SYNTHESIS OF p-NITROPHENYL 2-O-α-D-MANNOPYRANOSYL-α-D-MANNOPYRANOSIDE AND p-NITROPHENYL 6-O-α-D-MANNOPYRANOSYL-α-D-MANNOPYRANOSIDE

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ABSTRACT

Crystalline 2-O-α-D-mannopyranosyl-β-D-mannopyranose octaacetate was synthesized by condensation of tetra-O-acetyl-α-D-mannopyranosyl bromide (3) with 1,3,4,6-tetra-O-acetyl-β-D-mannopyranose. 6-O-α-D-Mannopyranosyl-α-D-mannopyranose octaacetate was prepared by condensation of 3 with 1,2,3,4-tetra-O-acetyl-6-O-trityl-α-D-mannopyranose. Fusion of each mannobiosyl octaacetate with p-nitrophenol was followed by deacetylation, to give the corresponding p-nitrophenyl (1→2)- and (1→6)-α-D-mannobioside.

INTRODUCTION

Early studies by Avery and co-workers[1,2] established that simple sugars, normally nonantigenic, acquire carbohydrate-specific antigenicity when conjugated to protein carriers. The p-nitrophenyl D-mannobiosides now described were reduced, and the amines were employed in the formation of mannobioside–protein conjugates; the conjugates serve as antigens for the induction of antibodies sharing the D-mannopyranose-binding specificity[3,4] of a lectin, concanavalin A.

RESULTS AND DISCUSSION

The reaction sequence was so devised that condensation of the monosaccharide units occurred with readily available starting-materials early in the synthesis of both disaccharides (see Schemes 1 and 2). Using the reaction conditions of Bahl and co-workers[5], 1,3,4,6-tetra-O-acetyl-β-D-mannopyranose (4, ref. 6) readily condensed with 3 to afford 2-O-α-D-mannopyranosyl-β-D-mannopyranose octaacetate (5).

A procedure developed by Lönngren and Svensson for the synthesis of 6-O-β-D-galactopyranosyl-D-mannose[7] was adapted for the synthesis of 6-O-α-D-mannopyranosyl-α-D-mannopyranose octaacetate (10) (see Scheme 2). The detritylation

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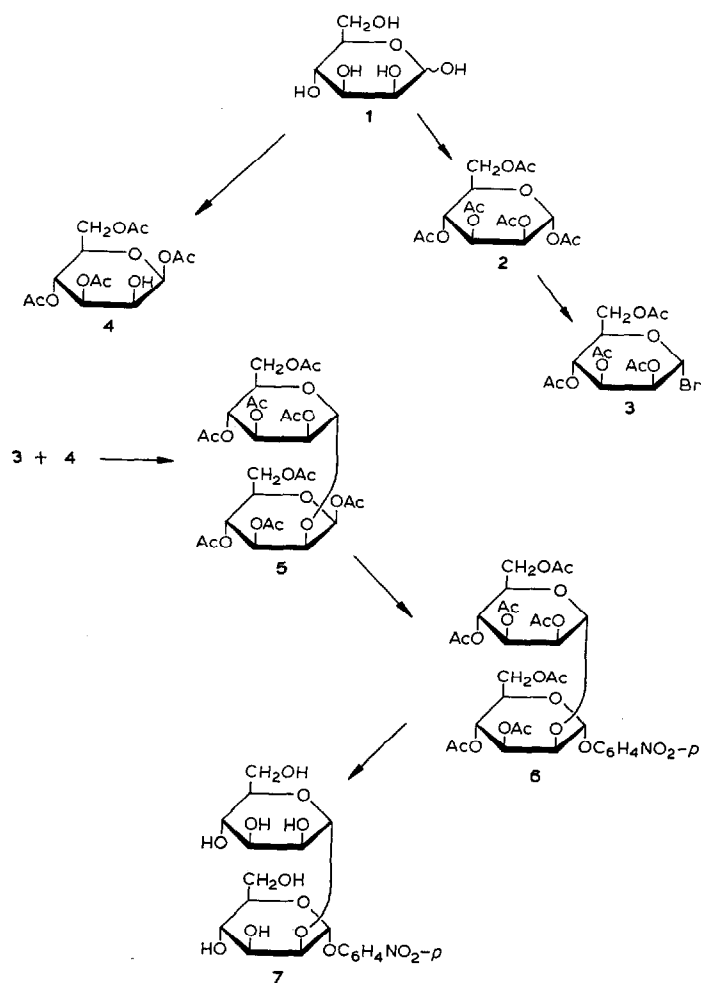
Scheme 1

step was incorporated into the condensation reaction of 1,2,3,4-tetra-O-acetyl-6-O-trityl-α-D-mannopyranose (9) with 3, to yield the corresponding (1→6)-α-D-mannobiose octaacetate (10) directly.

A zinc chloride-catalyzed, high-temperature, fusion technique[8] was employed for the condensation of p-nitrophenol with the (1→2)- and (1→6)-α-D-mannobiose octaacetate. The p-nitrophenyl hepta-O-acetyl-(1→2)- and -(1→6)-α-D-mannobiosides were subsequently deacetylated, and the free glycosides characterized. Following reduction to the corresponding amine, the (1→2)-α-D-mannobioside hapten was separately conjugated to bovine serum albumin and keyhole limpet hemocyanin via diazonium and phenyl isothiocyanate reactions[4].
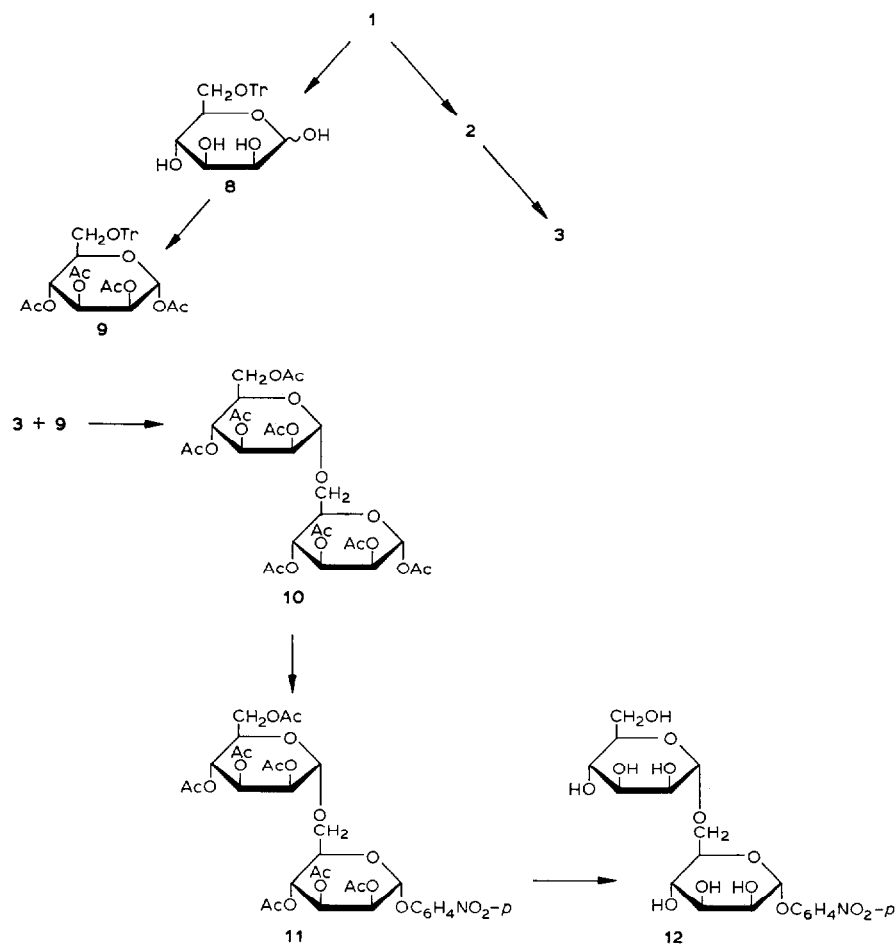
Scheme 2

EXPERIMENTAL

General. — Melting points were determined with a Fisher–Johns apparatus. Optical rotations were determined with a Rudolph model 80 polarimeter. Evaporations were conducted *in vacuo* at 35–40° in a Büchi Rotavapor. Compounds were dried by being kept for 3 to 6 h in a vacuum oven at 40–45°. Acid hydrolyses were performed in a Scientific Products module heater No. 2025. Elemental analyses were made in duplicate, either by Spang Microanalytical Lab., Ann Arbor, Michigan, or Galbraith Lab., Inc., Knoxville, Tennessee.

All commercial compounds used in this study were of A.C.S.-certified, reagent grade, or the best quality available. Chromatographic standards of 2-*O*-α-D-mannopyranosyl-β-D-mannopyranose octaacetate and 2-*O*-α-D-mannopyranosyl-D-mannose were provided by Dr. O. P. Bahl, SUNY, Buffalo, N.Y. D-Mannose was purchased

from Pfanstiehl Lab., Inc., Waukegan, Illinois. *p*-Nitrophenyl α-D-mannopyranoside was purchased from Calbiochem, San Diego, California, and *p*-nitrophenyl β-D-mannopyranoside from Sigma Chemical Co., St. Louis, Missouri. Dr. A. Misaki, Osaka University, Osaka, Japan, generously provided the jack-bean α-D-mannosidase and the methylated, reduced, and acetylated g.l.c. standards.

Infrared spectra were recorded with a Perkin–Elmer model 237 spectrophotometer. Circular dichroism studies were made, with the assistance of Dr. R. Zand, with a Jasco ORD/CD UV-5 spectrophotometer. Gas–liquid chromatography (g.l.c.) was performed on the methylated alditol acetates[9] with a Hewlett–Packard F and M model 402 gas chromatograph, in a column of 3% of ECNSS-M Gas Chrom Q (Applied Science Lab, Pa.) with helium as the carrier gas (40 mL.min^{-1}) and a column temperature of 180°.

Thin-layer chromatography was performed on glass plates coated with Silica Gel G (0.4 mm thick; E. Merck, Applied Science Lab., Inc.). The solvents employed were: (*A*) diethyl ether and (*B*) 9:6:3:1 butanol–acetic acid–diethyl ether–water. Silicic acid chromatography was performed on columns of Bio-Sil A (100–200 mesh; Bio-Rad, Richmond, Calif.). The solvents used were: (*C*) 5:2 benzene–ether, (*D*) 1:1 benzene–ether, and (*E*) 1:1 ethyl acetate–petroleum ether.

Whatman No. 1 paper was used for qualitative, descending, paper chromatography. Whatman No. 3MM paper was used for preparative work. The following solvents were used: (*F*) butanone–water azeotrope, (*G*) 10:4:3 ethyl acetate–pyridine–water, and (*H*) 5:3:2 butanol–pyridine–water.

Thin-layer chromatograms were routinely visibilized by spraying with 20% (v/v) conc. sulfuric acid–ethanol and then charring at 120°. Reversible visibilization of sugars was accomplished by development in a chromatography tank whose atmosphere was iodine-saturated. The *p*-nitrophenyl glycosides on thin-layer and paper chromatograms were detected with an ultraviolet lamp (model F-91, Ultra Violet Prod., Inc.). Reducing sugars were detected on the paper chromatograms with a silver nitrate spray[10]. Alternatively, acid-labile glycosides and reducing sugars were visibilized with a *p*-anisidine hydrochloride spray-reagent[11]. The 2-*O*-substituted disaccharides were detected with triphenyltetrazolium chloride spray-reagent[12].

2-*O*-α-D-*Mannopyranosyl-β*-D-*mannopyranose octaacetate* (**5**). — Freshly distilled acetonitrile (13 mL), Hg(CN)$_2$ (1.23 g), Hg(Br)$_2$ (1.78 g), and dry **4** (ref. 6) (3.65 g) were combined in a 100-mL, round-bottomed flask. Bromide[13,14] **3** (5.7 g, as a syrup from which purified acetonitrile had been three times evaporated) was added, and the mixture was stirred magnetically for 4.5 h at room temperature, evaporated to a thick syrup *in vacuo*, and extracted with anhydrous chloroform (3 × 50 mL). The extracts were combined, washed successively with cold, M potassium bromide (3 × 25 mL) and cold water (2 × 25 mL), dried (calcium chloride), and evaporated to a thick syrup. An aliquot (~3 g) of the syrup was chromatographed on a column (1.9 × 55 cm) of Bio-Sil A. The monosaccharides were eluted with solvent *C* (1.300 L), and the disaccharide octaacetate was eluted with solvent *D* (2.200 L), the fractionation being monitored by t.l.c. (solvent *A*). Compound **5**

crystallized from a solution of the pooled, purified fractions in absolute ethanol. After nucleating crystals had been obtained, the column-fractionation step could be eliminated; crystallization proceeded directly from absolute ethanol following addition of crystals to the absolute ethanol extract (3 × 100 mL) of the crude syrup. T.l.c. (solvent A) revealed a single, slow-moving spot that co-chromatographed with authentic 5. Crystalline 5 (2.65 g, 37% yield) had m.p. 102–103°, $[\alpha]_D^{20}$ +5.7° (c 0.78, chloroform).

Anal. Calc. for $C_{28}H_{38}O_{19}$: C, 49.51; H, 5.60. Found: C, 49.44; H, 5.80.

6-O-α-D-*Mannopyranosyl-α-D-mannopyranose octaacetate* (10). — The following procedure was adapted from a method developed by Lönngren and Svensson[7] for the synthesis of 6-O-β-D-galactopyranosyl-D-mannose. In a 100-mL, round-bottomed flask, 1,2,3,4-tetra-O-acetyl-6-O-trityl-α-D-mannopyranoside[15] (9) (2.9 g, dry) was dissolved in anhydrous nitromethane (20 mL) containing silver perchlorate (1.1 g). Drierite (1.5 g) was added, the flask was cooled to 0°, and a solution of fresh 3 (2.5 g) in ice-cold, anhydrous nitromethane (10 mL) was added. The flask containing the resulting, bright-yellow mixture was stoppered, and shaken vigorously in an ice bath for 5 min; the suspension was filtered with suction, and the solid (Drierite and triphenylmethanol) was washed with cold nitromethane (50 mL). The filtrate and washings were combined, washed successively with cold, aqueous M sodium hydrogen-carbonate (3 × 75 mL) and cold water (3 × 75 mL), dried (sodium sulfate), and evaporated *in vacuo* to dryness in a tared flask. The residue (3.4 g) was applied to a column (59 × 3.8 cm) of Bio-Sil A which was then irrigated with solvent E, the fractionation being monitored by t.l.c. (solvent A). Compound 10 was eluted in fractions 80–110 (20-mL fractions), after triphenylmethanol and the monosaccharide acetates. The yield of enriched product, which still contained an impurity, was 0.47 g (14%). [Overnight deacetylation of a few mg of the crude 10 gave a product shown by t.l.c. (solvent B) and paper chromatography (solvents G and H) to contain 6-O-α-D-mannopyranosyl-D-mannose and mannose. It was decided to postpone removal of the impurity until a later step in the synthetic sequence; the enriched (1→6)-α-D-mannobiose octaacetate was used directly in the subsequent fusion with p-nitrophenol.] Compound 10 crystallized from 95% ethanol, to give a chromatographically pure product (t.l.c., solvent A) that had m.p. 162°, $[\alpha]_D^{22}$ +68.0° (c 0.97, chloroform).

Anal. Calc. for $C_{28}H_{38}O_{19}$: C, 49.51; H, 5.60. Found: C, 49.49; H, 5.70.

p-*Nitrophenyl* 2-O-α-D-*mannopyranosyl-α-D-mannopyranoside heptaacetate* (6) *and* p-*nitrophenyl* 6-O-α-D-*mannopyranosyl-α-D-mannopyranoside heptaacetate* (11). — Fusion of each disaccharide with p-nitrophenol was patterned after the synthesis of p-nitrophenyl tetra-O-acetyl-α-D-mannopyranoside[8]. For the synthesis of 6, zinc chloride (200 mg; dry, freshly fused) was placed in a 15-mL, round-bottomed flask containing p-nitrophenol (1 g) and crystalline 5 (1 g, dry), and the mixture was fused as previously reported[8]. T.l.c. (solvent A) of the neutral, washed, chloroform extract revealed the presence of starting materials, a trace of p-nitrophenyl α-D-mannopyranoside tetraacetate, and the desired 6. The dried syrup (1.79 g) was deacetylated[16] prior to purification and characterization.

Compound **11** was similarly prepared, but on a smaller scale. Zinc chloride (60 mg), *p*-nitrophenol (250 mg), and **10** (297 mg, partially purified, column-fractionated syrup) reacted to yield a heterogeneous syrup (202 mg) which was de-acetylated[16], and the product purified, and characterized.

p-*Nitrophenyl* 2-O-α-D-*mannopyranosyl*-α-D-*mannopyranoside* (**7**) *and* p-*nitro-phenyl* 6-O-α-D-*mannopyranosyl*-α-D-*mannopyranoside* (**12**). — Deacetylation of **6** and **11** proceeded satisfactorily, provided that no water or acid was present. In each case, the deacetylated syrup was applied in a narrow band to pre-washed, preparative paper-chromatograms (Whatman No. 3MM) which were then developed twice (*F*). The D-mannobiosides were located on the chromatograms by their u.v. absorbance and relatively low partition coefficients (they trailed behind *p*-nitrophenyl α-D-mannopyranoside and *p*-nitrophenol). The paper chromatograms were cut into strips, and these were eluted with water by descending chromatography. Both compound **7** (304 mg, 45% yield from the octaacetate) and **12** (18 mg, 9% yield from the octaacetate) were chromatographically pure (t.l.c., solvent *B*). The structure and anomeric configuration of each compound were determined by circular dichroism, enzymic studies, and g.l.c. of the corresponding alditol acetates[9] obtained by methylation, hydrolysis, reduction, and acetylation. Neither the *p*-nitrophenyl (1→2)- nor the (1→6)-α-D-mannobioside crystallized. The purified syrups had: **7**, $[\alpha]_D^{20}$ +85.1° (*c* 1.2, water); and **12**, $[\alpha]_D^{20}$ +114° (*c* 0.29, water).

Methylated, hydrolyzed, and acetylated *Penicillium* galactomannan was used as the standard for g.l.c. Detection of equimolar amounts of 1,5-di-*O*-acetyl-2,3, 4,6-tetra-*O*-methylmannitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol provided evidence for a (1→2)-linkage in **7**. The presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol in a separate g.l.c. study provided similar evidence for a (1→6)-linkage in **12**.

Circular dichroism spectra were recorded at ambient temperature in cells having a 1-cm path-length. The *p*-nitrophenyl derivatives (0.1mM in distilled water) exhibited absorption maxima near 310 nm. Positive Cotton-effects of *p*-nitrophenyl α-D-mannopyranoside and the two *p*-nitrophenyl mannobiosides (**7** and **12**) contrasted with a negative Cotton-effect for *p*-nitrophenyl β-D-mannopyranoside, providing evidence[17] for the α configuration of the anomeric carbon atom to which the *p*-nitrophenoxy group was attached in the biosides.

Enzymic studies. — Purified, jack-bean α-D-mannosidase, prepared by Dr. A. Misaki, was employed in the enzymic analysis; this enzyme is specific for α-D-mannosidic linkages; and β-D-mannosides, α- or β-D-glucosides, and α- or β-D-galactosides are not hydrolyzed by the enzyme[18]. Compounds **7**, **12**, *p*-nitrophenyl α-D-mannopyranoside, and *p*-nitrophenyl β-D-mannopyranoside were each incubated in the presence of the enzyme in 0.05M citrate buffer (pH 4.5) for 24 h at room temperature, prior to heat inactivation during 10 min at 100° (boiling-water bath). The enzymic digest was concentrated, the concentrate extracted with methanol, and the extract chromatographed by t.l.c. (solvent *B*) and by paper chromatography. The paper chromatograms were developed by two-dimensional, descending, paper chromato-

graphy, twice with solvent *F* (to separate the *p*-nitrophenyl derivatives), and once at an angle of 90° with solvent *G* (to separate mannose from any mannobiose that might be present). The enzymic hydrolyses of the *p*-nitrophenyl mannobiosides proceeded all the way to *p*-nitrophenol and mannose, indicating the α configuration both of the inter-D-mannosidic and the anomeric linkages. As expected, the β-D-linked *p*-nitrophenyl mannopyranoside was not hydrolyzed by the jack-bean α-D-mannosidase.

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