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THE SYNTHESIS, BINDING, AND AGGLUTINATING ACTIVITY OF 6-AMINOHEXYL β-D-MANNOPYRANOSIDE*

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ABSTRACT

6-Aminohexyl β-D-mannopyranoside was synthesized by inversion of configuration at C-2 of the D-glucoside moiety of 6-aminohexyl β-D-glucopyranoside, and coupled to beads of cyanogen bromide-activated Sepharose 4-B. This polymer was found to be inactive in the affinity chromatography of concanavalin A.

INTRODUCTION

For enzyme purification by affinity chromatography, R. Barker *et al.*[1] introduced agarose derivatives in which a glycoside ligand is attached through an extended, linear C_6-chain to the matrix, thus decreasing the possible interactions between matrix and enzyme proteins. Chipowsky and Lee[2] applied a similar synthesis to 1-thio-β-D-glucopyranosides and -D-galactopyranosides which were subsequently linked to crosslinked dextran[3]. For the preparation of ligand–resins for the purification of lectins by affinity chromatography, the synthesis by Barker *et al.*[1] was extended to α- and β-D-glucopyranoside, α-D-mannopyranoside, and 2-acetamido-2-deoxy-α-D-glucopyranoside derivatives[4].

Most *N*-glycoproteins [glycoproteins having a 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-D-glucopyranosylamine carbohydrate–protein linkage] have a structure that includes a β-D-mannopyranosyl group linked at O-4 of the di-*N*-acetylchitobiosyl

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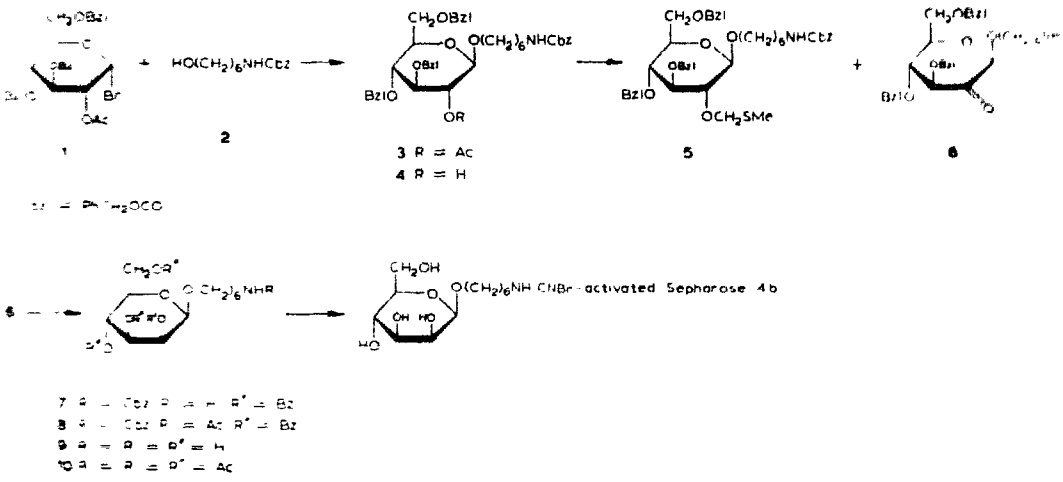
residue[5]. Until now, no lectin has been known to react with this residue and, in order to search for such a biological reagent, 6-aminohexyl β-D-mannopyranoside was synthesized and coupled with a Sephadex polymer, and the binding and agglutinating activity of the resulting product was determined.

Due to the difficulties encountered in the synthesis of 1,2-*cis*-glycosides, the 6-aminohexyl β-D-mannopyranoside was prepared from the suitably protected and conveniently obtained 1,2-*trans*-glycoside derivative **3** by inversion of configuration at C-2.

RESULTS AND DISCUSSION

Koenigs–Knorr condensation of 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl bromide[6,7] (**1**) with 6-(benzyloxycarbonylamino)-1-hexanol[2] (**2**) in the presence of mercuric cyanide gave **3** in 62% yield. *O*-Deacetylation of **3** with a catalytic amount of sodium methoxide afforded the crystalline derivative **4**, the i.r. spectrum of which showed a hydroxyl-group absorption at 3450 cm^{-1} and no *O*-acetyl group absorption. Oxidation of **4** with dimethyl sulfoxide–acetic anhydride according to the method of Albright and Goldman[8] gave a mixture of the D-*arabino*-hexopyranosid-2-ulose (**6**) and the 2-*O*-(methylthio)methyl derivative **5**. The latter kind of derivative is usually[9] a by-product of this method of oxidation. Separation of **5** from **6** was not achieved by repeated chromatography on a column of silica gel, but was obtained by preparative, thin-layer chromatography on plates of silica gel. The hexosid-2-ulose **6** showed characteristic, carbonyl-group absorption at 1745 cm^{-1}. Stereospecific[10] reduction of **6** with sodium borohydride gave the β-D-mannopyranoside **7**, which showed R_F and optical rotation values different from those of the corresponding β-D-glucoside derivative **4**. The i.r. spectrum of **7** lacked the carbonyl-group absorption of the parent hexosidulose, and showed a hydroxyl-group absorption at 3350 cm^{-1}. Acetylation of **7** gave the 2-*O*-acetyl derivative **8**, having physical constants markedly different from those of the β-D-glucoside analog **3**



Hydrogenolysis of the *N*-benzyloxycarbonyl and *O*-benzyl groups of **7** in the presence of palladium-on-charcoal gave 6-aminohexyl β-D-mannopyranoside (**9**) as an unstable, amorphous material giving a positive ninhydrin reaction[11]. Compound **9** was characterized as the *N*-acetyl-tetra-*O*-acetyl derivative (**10**).

Methanolysis of **9**, followed by per(trimethylsilyl)ation and g.l.c analysis, indicated the presence of methyl α- and β-D-mannopyranoside in the methanolyzate. This result, together with the difference in physical constants of **3** and **4** (compared to those of **8** and **7**, respectively), indicates the stereoselectivity of the borohydride reduction of the hexosid-2-ulose **6** to the β-D-mannoside **7**. This is in agreement with results previously described[6,10].

The 6-aminohexyl derivative **9** was immediately coupled to beads of cyanogen bromide-activated Sepharose 4B according to the method of Barker *et al.*[1]. The ligand–polymer was tested with concanavalin A, both for binding and for agglutinating properties. It was found to be inactive, thus indicating that the lectins isolated with this reagent would be specific for the β anomer of D-mannose.

EXPERIMENTAL

General methods. — Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points". Optical rotations were determined in 1-dm, semimicro tubes, with a Perkin–Elmer Model 141 polarimeter; the chloroform used was analytical-reagent grade, and contained ~0.75% of ethanol. I r. spectra were recorded with a Perkin–Elmer Model 237 spectrophotometer. G.l.c. was performed with a Perkin–Elmer Model 900 gas chromatograph equipped with a flame-ionization detector, on a column of 3% of OV-17 on Gas Chrom Q, with nitrogen as the carrier gas. Column chromatography was performed on Silica gel Merck (70–325 mesh, E. Merck, Darmstadt, Germany), used without pretreatment. The ratio of weight of substance to weight of silica gel was 1:80 to 1:120. The ratio of the column diameter to its length was 1:8 to 1:18. The volume of the fractions eluted was 3–4 ml per g of substance to be chromatographed. The homogeneity of the products was verified by t.l.c. on plates precoated with Silica Gel G (E. Merck; layer thickness 0.25 mm); the solvent travel-distance was ~5 cm. The spots were detected by spraying the plates with 1:1:18 (v/v) anisaldehyde–conc. sulfuric acid–ethanol[12], and heating them on a hot plate for a few minutes. Evaporations were conducted *in vacuo*, with the bath temperature below 45°. Solutions (<5 ml) in volatile solvents were evaporated under a stream of nitrogen. Microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

6-(Benzyloxycarbonylamino)hexyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranoside (**3**). — To a solution of 6-(benzyloxycarbonylamino)-1-hexanol[2] (**2**, 1.325 g) in 1:1 (v/v) benzene–nitromethane (180 ml) was added mercuric cyanide (2 g), and 50 ml of the solvents were distilled at atmospheric pressure. The mixture was cooled to room temperature, treated with a solution of 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl bromide[6,7] (**1**, 3 g) in dry benzene (20 ml), and stirred for

72 h. The mixture was filtered on a Celite layer, and the inorganic residue washed with dichloromethane (20 ml). The filtrate was diluted with dichloromethane (300 ml), and successively washed with water (2 × 100 ml), saturated $NaHCO_3$ (2 × 50 ml), saturated KI (2 × 50 ml), and water (2 × 100 ml), dried (Na_2SO_4), and evaporated, to give a syrup, which was chromatographed on a column of silica gel with dichloromethane, affording 2.25 g of **3** (62%) as a syrup, $[\alpha]_D^{20}$ −1.1° (*c* 1.13, chloroform): ν_{max}^{film} 3350 (NH), 1740 (OAc), 1715 (Amide I), 1525 (Amide II), 750, and 680 cm^{-1} (Ph); t.l.c. in 49:1 (v/v) chloroform–ethanol: R_F 0.44.

Anal. Calc. for $C_{43}H_{51}NO_9 \cdot 0.5H_2O$: C, 70.27; H, 7.13, N, 1.91; O, 20.68. Found: C, 69.90, H, 6.90; N, 1.81; O, 21.06.

*6-(Benzyloxycarbonylamino)hexyl 3,4,6-tri-*O*-benzyl-β-*D*-glucopyranoside* (**4**). — A solution of **3** (1.815 g) in 1:1 (v/v) dichloromethane–methanol (30 ml) was treated with 0.1M sodium methoxide in methanol (1 ml) for 6 h at room temperature. The solution was passed through a column of Dowex 50 (H^+) cation-exchange resin, and the eluate evaporated. Crystallization of the residue from methanol gave 1.47 g (86%) of **4**, m.p. 81–83°, $[\alpha]_D^{22}$ −4.0° (*c* 1.8, chloroform); ν_{max}^{KBr} 3450 (shoulder, OH), 3310 (NH), 1680 (Amide I), 1550 (Amide II), 730, and 680 cm^{-1} (Ph); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.62.

Anal. Calc. for $C_{41}H_{49}NO_8$: C, 72.01; H, 7.23; N, 2.05; O, 18.72. Found: C, 71.91; H, 7.22; N, 2.11; O, 18.65.

*6-(Benzyloxycarbonylamino)hexyl 3,4,6-tri-*O*-benzyl-2-*O*-[(methylthio)methyl]-β-*D*-glucopyranoside* (**5**) *and 6-(benzyloxycarbonylamino)hexyl 3,4,6-tri-*O*-benzyl-β-*D*-arabino-hexopyranosid-2-ulose* (**6**). — A solution of **4** (2.0 g) in 1:2 (v/v) acetic anhydride–dimethyl sulfoxide (48 ml) was kept for 20 h at room temperature under an atmosphere of nitrogen. The mixture was then evaporated, and a solution of the residue in chloroform (200 ml) was washed with water (3 × 50 ml), dried (Na_2SO_4), and evaporated. The residue was chromatographed on a column of silica gel, with chloroform as the eluent, but only partial separation of **5** from **6** was obtained. Successful separation was achieved by preparative, thin-layer chromatography on plates (20 × 20 cm) precoated with Silica Gel G (E. Merck; 2-mm layer thickness) with 49:1 (v/v) chloroform–ethanol, to give 48 mg (22%) of **5** as the fast-moving fraction, and 1.2 g (61%) of **6** as the slow-moving fraction. Crystallization of **5** from methanol gave needles, m.p. 90–91°, $[\alpha]_D^{20}$ +3.6° (*c* 3.9, chloroform); ν_{max}^{KBr} 3310 (NH), 1680 (Amide I), 1545 (Amide II), 745, and 680 cm^{-1} (Ph); t.l.c. in 29:1 chloroform–ethanol: R_F 0.56.

Anal. Calc. for $C_{43}H_{53}NO_8S$: C, 69.42; H, 7.18; N, 1.88; S, 4.31. Found: C, 69.49; H, 7.18; N, 1.95; S, 4.40.

Crystallization of **6** from ethanol gave needles, m.p. 70–71°, $[\alpha]_D^{20}$ −28° (*c* 2.9, chloroform); ν_{max}^{KBr} 3305 (NH), 1745 (C=O), 1680 (Amide I), 1550 (Amide II), 7.25, and 680 cm^{-1} (Ph); t.l.c. in 29:1 (v/v) chloroform–ethanol: R_F 0.36.

Anal. Calc. for $C_{41}H_{47}NO_8$: C, 72.23; H, 6.95; N, 2.05; O, 18.77. Found: C, 72.74; H, 6.98; N, 2.04; O, 18.68.

6-(Benzyloxycarbonylamino)hexyl 3,4,6-tri-O-benzyl-β-D-mannopyranoside (**7**). — A solution of **6** (1.36 g) in *N,N*-dimethylfornamide (15 ml) was treated with sodium borohydride (0.3 g) for 10 h at room temperature. The mixture was diluted with methanol (50 ml), and passed through a column of Dowex 50 (H^+) cation-exchange resin. Evaporation of the solvents, followed by repeated addition and distillation of methanol, gave a syrup that was chromatographed on a column of silica gel with 49:1 (v/v) chloroform–ethanol, to give 1.135 g (83%) of **7** as a syrup, $[\alpha]_D^{20}$ −16° (*c* 2.5, chloroform); ν_{max}^{film} 3550 (OH), 3350 (NH), 1670 (Amide I), 1530 (Amide II), 740, and 685 cm^{-1} (Ph); t.l.c. in 49:1 (v/v) chloroform–ethanol: R_F 0.27.

Anal. Calc. for $C_{41}H_{49}NO_8 \cdot H_2O$: C, 70.16; H, 7.32; N, 2.00; O, 20.52. Found: C, 70.37; H, 7.13; N, 2.05; O, 20.58.

6-(Benzyloxycarbonylamino)hexyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-mannopyranoside (**8**). — A solution of **7** (175 mg) in pyridine (3 ml) was treated with acetic anhydride (2 ml) for 16 h at room temperature. The mixture was evaporated, and the residue dried by several additions and distillations of toluene. Chromatography of the syrupy residue on a column of silica gel with 49:1 (v/v) chloroform–ethanol gave 148 mg (82%) of **8** as a syrup, $[\alpha]_D^{20}$ −31° (*c* 2.8, chloroform); ν_{max}^{film} 3350 (NH), 1735 (OAc), 1710 (Amide I), 1525 (Amide II), 730, and 680 cm^{-1} (Ph); t.l.c. in 49:1 (v/v) chloroform–ethanol: R_F 0.40.

Anal. Calc. for $C_{43}H_{51}NO_9$: C, 71.15; H, 7.08; N, 1.93; O, 19.84. Found: C, 71.00; H, 7.08; N, 1.87; O, 19.68.

6-Aminohexyl β-D-mannopyranoside (**9**). — A solution of **7** (900 mg) in 96% ethanol (50 ml) was hydrogenolyzed with hydrogen in the presence of 10% Pd/C (200 mg) for 24 h at room temperature and 2 atm. The mixture was filtered on a Celite layer, and the filtrate was hydrogenolyzed twice more, as just described. Evaporation of the solvent gave a foam (294 mg, 82%) that showed a positive ninhydrin[11] reaction; ν_{max}^{KBr} 3375 (broad, OH and NH) and 1625 cm^{-1} (NH_2). Compound **9** was unstable, and became brown when kept at room temperature. It was, therefore, used directly for the preparation of the *N*-acetyl-tetra-*O*-acetyl derivative **10**, and for coupling with cyanogen bromide-activated Sepharose 4B.

Methanolysis of **9** (~50 μg) with M HCl in methanol (1 ml) for 16 h at 65° was followed by evaporation. The residue was per-*O*-(trimethylsilyl)ated, and the product injected onto a stainless-steel column (300 × 0.2 cm) packed with Gas Chrom Q (80–100 mesh) coated with 3% of OV-17 (Applied Science Laboratories Inc., P.O. Box 440, State College, PA 16801) and programmed for a rise of 6.5°/min from 120 to 300°. A major and a minor peak (ratio ~11:1) at 15.75 and 16.25 min, respectively, corresponding to those of methyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-α- and -β-D-mannopyranoside, respectively, were observed.

6-Acetamidohexyl 2,3,4,6-tetra-O-acetyl-β-D-mannopyranoside (**10**). — To a solution of **9** (70 mg) in pyridine (2 ml) was added acetic anhydride (2 ml); the mixture was kept for 16 h at room temperature, and then evaporated under diminished pressure. The residue was dried by several additions and distillations of toluene. Chromatography on a column of silica gel with 19:1 (v/v) chloroform–ethanol gave

89 mg (73%) of **10** as a syrup, $[\alpha]_D^{21}$ −32° (*c* 3.8, chloroform): ν_{max}^{film} 3300 (NH), 1740 (OAc), 1650 (Amide I), and 1550 cm^{-1} (Amide II); t.l.c. in 19:1 (v/v) chloroform–ethanol: R_F 0.26.

Anal. Calc. for $C_{22}H_{35}NO_{11}$: C, 53.93; H, 7.21; N, 2.86; O, 35.95. Found: C, 53.88; H, 7.21; N, 2.96; O, 35.87.

Coupling of 6-aminohexyl β-D-mannopyranoside (**9**) *to Sepharose 4B.* — A solution of **9** (10 mg) in a buffer consisting of 1:1 (v/v) 0.5M $NaHCO_3$ and 0.5M NaCl (5 ml) was added to swollen, cyanogen bromide-activated Sepharose 4B (3.5 ml, Pharmacia Fine Chemicals, Piscataway, N.J. 08854). The suspension was kept for 3 h at room temperature with constant, end-over-end mixing. Coupling was monitored by disappearance of **9** from the supernatant liquor, as determined by the ninhydrin reaction[11]. After coupling was complete, the beads were washed with the same buffer, and the unreacted sites were blocked by treatment with M 2-aminoethanol (15 ml, pH 8) for 2 h at room temperature. The beads were alternately washed with 0.1M sodium acetate–M NaCl (pH 5.0) and 0.1M $Na_2B_4O_7$–M NaCl (pH 8.0) buffers for three complete cycles. The ninhydrin reaction demonstrated a final concentration of coupled ligand of 6.86 mmoles/liter of wet beads. This value was consistent with the determination of the ligand released by alkaline hydrolysis[3].

Determination of binding and agglutination of concanavalin A by Sepharose 4B-6-aminohexyl β-D-mannopyranoside. — Tritium-labeled concanavalin A (New England Nuclear, Boston, Massachusetts 02118) was diluted with unlabeled lectin (Miles Laboratories Inc., Kankakee, Ill. 60901), and subsequently purified by affinity chromatography[13] on Sephadex G-200. [^{3}H]Concanavalin A (10^5 c.p.m., 100 μg) in 0.15M NaCl–6.5mM NaH_2PO_4–14.5mM Na_2HPO_4 (pH 7.2, 100 μl) was added to a suspension of the Sepharose 4B–6-aminohexyl β-D-mannopyranoside beads (1×10^6) in the same buffer (400 μl), and incubated for 15 min at 37°. The beads were washed with an excess of the buffer, collected by centrifugation at low speed, and dried. Bound lectin was measured by counting in toluene–POPOP scintillator (New England Nuclear), and nonspecific lectin-binding was determined by measurement of bound lectin in the presence of 50 mmoles of methyl α-D-mannopyranoside in the reaction buffer. No specific binding of the lectin to the beads could be detected.

Similarly, no agglutination could be detected, either macro- or microscopically, after incubating the beads (1×10^6) with concanavalin A (250 μg) in NaCl–phosphate buffer (pH 7.2, 500 μl).

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