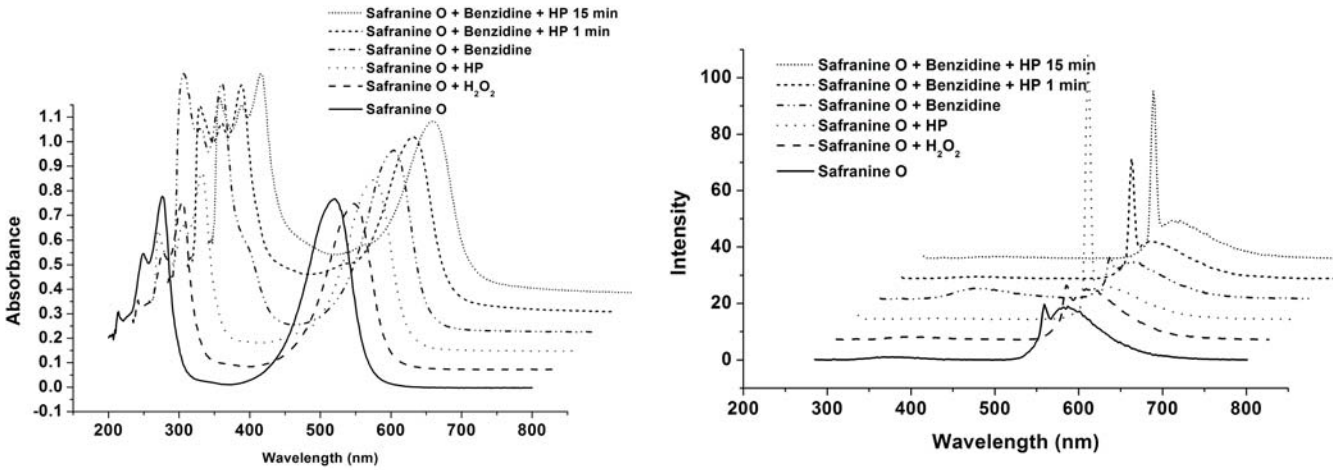


Figure 5. UV-Vis and Fluorescence spectra for reaction of Safranine O **2** with DAB, exc 280 nm.

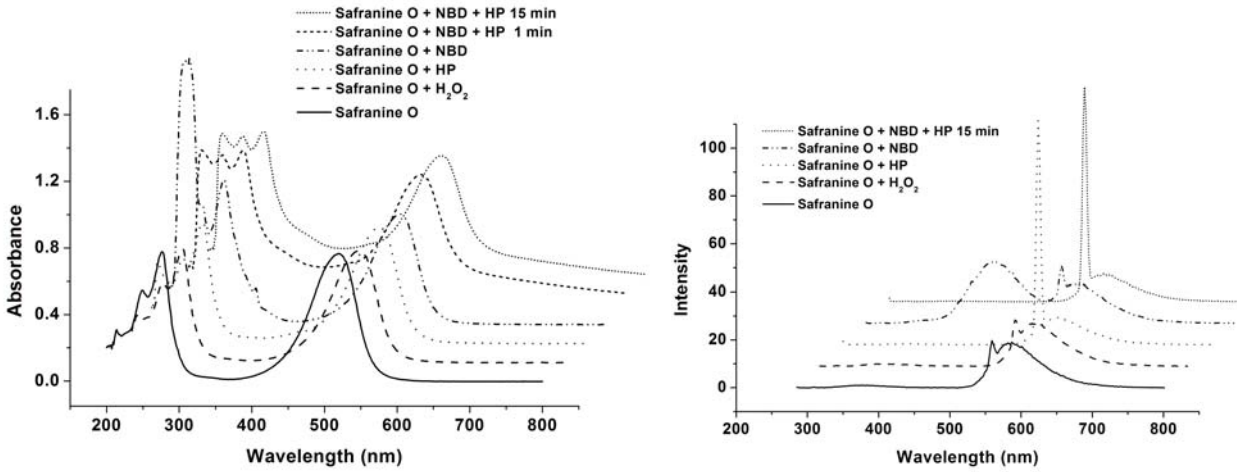


Figure 6. UV-Vis and Fluorescence spectra for reaction of Safranine O **2** with NBD, exc 280 nm.

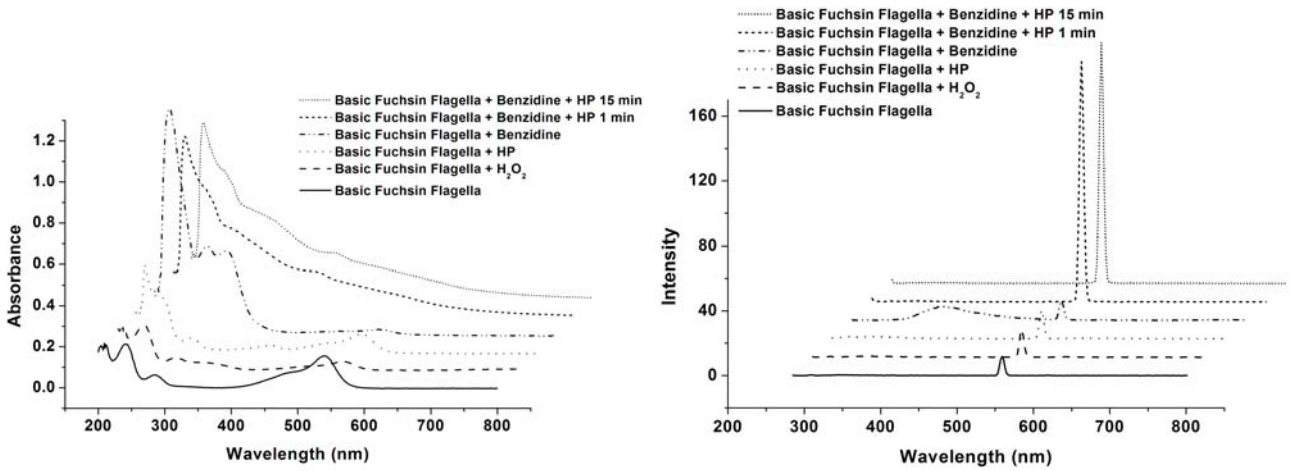


Figure 7. UV-Vis and Fluorescence spectra for reaction of Fuchsin Basic Flagella **3**with DAB, exc 280 nm.

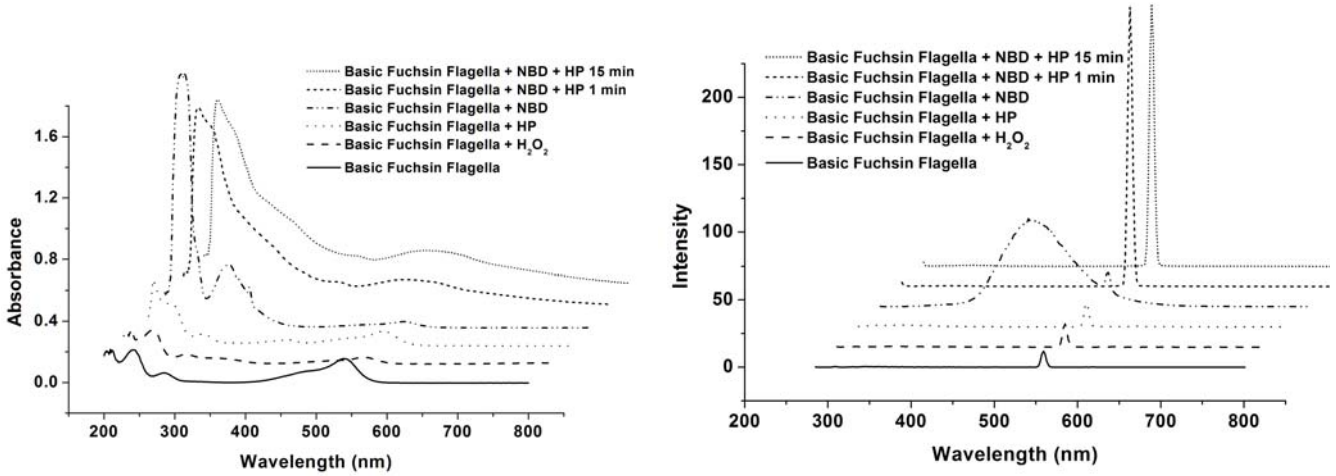


Figure 8. UV-Vis and Fluorescence spectra for reaction of Fuchsin Basic Flagella **3** with NBD, exc 280 nm.

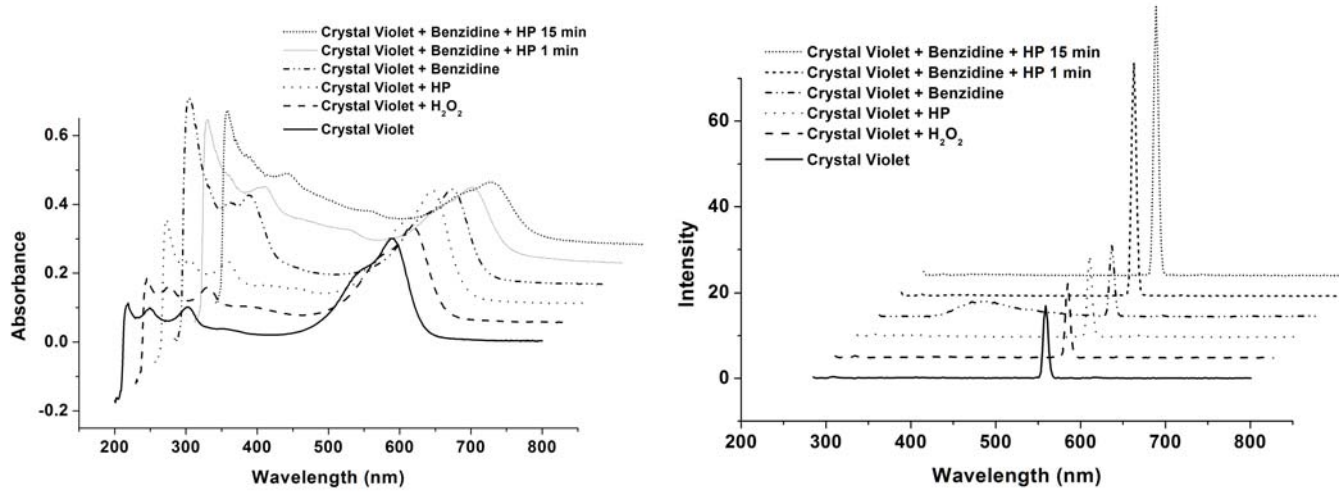


Figure 9. UV-Vis and Fluorescence spectra for reaction of Crystal Violet **7** with DAB, exc 280 nm.

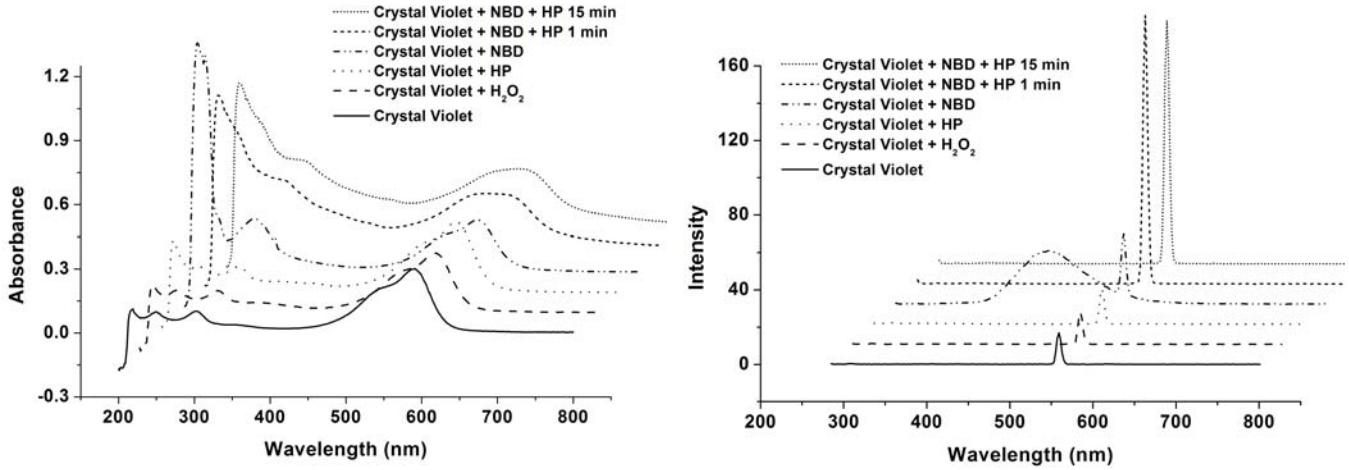


Figure 10. UV-Vis and Fluorescence spectra for reaction of Crystal Violet **7** with NBD, exc 280 nm.

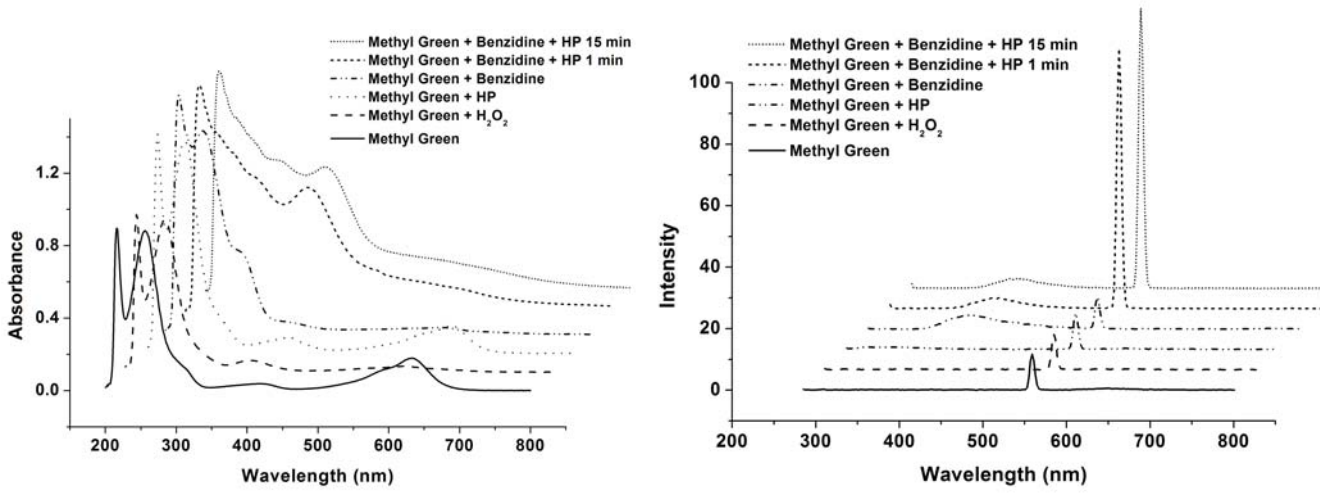


Figure 11. UV-Vis and Fluorescence spectra for reaction of Methyl Green **9** with DAB, exc 280 nm.

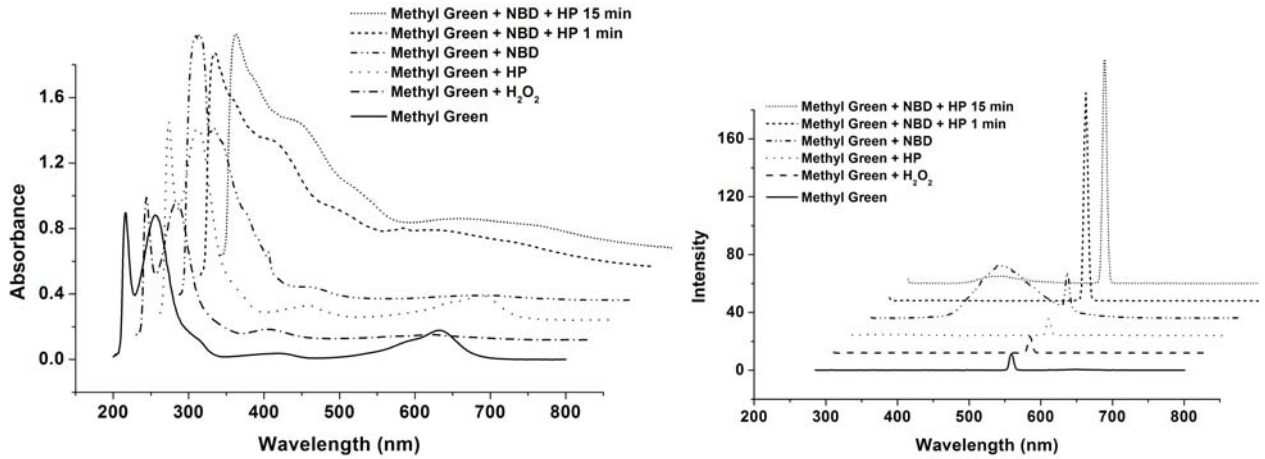


Figure 12. UV-Vis and Fluorescence spectra for reaction of Methyl Green **9** with NBD, exc 280 nm.

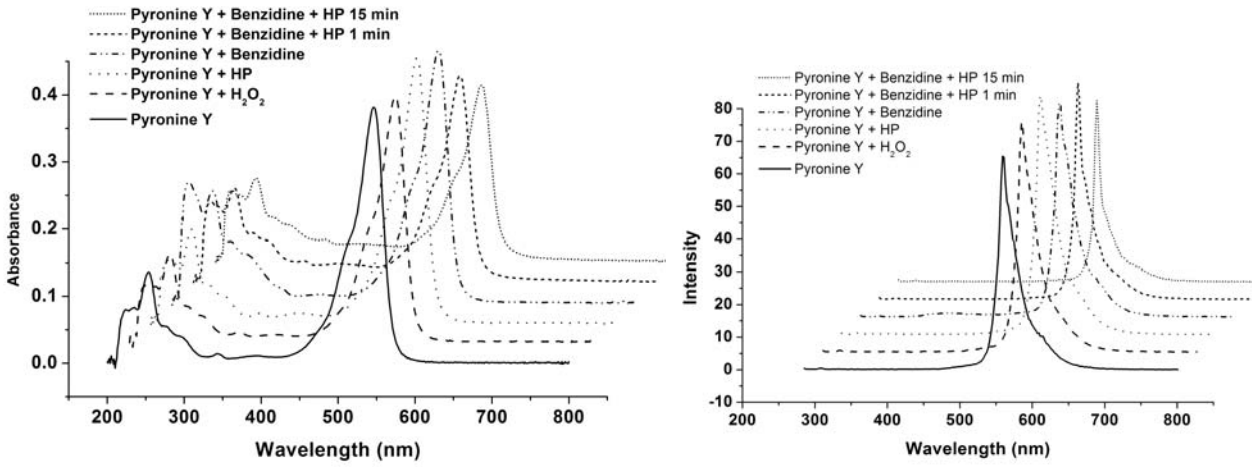


Figure 13. UV-Vis and Fluorescence spectra for reaction of Pyronin Y with DAB, exc 280 nm.

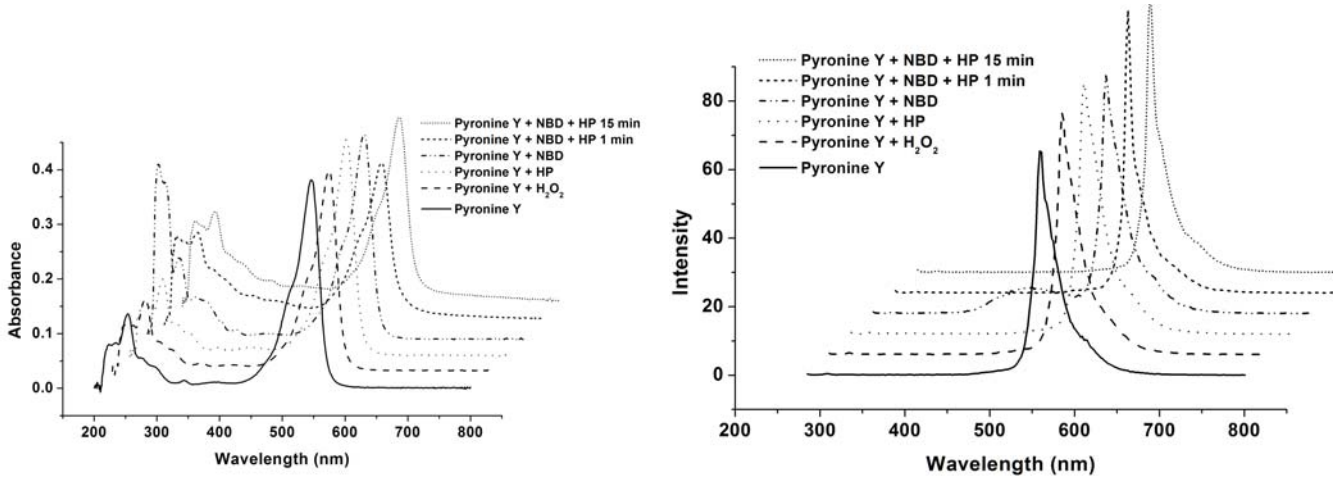


Figure 14. UV-Vis and Fluorescence spectra for reaction of Pyronin Y with NBD, exc 280 nm.

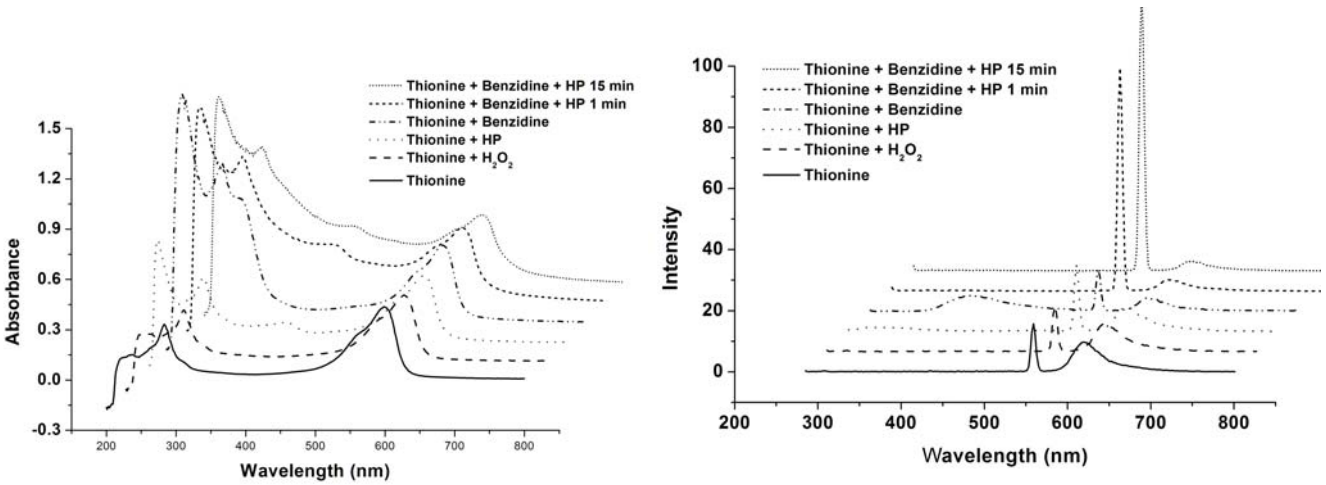


Figure 15. UV-Vis and Fluorescence spectra for reaction of Thionine **6** with NBD, exc 280 nm.

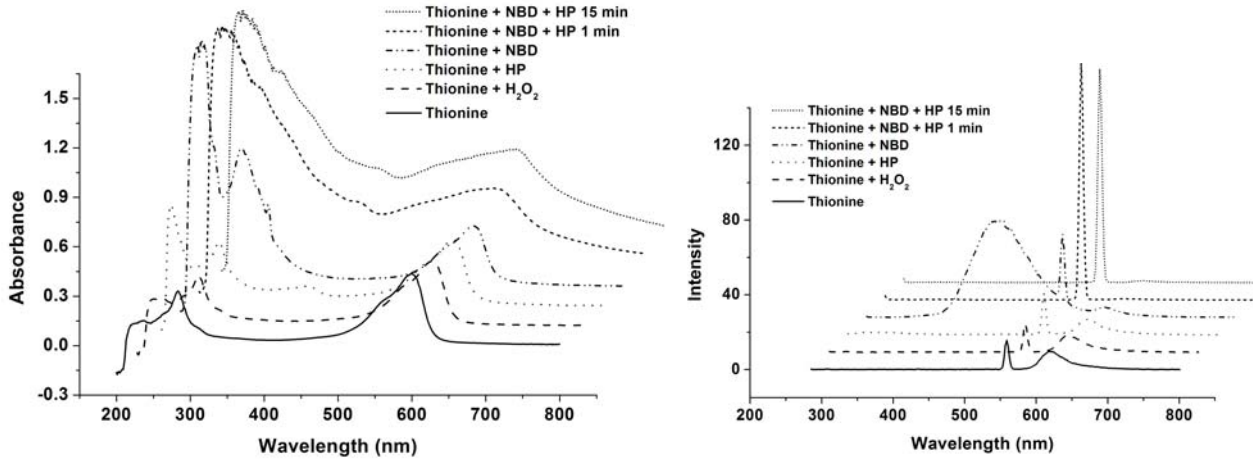


Figure 16. UV-Vis and Fluorescence spectra for reaction of Thionine **6** with NBD, exc 280 nm.

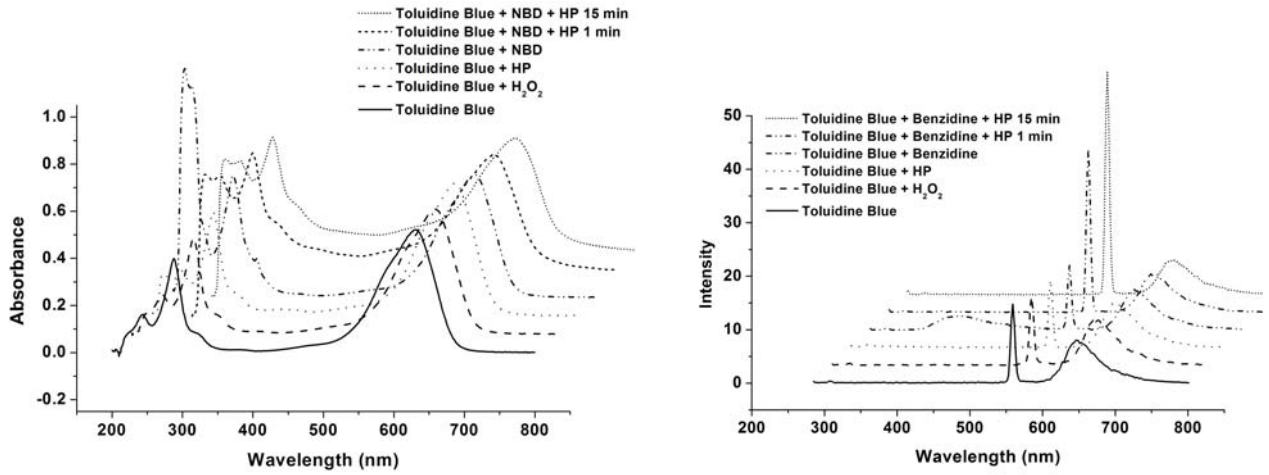


Figure 17. UV-Vis and Fluorescence spectra for reaction of Toluidine Blue **12** with NBD, exc 280 nm.

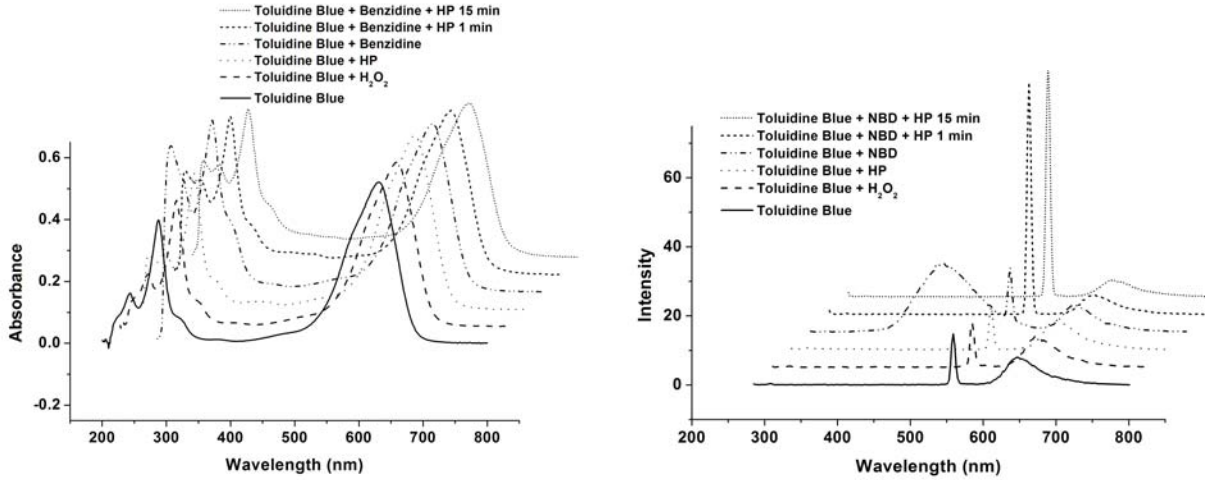


Figure 18. UV-Vis and Fluorescence spectra for reaction of Toulidine Blue **12** with NBD, exc 280 nm.

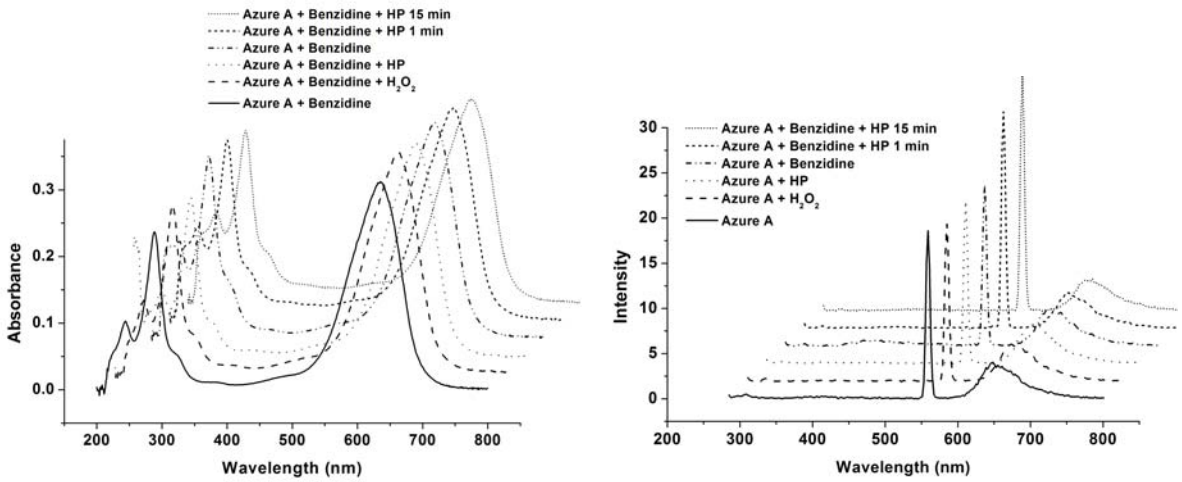


Figure 19. UV-Vis and Fluorescence spectra for reaction of Azure A with NBD, exc 280 nm.

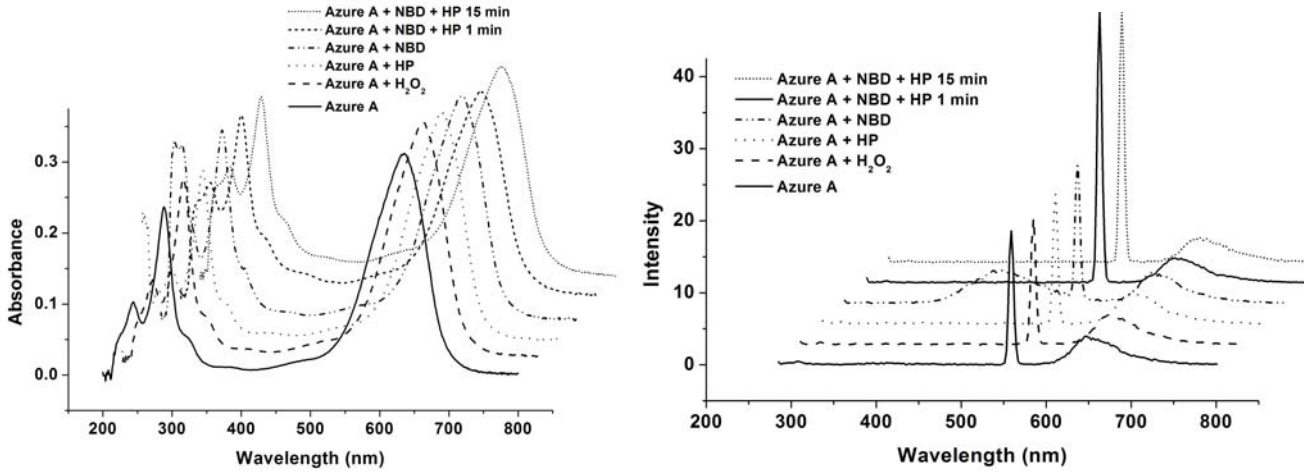


Figure 20. UV-Vis and Fluorescence spectra for reaction of Pararosaniline with NBD, exc 280 nm.

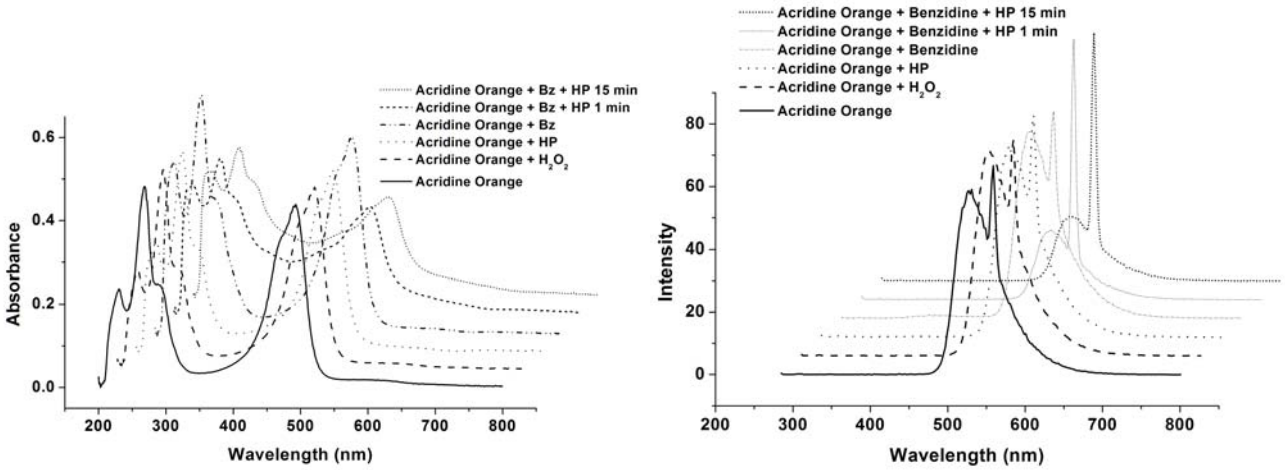


Figure 21. UV-Vis and Fluorescence spectra for reaction of Acridine Orange **7** with NBD, exc 280 nm.

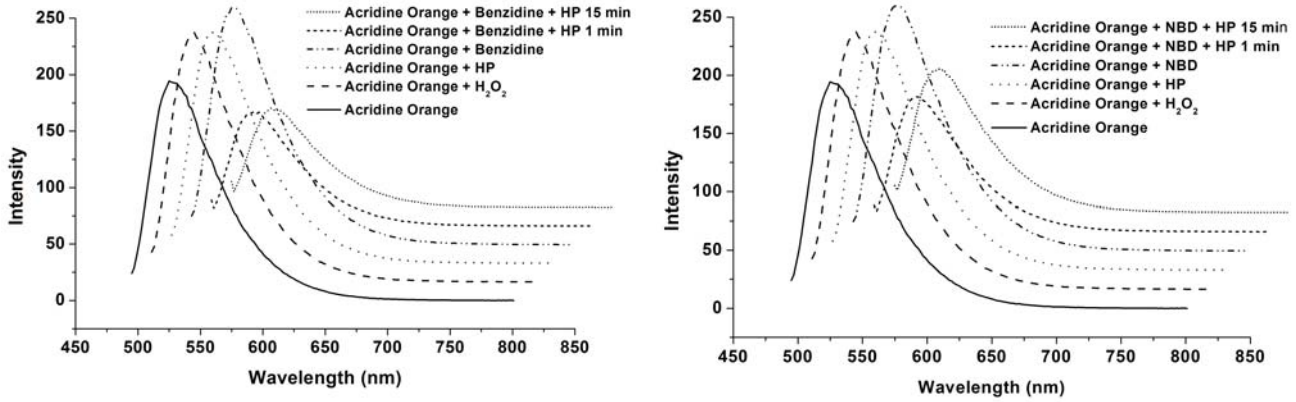


Figure 22. Fluorescence spectra for reaction of Acridine Orange **7** with DAB and NBD, exc 490 nm.

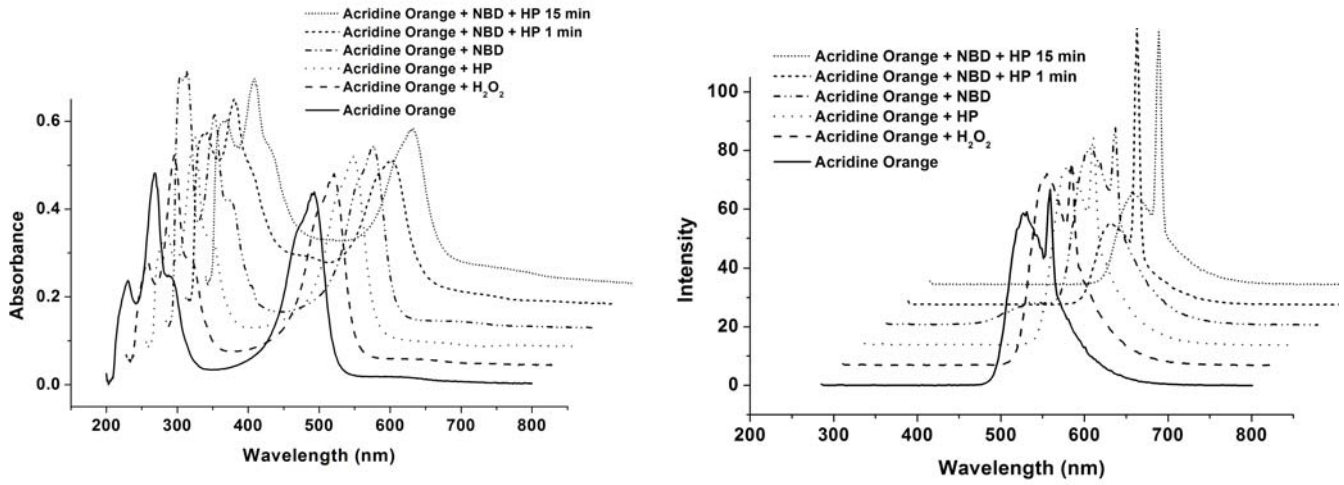


Figure 23. UV-Vis and Fluorescence spectra for reaction of Acridine Orange **7** with NBD, exc 280 nm.

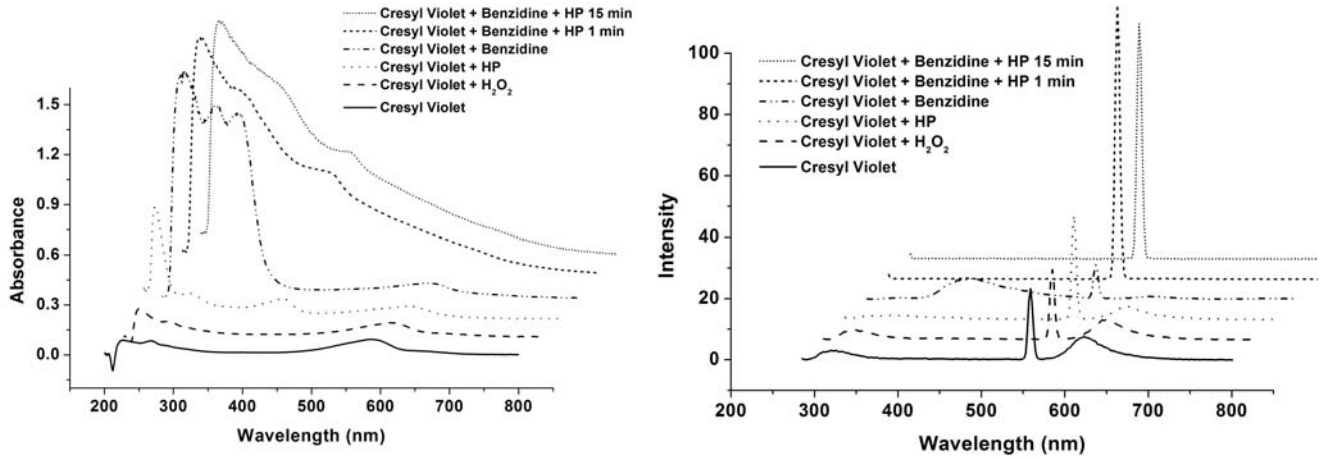


Figure 24. UV-Vis and Fluorescence spectra for reaction of Cresyl Violet **4** with DAB, exc.280 nm.

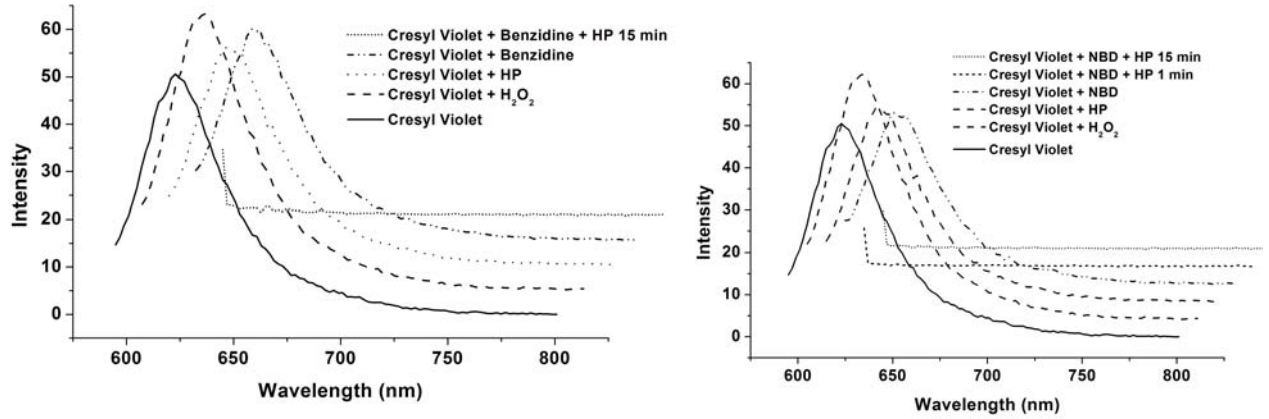


Figure 25. Fluorescence spectra for reaction of Cresyl Violet **4** with DAB and NBD, exc. 590 nm.

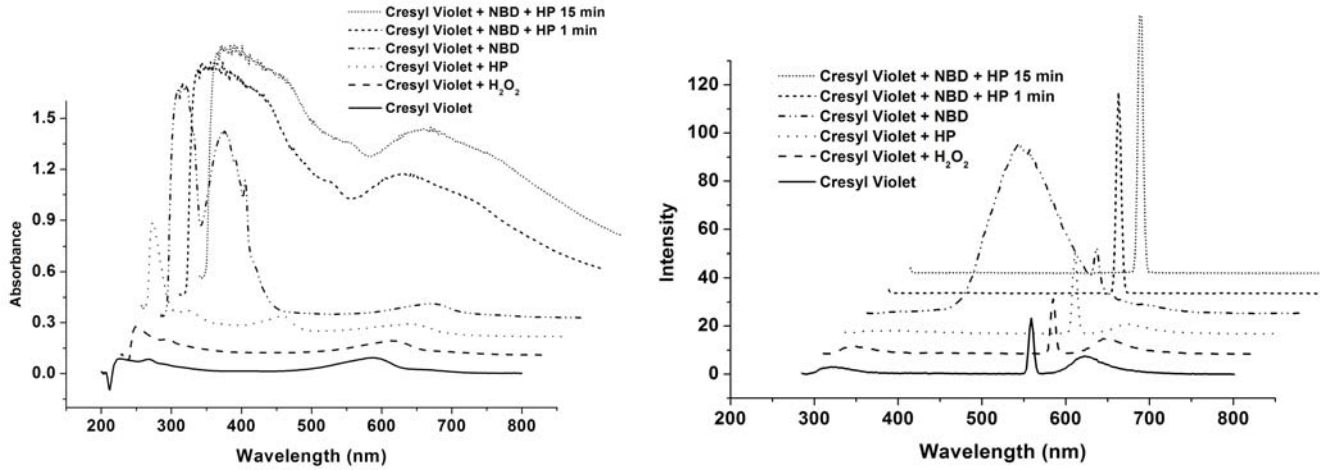


Figure 26. UV-Vis and Fluorescence spectra for reaction of Cresyl Violet **4** with NBD, exc 280 nm.

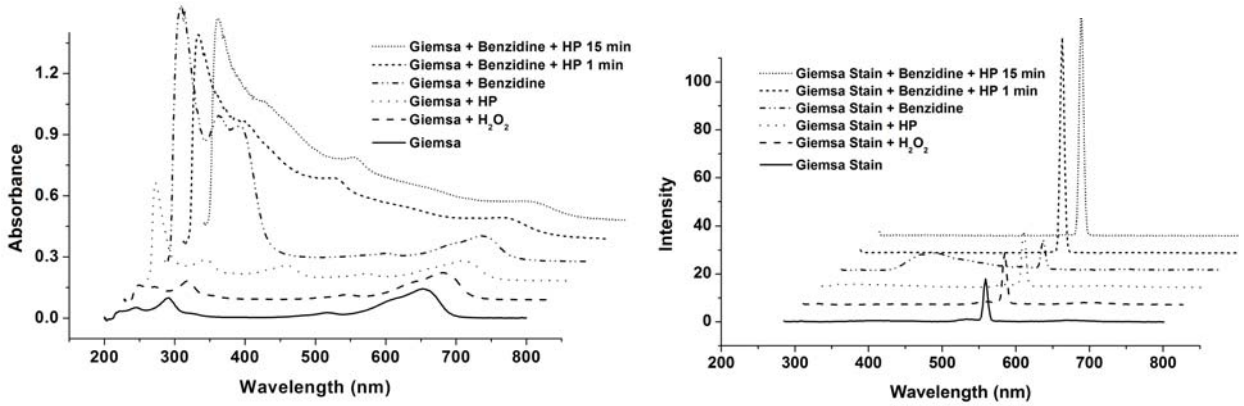


Figure 27. UV-Vis and Fluorescence spectra for reaction of Giemsa Stain with DAB, exc 280 nm.

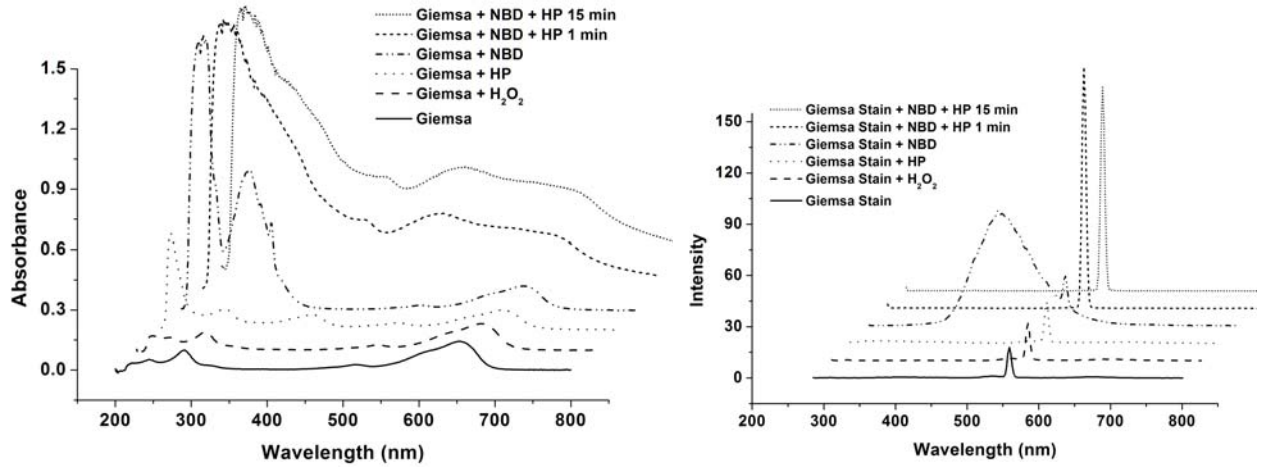


Figure 28. UV-Vis and Fluorescence spectra for reaction of Giemsa Stain with NBD, exc 280 nm.

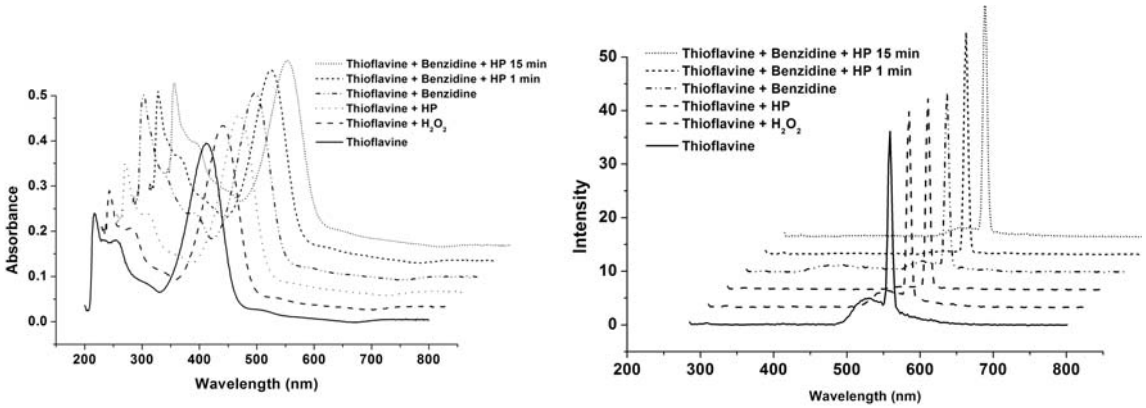


Figure 29. UV-Vis and Fluorescence spectra for reaction of Thioflavine T **8** with DAB, exc 280 nm.

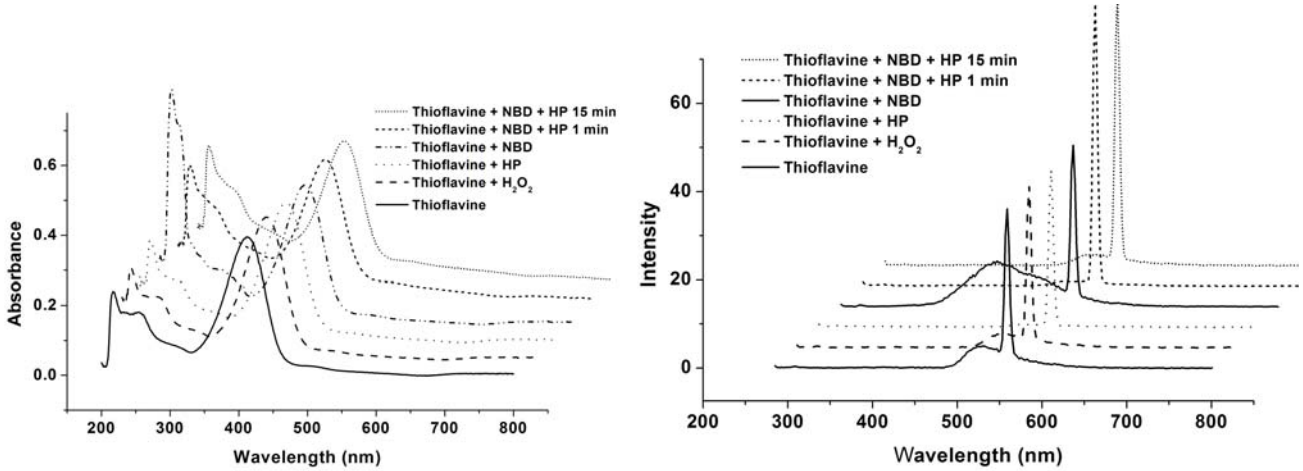


Figure 30. UV-Vis and Fluorescence spectra for reaction of Thioflavine T **8** with NBD, exc 280 nm.

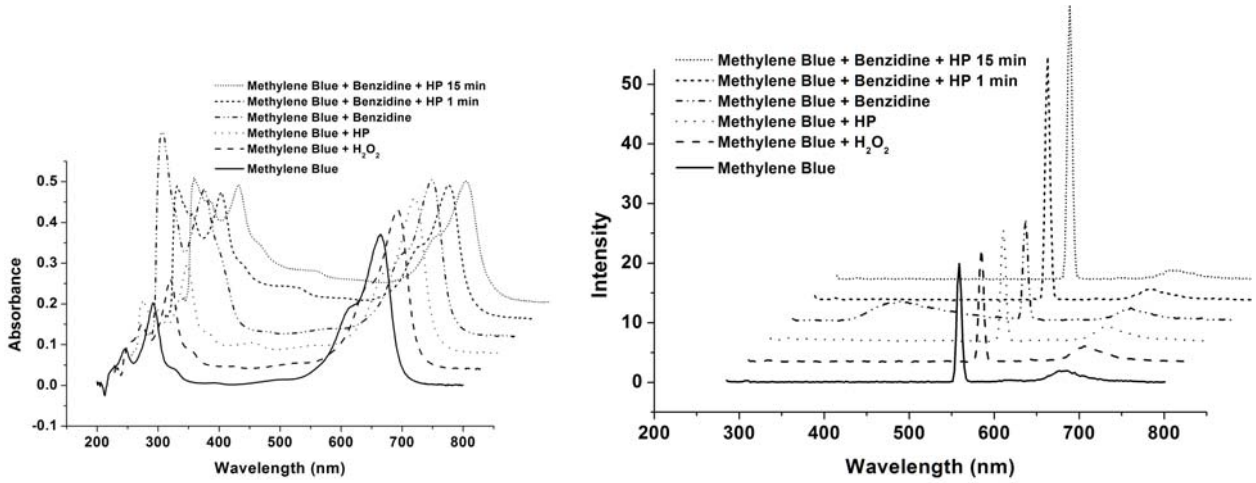


Figure 31. UV-Vis and Fluorescence spectra for reaction of Methylene Blue **11** with DAB, exc 280 nm.

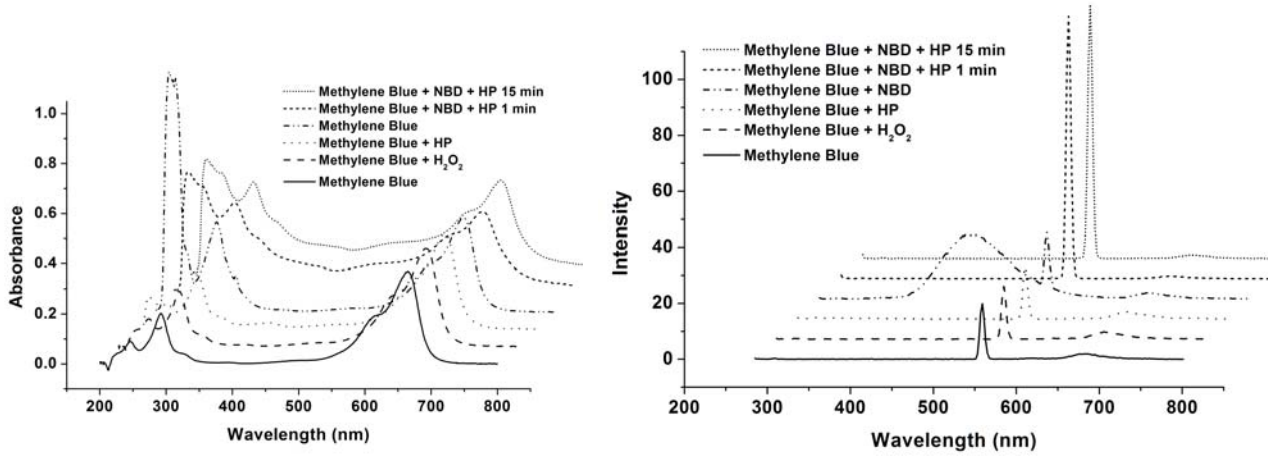


Figure 32. UV-Vis and Fluorescence spectra for reaction of Methylene Blue **11** with NBD, exc 280 nm.

Appendix II

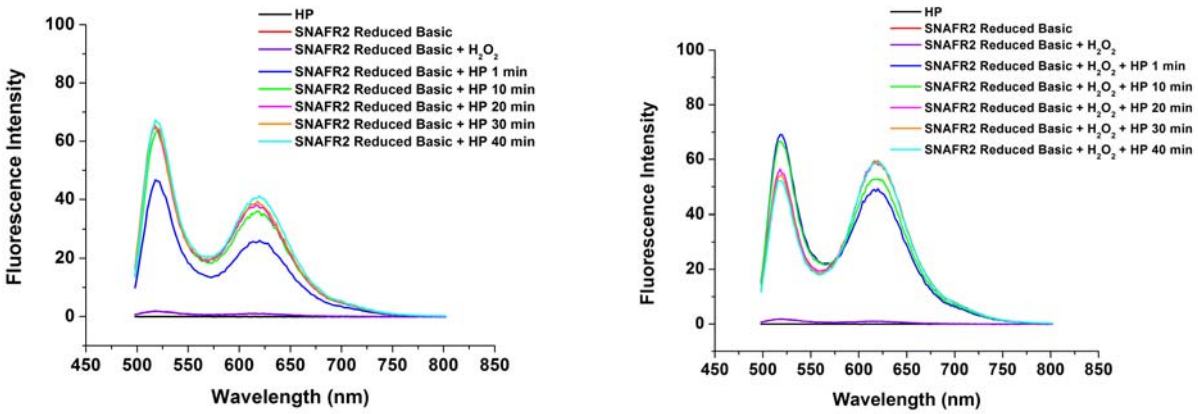


Figure 1. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Basic conditions, exc. 488 nm. SNAFR2 = Compound **19**

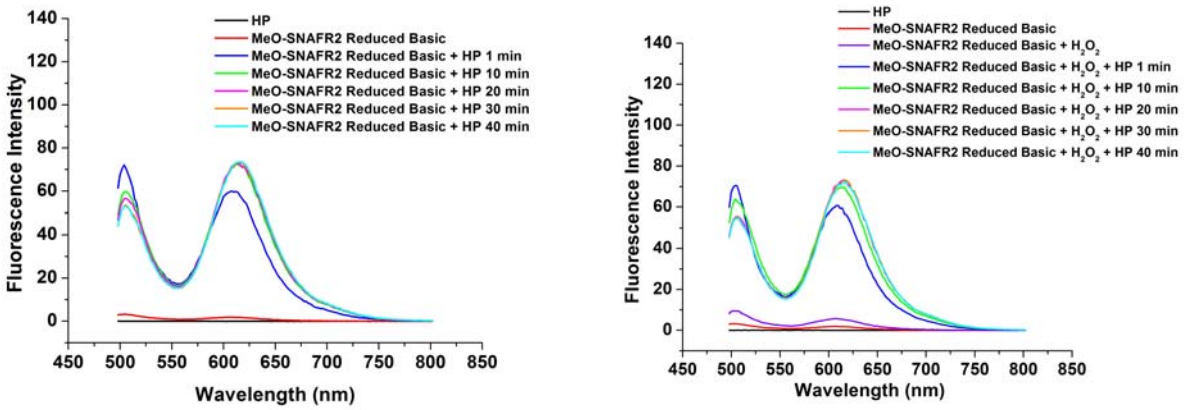


Figure 2. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Basic conditions, exc. 488 nm. MeO-SNAFR2 = Compound **20**

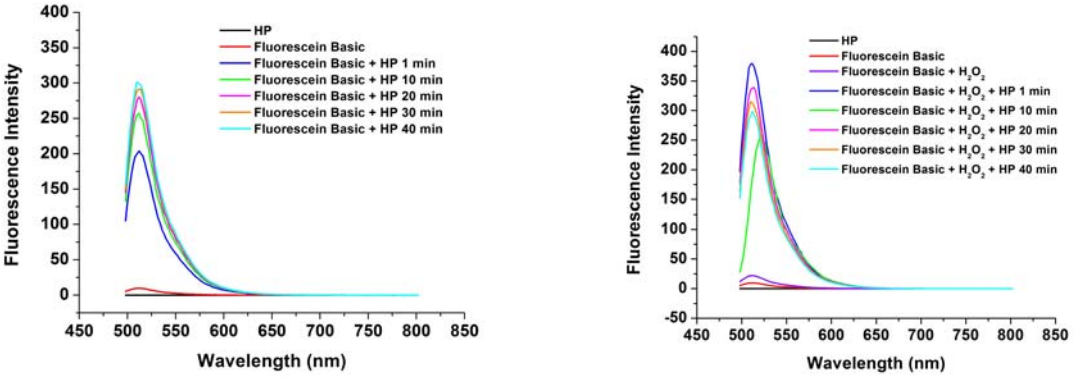


Figure 3. Fluorescence spectra for Peroxidase reaction with Fluorescein reduced under Basic conditions, exc. 488 nm.

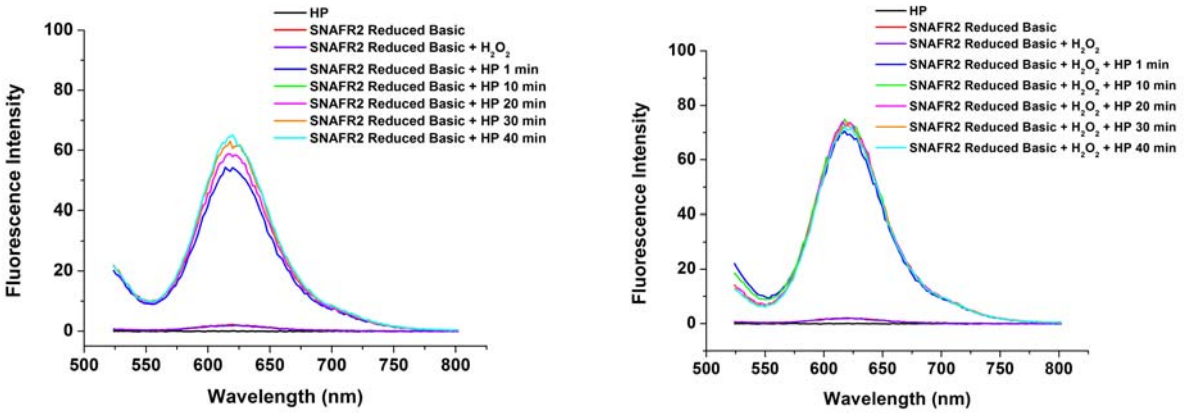


Figure 4. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Basic conditions, exc. 514 nm. SNAFR2 = Compound **19**

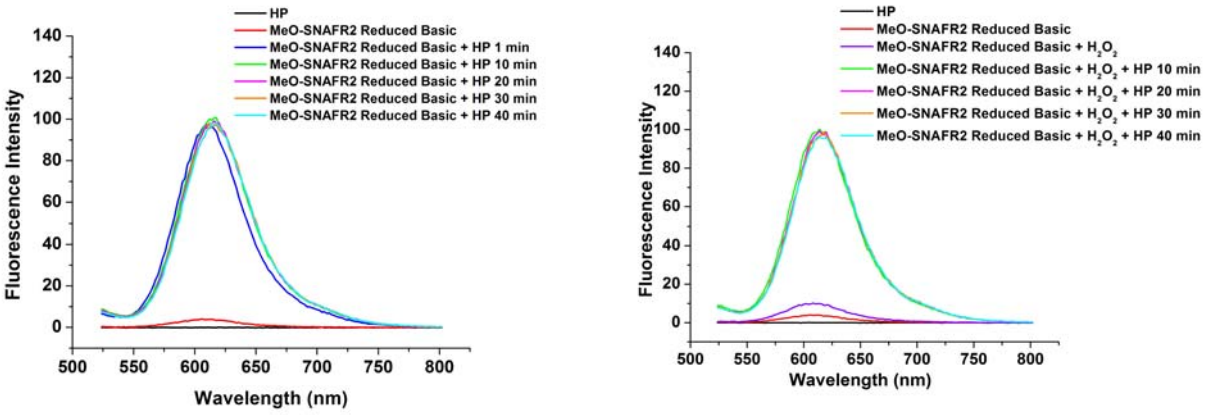


Figure 5. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Basic conditions, exc. 514 nm. MeO-SNAFR2 = Compound **20**

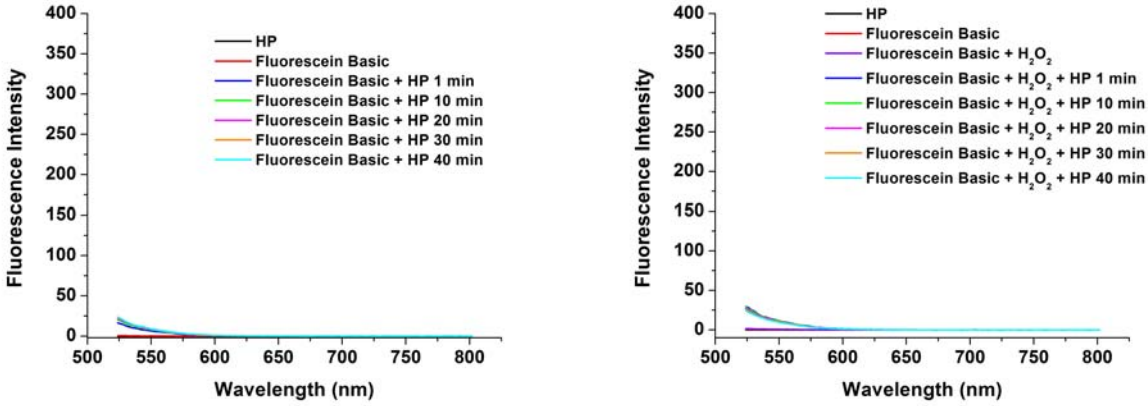


Figure 6. Fluorescence spectra for Peroxidase reaction with Fluorescein reduced under Basic conditions, exc. 514 nm.

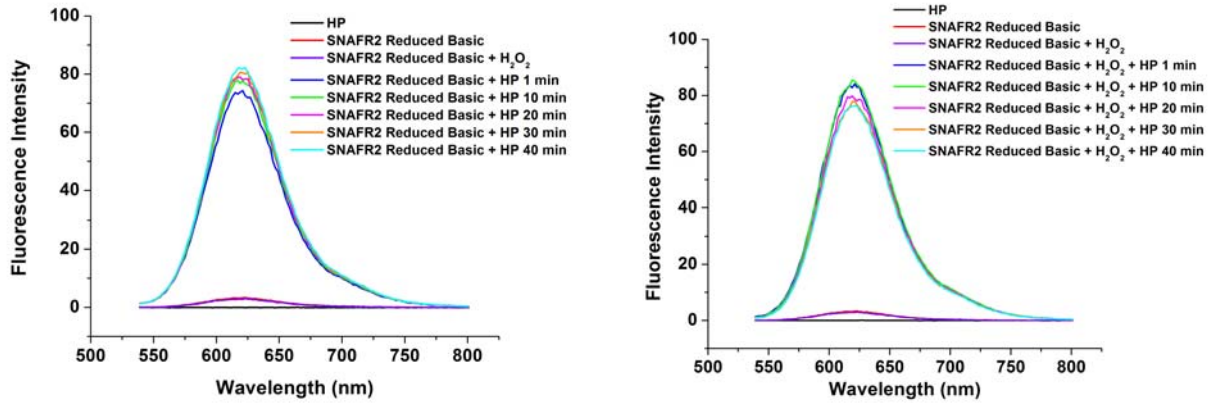


Figure 7. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Basic conditions, exc. 529 nm. SNAFR2 = Compound **19**

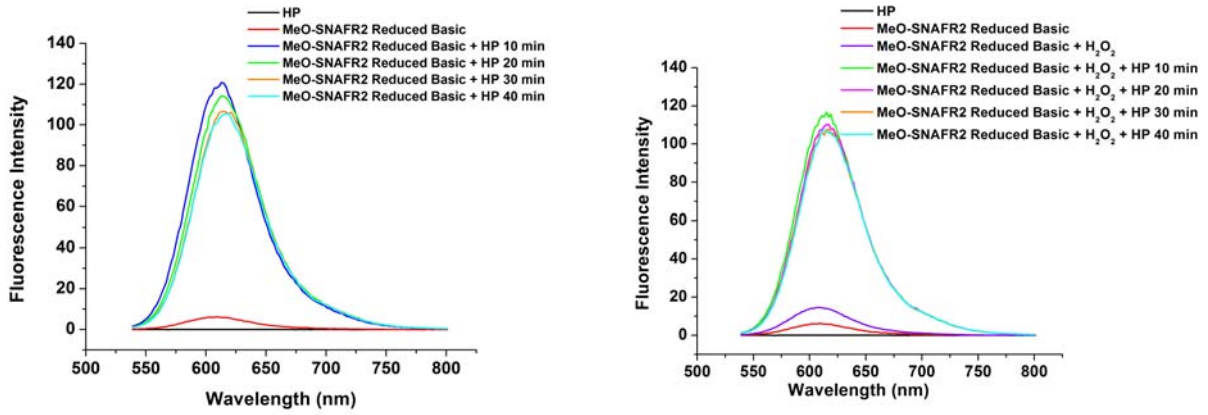


Figure 8. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Basic conditions, exc. 529 nm. MeO-SNAFR2 = Compound **20**

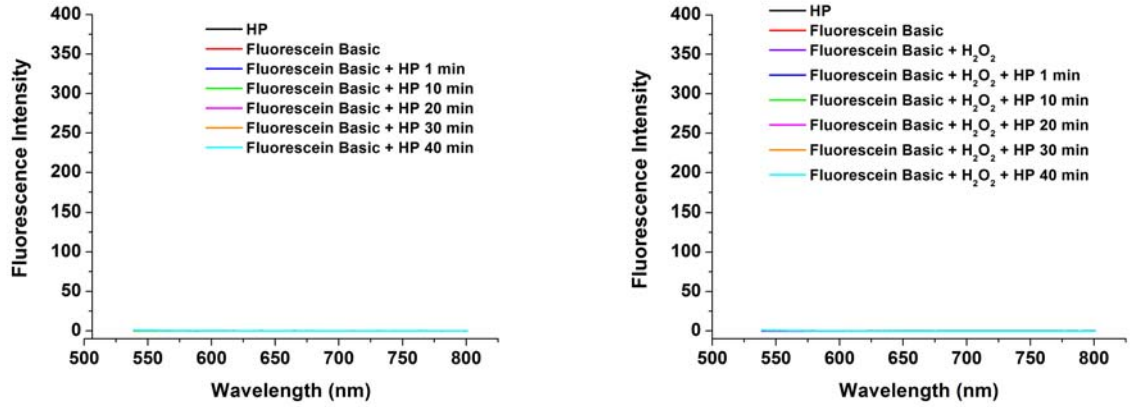


Figure 9. Fluorescence spectra for Peroxidase reaction with Fluorescein reduced under Basic conditions, exc. 529 nm.
543

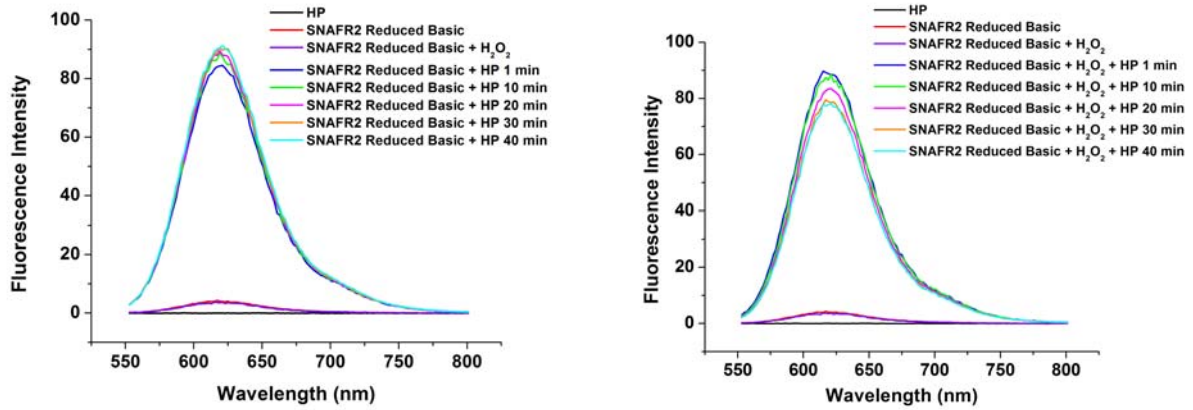


Figure 10. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Basic conditions, exc. 543 nm. SNAFR2 = Compound **19**

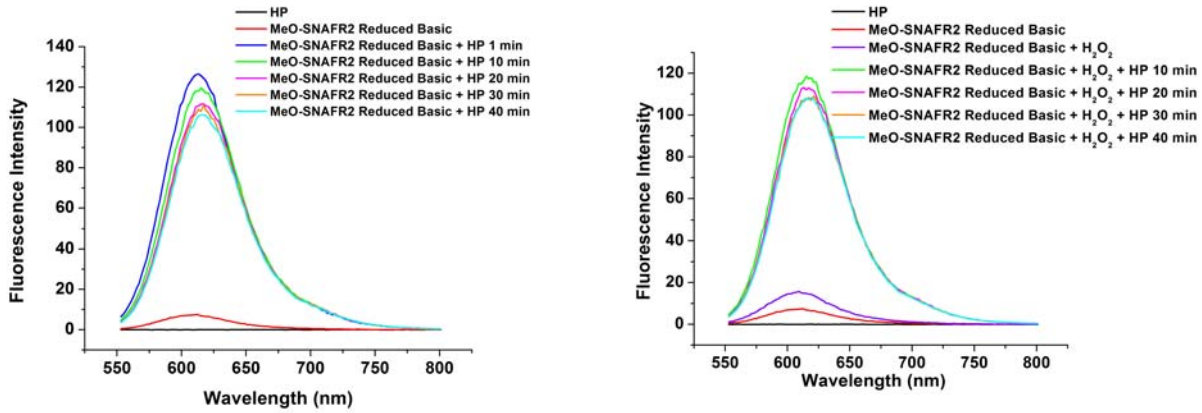


Figure 11. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Basic conditions, exc. 543 nm. MeO-SNAFR2 = Compound **20**

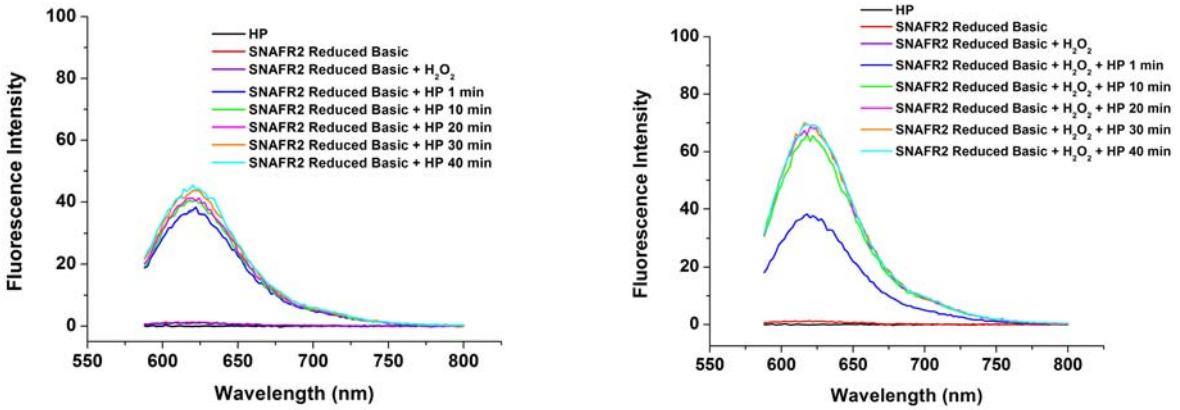


Figure 12. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Basic conditions, exc. 578 nm. SNAFR2 = Compound **19**

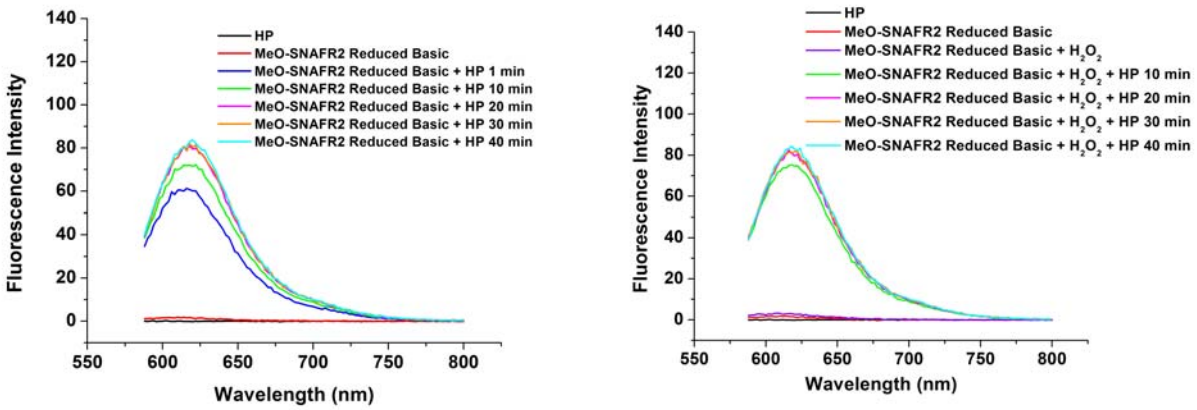


Figure 13. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Basic conditions, exc. 578 nm. MeO-SNAFR2 = Compound **20**

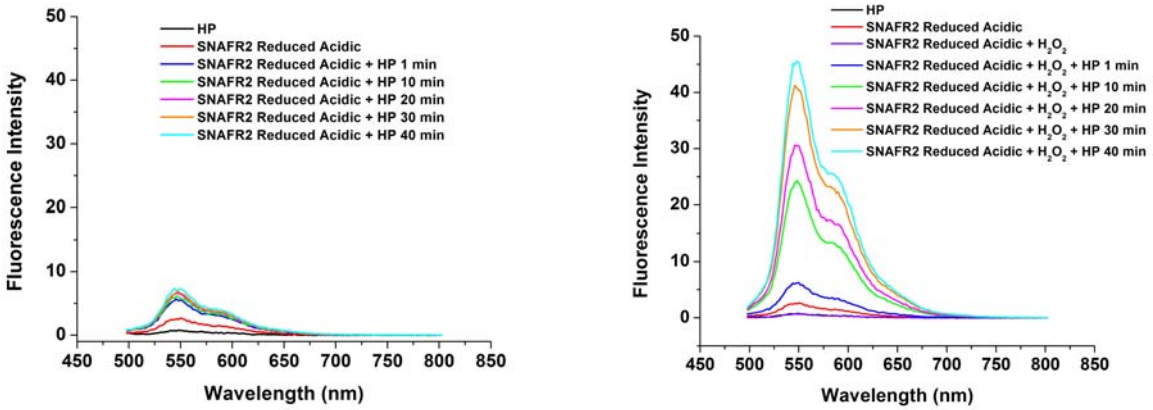


Figure 14. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Acidic conditions, exc. 488 nm. SNAFR2 = Compound **19**

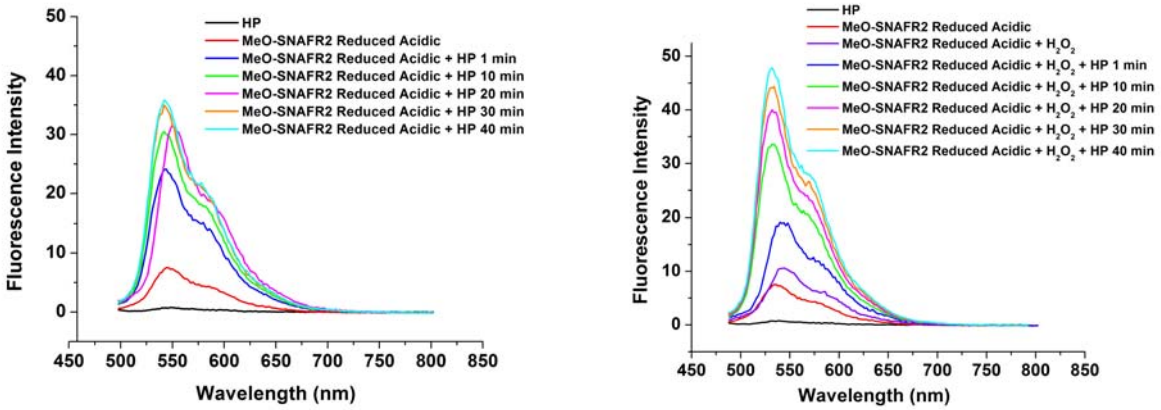


Figure 15. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Acidic conditions, exc. 488 nm. MeO-SNAFR2 = Compound **20**

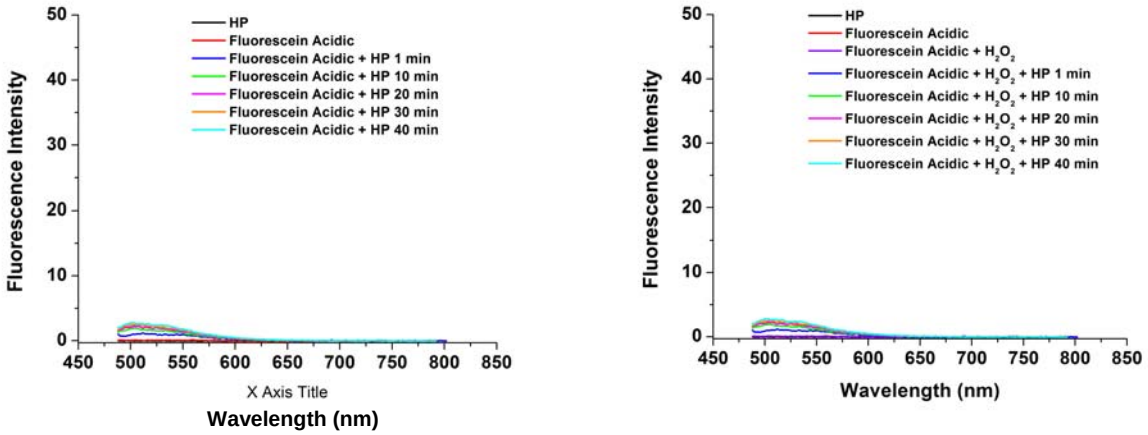


Figure 16. Fluorescence spectra for Peroxidase reaction with Fluorescein reduced under Acidic conditions, exc. 488 nm.

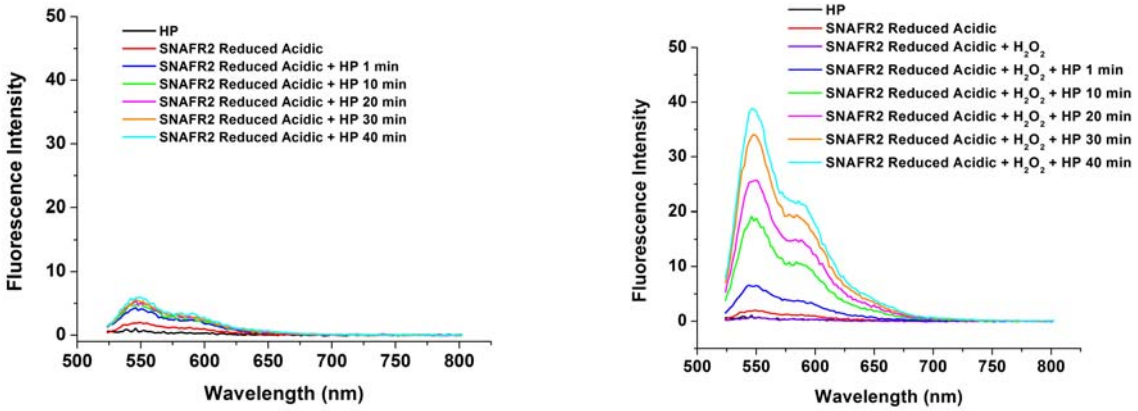


Figure 17. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Acidic conditions, exc. 514 nm. SNAFR2 = Compound **19**

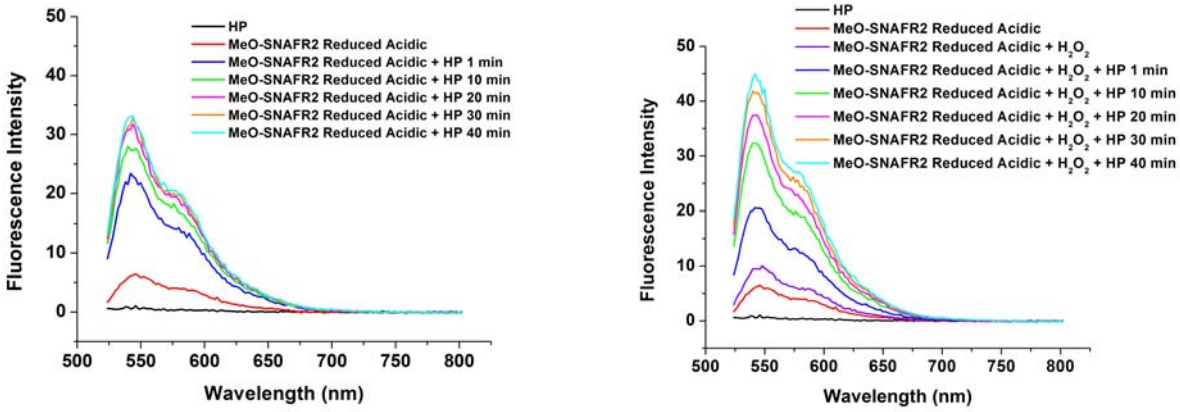


Figure 18. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Acidic conditions, exc. 514 nm. MeO-SNAFR2 = Compound **20**

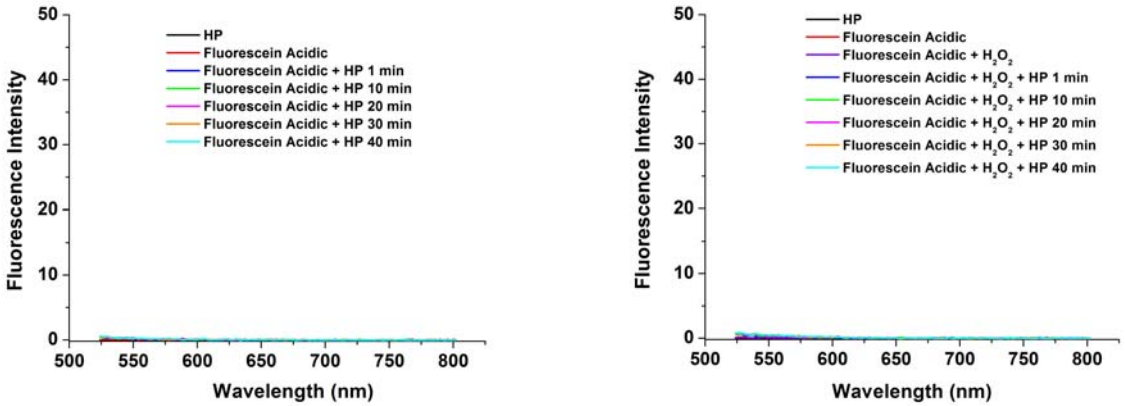


Figure 19. Fluorescence spectra for Peroxidase reaction with Fluorescein reduced under Acidic conditions, exc. 514 nm.

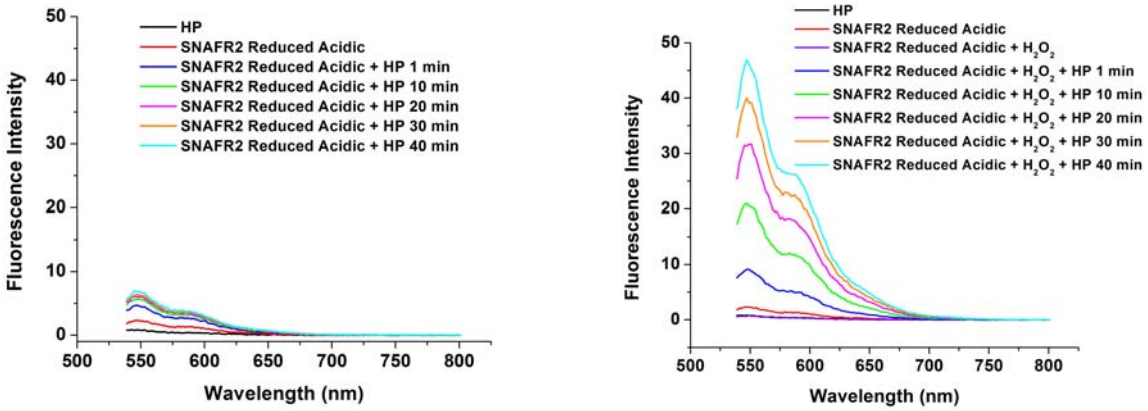


Figure 20. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Acidic conditions, exc. 529 nm. SNAFR2 = Compound **19**

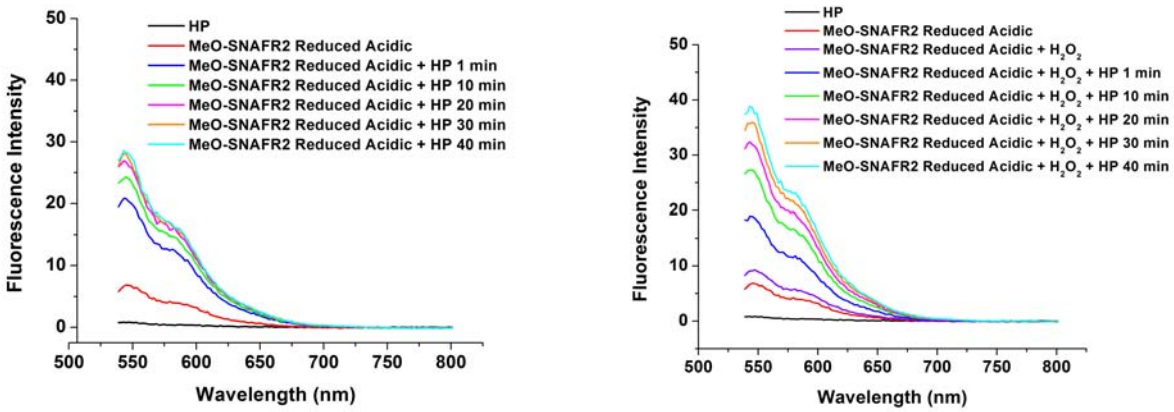


Figure 21. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Acidic conditions, exc. 529 nm. MeO-SNAFR2 = Compound **20**

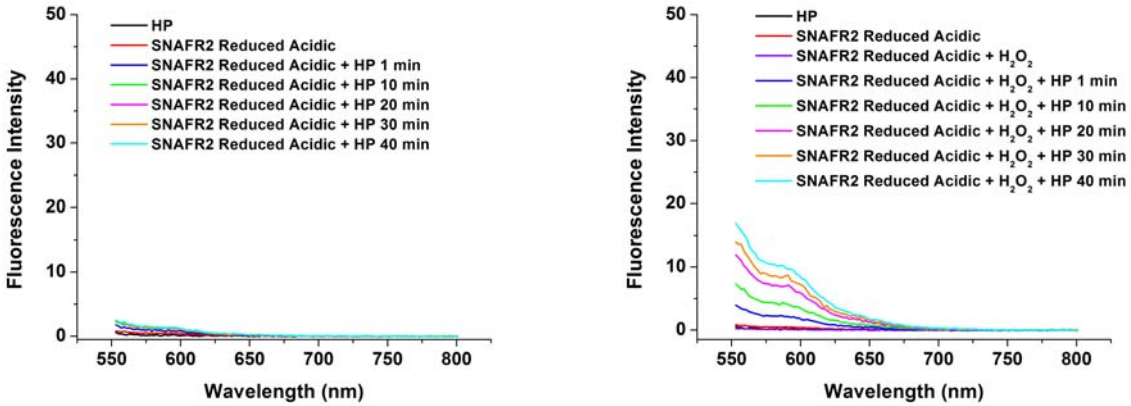


Figure 22. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Acidic conditions, exc. 543 nm. SNAFR2 = Compound **19**

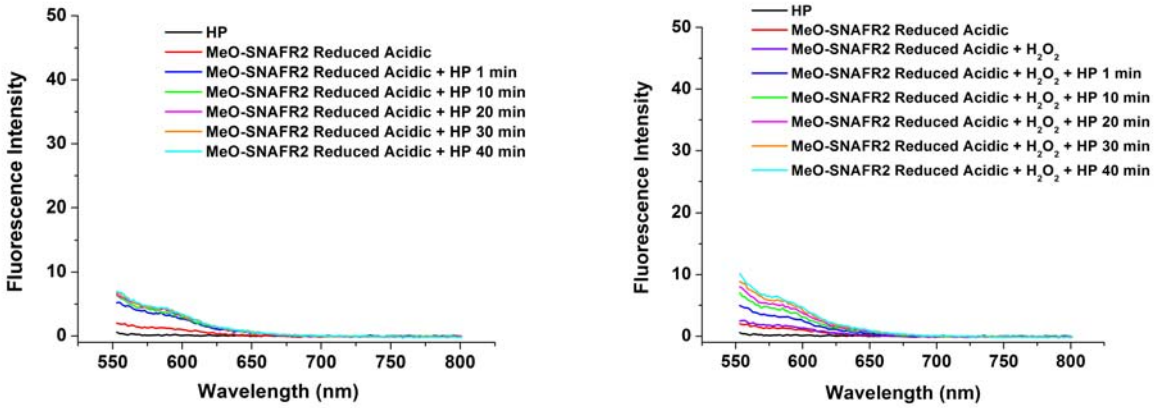


Figure 23. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Acidic conditions, exc. 543 nm. MeO-SNAFR2 = Compound **20**

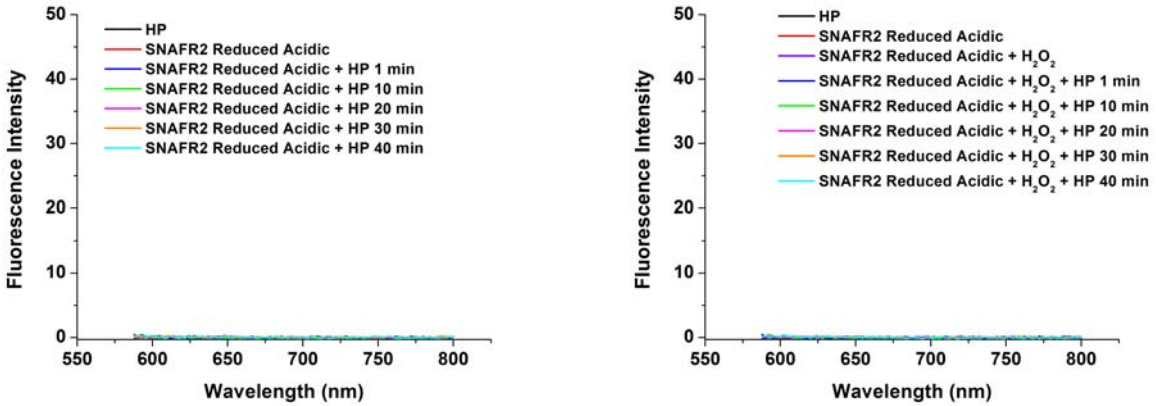


Figure 24. Fluorescence spectra for Peroxidase reaction with compound **19** reduced under Acidic conditions, exc. 578 nm. SNAFR2 = Compound **19**

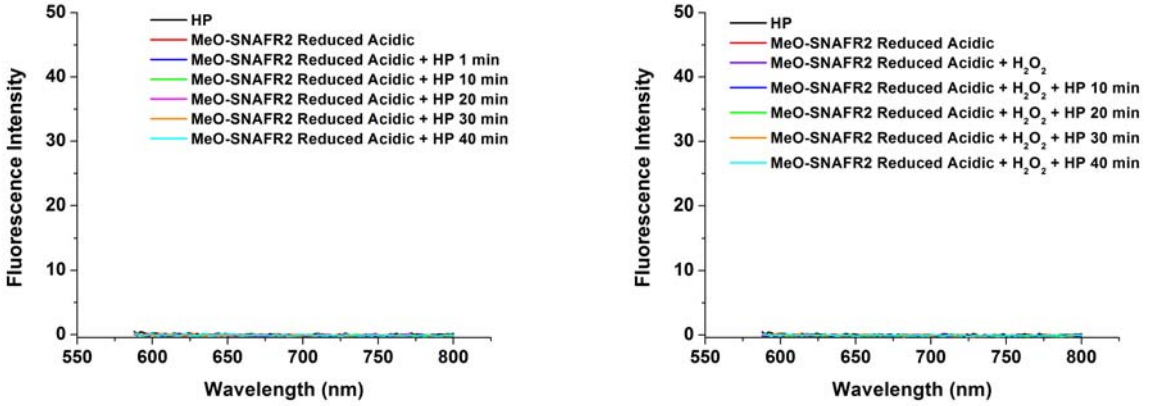
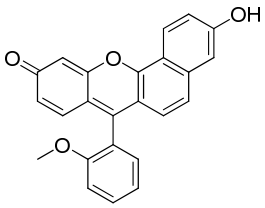


Figure 25. Fluorescence spectra for Peroxidase reaction with compound **20** reduced under Acidic conditions, exc. 578 nm. MeO-SNAFR2 = Compound **20**

Appendix III



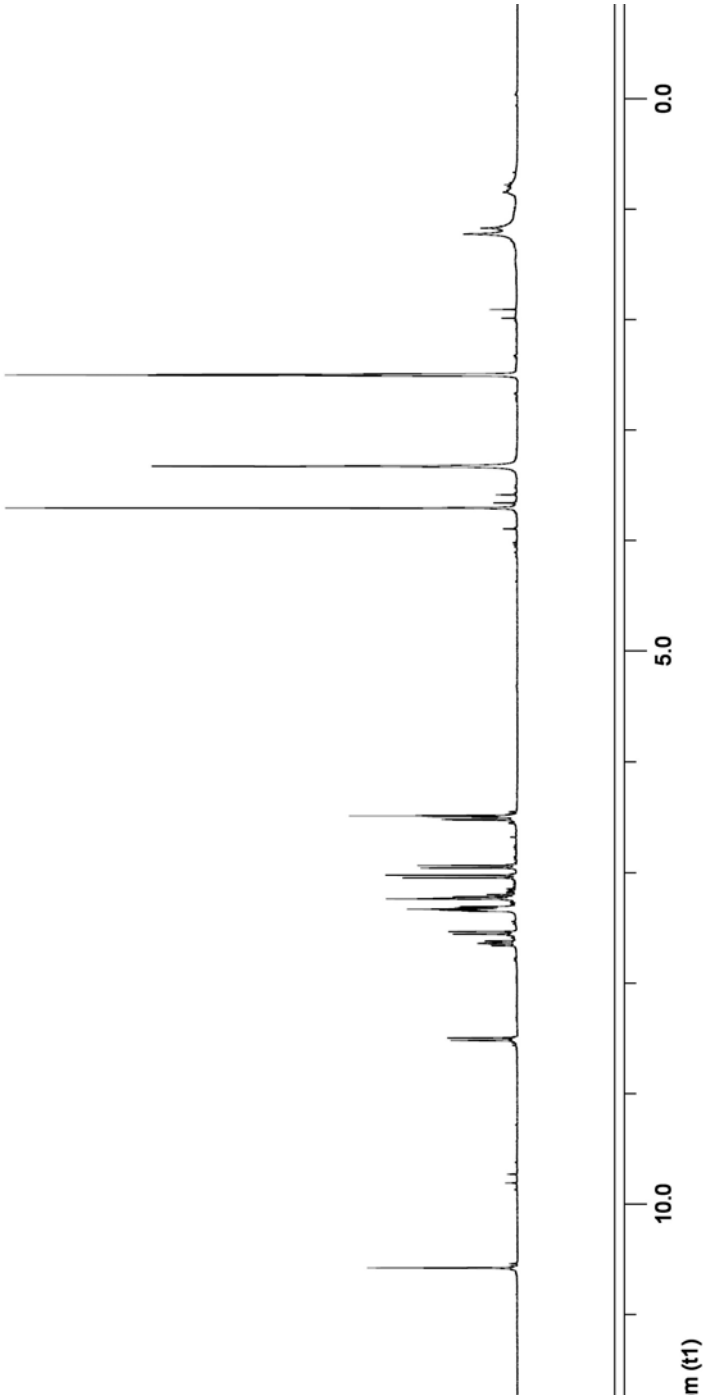


Figure 1. ^{1}H NMR of compound **20** in d_6 –DMSO.

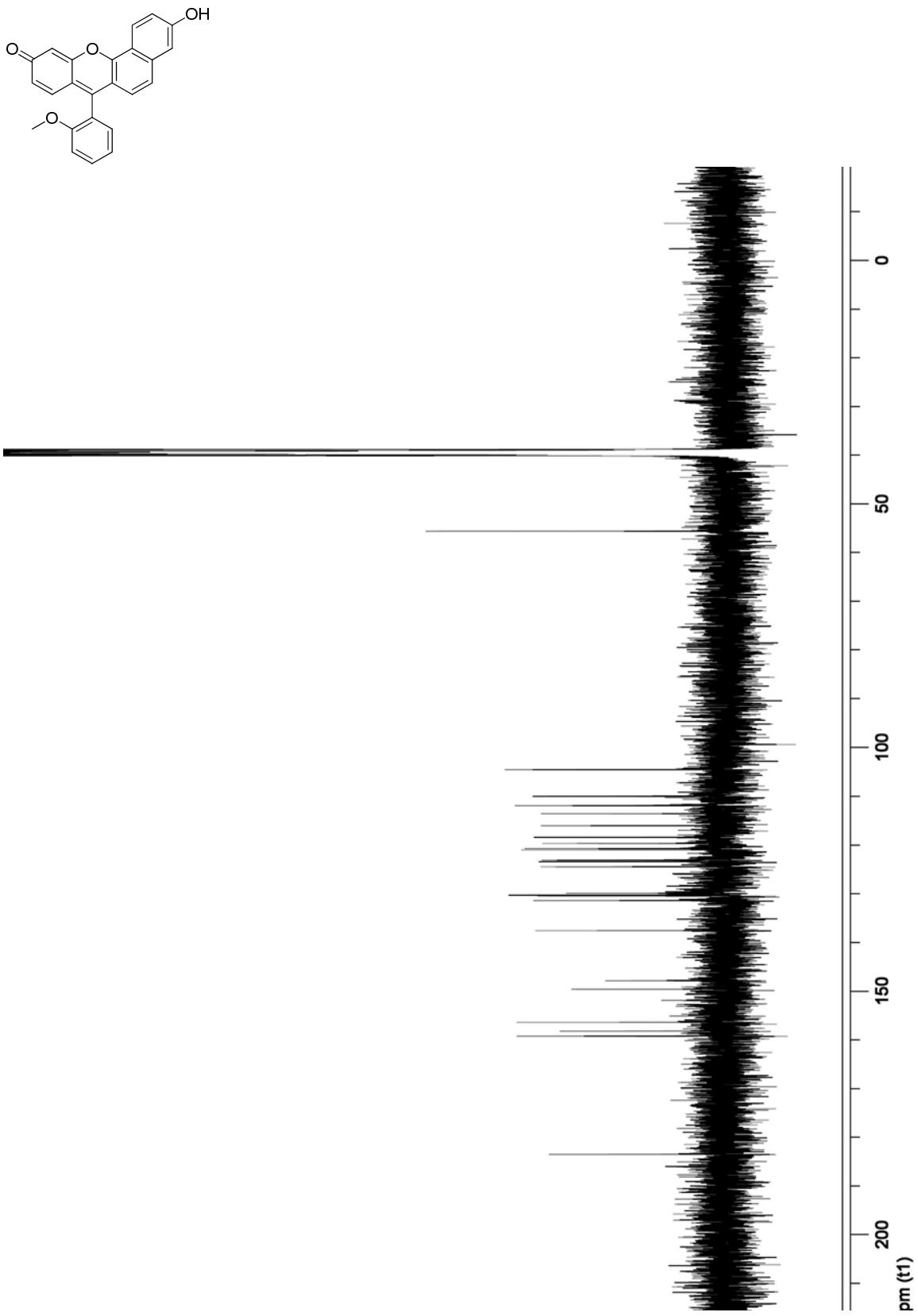


Figure 2. ^{13}C NMR of compound **20** in d_6 –DMSO.

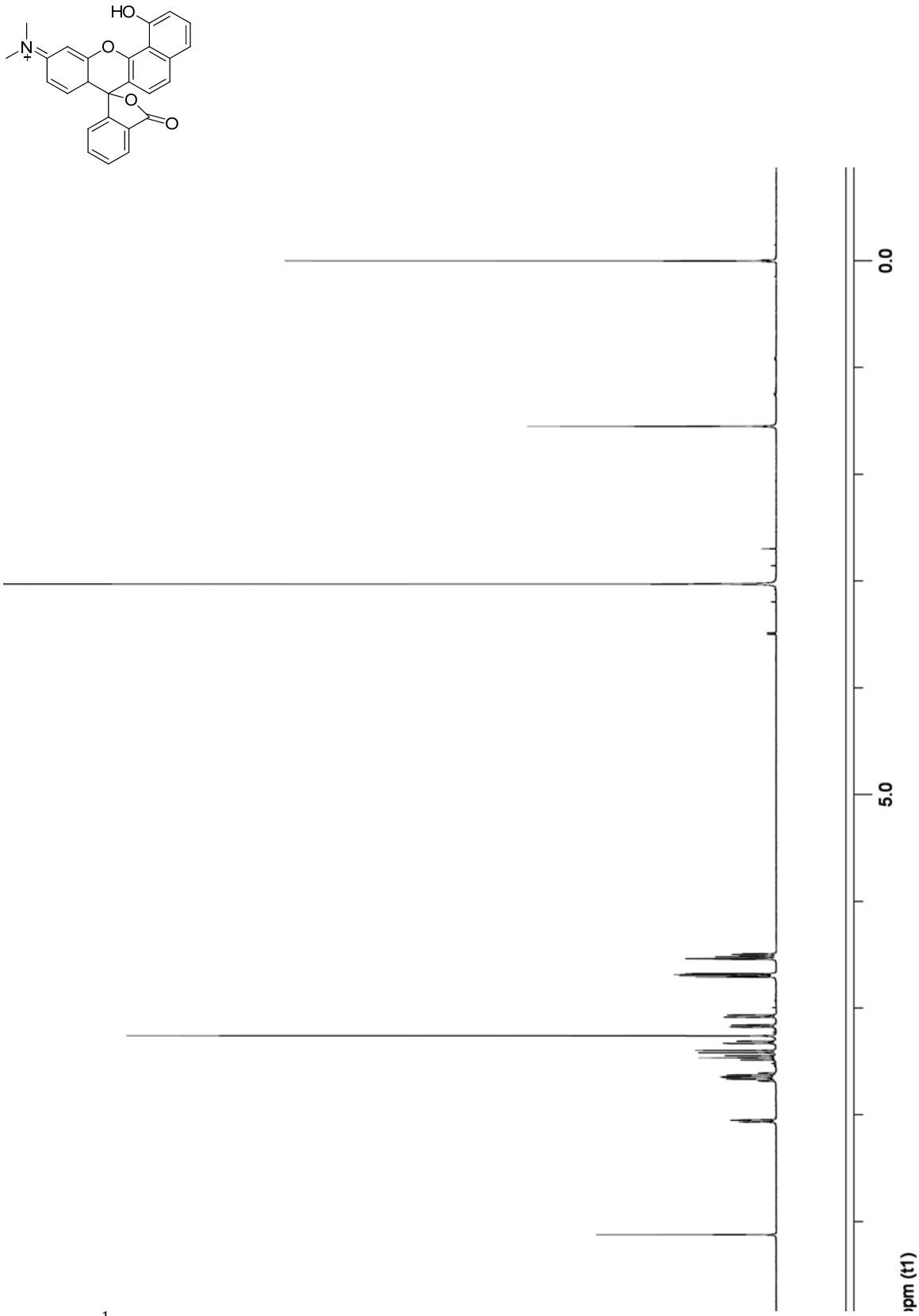


Figure 3. ^{1}H NMR of compound **40** in $CDCl_3$.

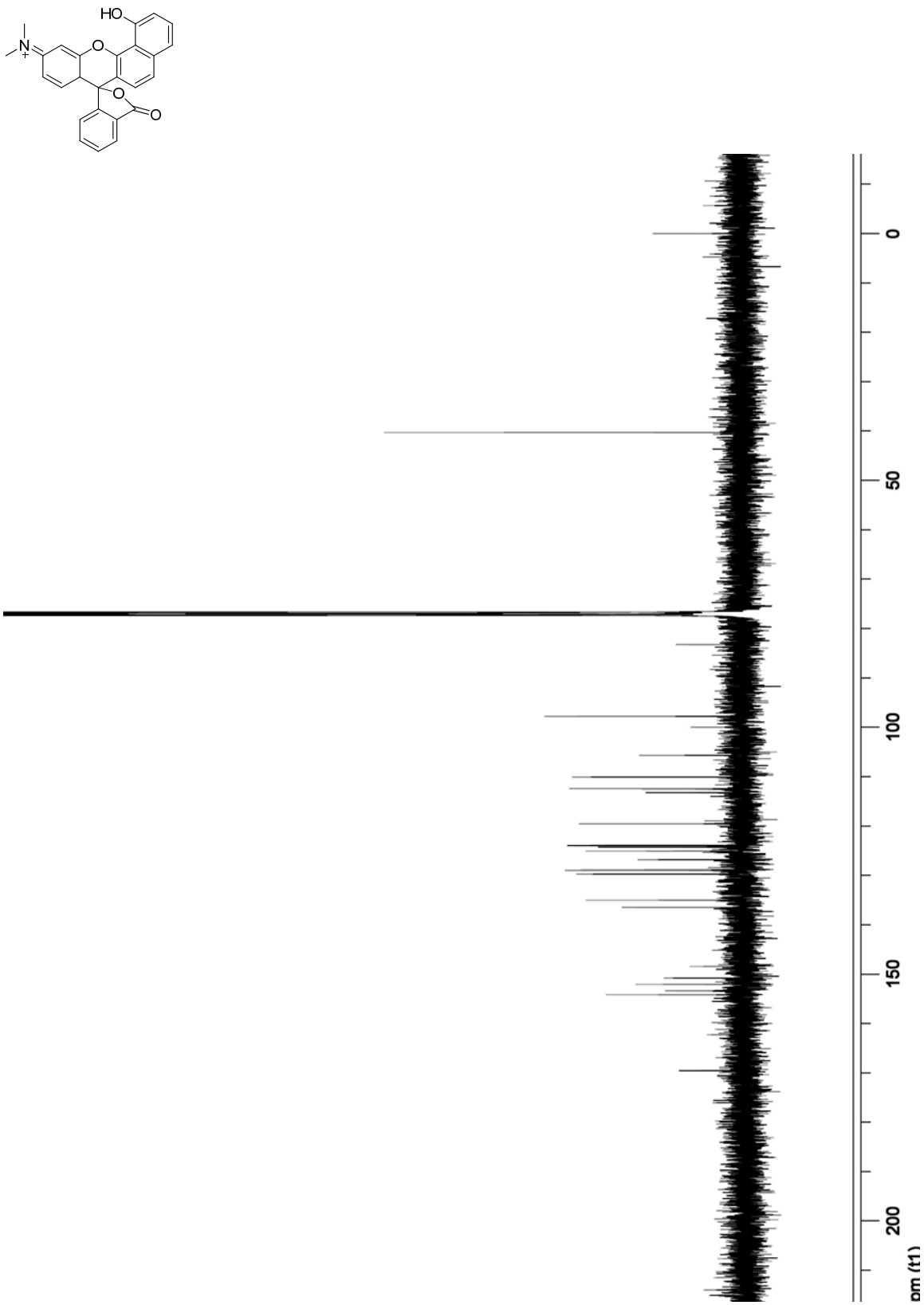


Figure 4. ^{13}C NMR of compound **40** in $CDCl_3$.