Carbohydrate Research, 89 (1981) 301–308
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

STUDIES ON THE STRUCTURE OF A POLYSACCHARIDE FROM *Epidermophyton floccosum* AND APPROACH TO A SYNTHESIS OF THE BASIC TRISACCHARIDE REPEATING UNITS

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(Received February 5th, 1980; accepted for publication in revised form, June 17th, 1980)

ABSTRACT

An alkali-soluble polysaccharide, designated as S-Iawe, has been isolated from the mycelia of *Epidermophyton floccosum*. Methylation, periodate oxidation, and acetolysis studies suggested that S-Iawe is composed of (1→6)-*O*-α-D-mannopyranosyl-(1→6)-*O*-[α-D-mannopyranosyl-(1→2)]-*O*-α-D-mannopyranosyl repeating units. Condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide with methyl 3-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside in the presence of mercuric cyanide gave in 70% yield methyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside. Condensation of the debenzylidenated disaccharide with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide afforded the corresponding trisaccharide repeating unit.

INTRODUCTION

Our previous paper[1] on the components of the cell wall of *Trichophyton mentagrophytes*, which is a dermatophyte that causes tinea pedis and other cutaneous lesions in humans[2], reported the isolation of a D-mannan composed of (1→6)-*O*-α-D-mannopyranosyl-(1→6)-*O*-[α-D-mannopyranosyl-(1→2)]-*O*-α-D-mannopyranosyl repeating units, the average chain-length of which was determined to be ~150 mannose units. We now report structural studies on the polysaccharide component of the cell wall of *Epidermophyton floccosum*, which has an infectivity weaker than that of *T. mentagrophytes*. The polysaccharides of both dermatophytes gave a single precipitin band on immunodifusion against antiserum. In addition, we report the synthesis of a trisaccharide corresponding to the repeating unit, and also that of a tetrasaccharide.

RESULTS AND DISCUSSION

The S-Iawe fraction was isolated from *Epidermophyton floccosum* by use of Fehling solution as previously described[1]. Recovery of the polysaccharides from their insoluble copper complexes yielded compounds showing a single, sharp peak in

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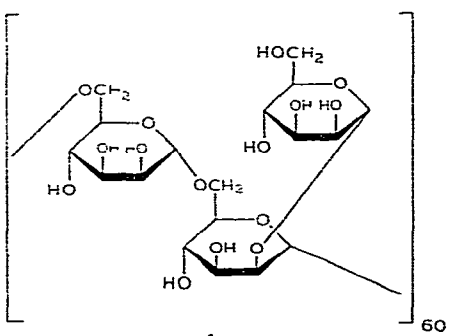
electrophoresis and high-performance liquid chromatography. The high, positive specific rotations indicated that the majority of the linkages in the S-Iawe were in the α-D configuration. The major constituent monosaccharide was D-mannose.

Methylation analysis of S-Iawe yielded approximately equal amounts of methyl 2,3,4,6-tetra-*O*-methylmannopyranoside, methyl 2,3,4-tri-*O*-methylmannopyranoside, and methyl 3,4-di-*O*-methylmannopyranoside, which were identified by comparison with authentic samples. S-Iawe was acetolyzed, and the resulting acetates were chromatographed on silica gel to give a disaccharide in 50% yield and the same amount of D-mannose pentaacetate. The disaccharide was *O*-deacetylated and converted into a per-*O*-methyl derivative. Methanolysis of this derivative gave methyl 2,3,4,6-tetra- and 3,4,6-tri-*O*-methylmannopyranosides in a 1:1 ratio. When S-Iawe was subjected to periodate oxidation, 1.70 mol of periodate was consumed and 0.66 mol of formic acid liberated per D-mannose residue. These results are in good agreement with the theoretical values deduced from methylation analysis, which indicates a periodate consumption of 1.67 mol/mol. From these results, it is evident that the immunologically active polysaccharides from *E. floccosum* contain the same repeating structural unit **1**.

The synthesis of the oligosaccharide **8** corresponding to the repeating unit **1** was started by condensation of methyl 3-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside (**2**), which was prepared from methyl *exo*-2,3:4,6-di-*O*-benzylidene-α-D-mannopyranoside according to the method of Lipták *et al.*[3], with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**3**) in the presence of mercuric cyanide. This gave the fully protected, α-D-linked disaccharide **4**, in 70% yield from **2**. The ^{1}H-n.m.r. spectrum of **4** showed signals characteristic for two phenyl groups, one benzylidene proton, and four acetyl groups. Compound **4** was *O*-debenzylidenated with 50% acetic acid to give methyl 3-*O*-benzyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**5**) in 95% yield.

Similarly, **5** and an equivalent amount of bromide **3** in nitromethane containing mercuric cyanide gave, after column chromatography, a 60% yield (based on **5**) of the trisaccharide, methyl 3-*O*-benzyl-2,6-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**6**). Hydrogenolysis of **6** in tetrahydrofuran gave, in 95% yield, methyl 2,6-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-α-



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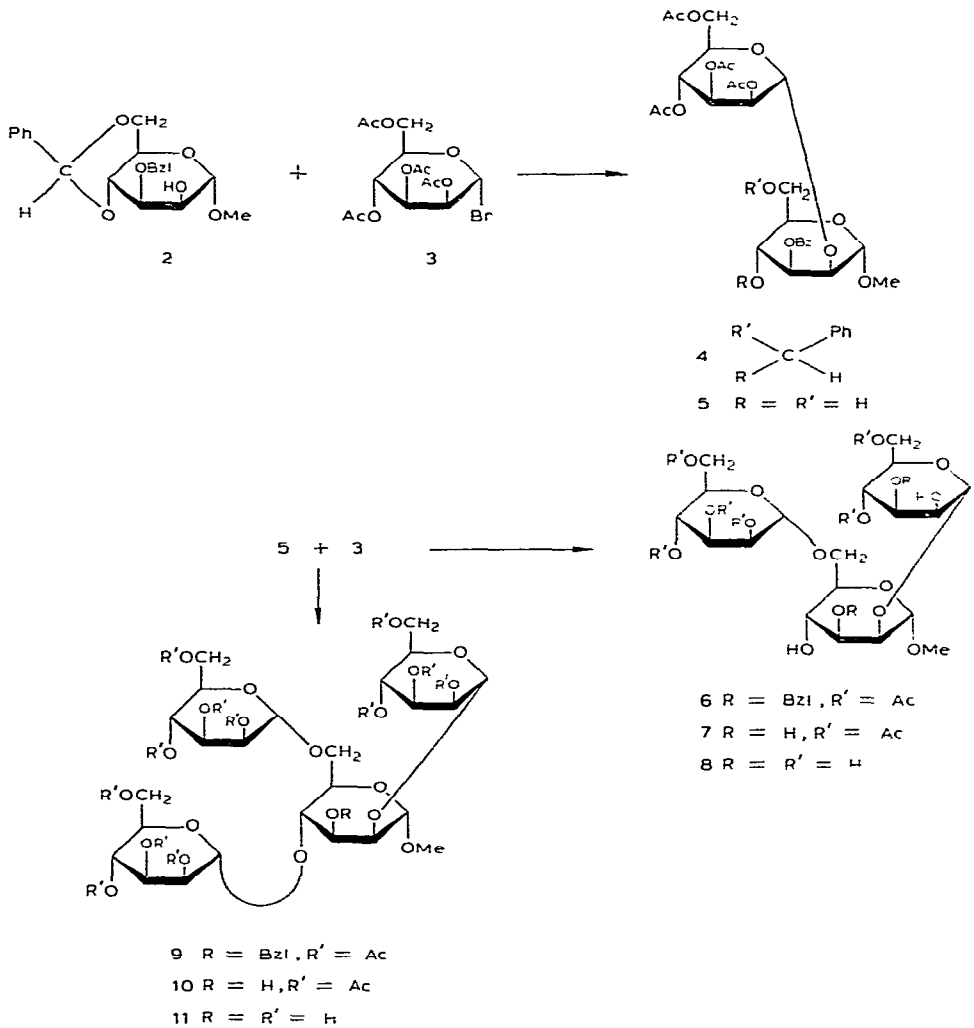

D-mannopyranoside (7), further *O*-deacetylated with triethylamine in 90% yield into methyl 2,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (8). The ^{1}H-n.m.r. data indicated three anomeric protons, and the ^{13}C-n.m.r. data (Table I) three anomeric carbon atoms at δ 102.9 (C-1′), 100.4 (C-1), and 100.3 (C-1″), respectively. Introduction of α-D-mannopyranosyl groups at O-2 and O-6 deshielded C-2 by δ 8.4, and C-6 by 4.4, as compared with methyl α-D-mannopyranoside[4].

The ^{13}C-n.m.r. data provide substantial confirmation that the synthetic trisaccharide **8** is indeed methyl 2,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside. The α-D configuration was indicated by comparison of the molecular rotation with the sum of the molecular rotations of the components[5] (Table II). Permethylation of **8** and methanolysis gave two methylated sugars, which were identified by g.l.c.

TABLE I

^{13}C-N.M.R. SPECTRAL DATA (δ) FOR **8**, **11**, AND THEIR DERIVATIVES

Carbon atom	*Chemical shifts of compounds*						
	4	**6**	**7**	**8**	**9**	**10**	**11**
C-1	99.5	99.3	99.9	100.4	99.4	99.2	101.9
C-2	76.7	79.7	80.0	79.6	79.7	79.4	80.3
C-3	75.6	75.1	69.4	71.3	74.9	70.8	70.9
C-4	79.0	69.8	69.8	70.9	77.5	76.3	75.2
C-5	63.8	69.5	69.1	74.2	69.8	69.7	74.4
C-6	68.6	71.2	71.3	66.5	70.1	69.8	67.4
C-1′	101.5	99.8	99.9	102.9	99.7	99.9	103.4
C-2′	68.9	69.1	69.1	71.5	69.3	69.3	71.2
C-3′	68.9	68.5	66.7	71.2	69.1	69.1	70.9
C-4′	66.3	66.3	66.3	67.4	66.5	66.2	67.4
C-5′	69.2	69.1	69.1	73.7	69.6	69.6	74.2
C-6′	62.5	62.7	62.7	61.8	62.8	62.7	61.8
C-1″		97.3	97.3	100.3	97.3	97.5	99.9
C-2″		69.1	68.6	71.3	69.1	69.1	71.2
C-3″		67.1	66.7	70.9	68.7	68.9	70.9
C-4″		66.2	66.3	67.4	66.2	66.2	67.4
C-5″		69.1	69.1	73.7	69.3	69.4	73.7
C-6″		62.4	62.5	61.8	62.8	62.7	61.8
C-1‴					99.3	98.2	100.8
C-2‴					69.1	69.1	71.2
C-3‴					67.5	68.7	70.9
C-4‴					66.2	66.2	70.9
C-5‴					69.3	69.3	71.2
C-6‴					62.3	62.3	61.8

TABLE II

COMPARISON OF MOLECULAR ROTATIONS OF **2**, **4**, **8**, **9**, AND **11**

Compound	$[M]_D$ *(degrees)* $\times$ 10^{-2}
Compound **2**[a]	+138
Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside[a]	+178
2 + Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside[a]	+316
Compound **4**[a]	+353
Compound **8**[b]	+396
Methyl α-D-mannopyranoside[b]	+154
Compound **9**[a]	+435
3 + Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside	+371
Compound **11**[b]	+526
8 + Methyl α-D-mannopyranoside	+560

[a]Optical rotation determined on solution in chloroform. [b]In water.

as the methyl pyranosides of 2,3,4,6-tetra-*O*-methyl- and 3,4-di-*O*-methyl-D-mannose in a ratio of 2:1.

Reaction of **5** with twice the molar amount of **3** in the presence of mercuric cyanide in nitromethane afforded, in 85% yield, the corresponding tetrasaccharide **9**. *O*-Debenzylation of **9** in tetrahydrofuran in the presence of platinum oxide gave a 92% yield of **10**, which was *O*-deacetylated with triethylamine in aqueous methanol to afford methyl 2,4,6-tri-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**11**). The ^{13}C-n.m.r. spectrum of **11** showed signals for four anomeric carbon atoms, the assignments of which were based on the work of Gorin[6].

EXPERIMENTAL

General methods. — Melting points were determined with a Yanagimoto micro-apparatus and are uncorrected. ^{1}H-N.m.r. spectra were recorded with a JNM MH-100 spectrometer, and ^{13}C-n.m.r. spectra with a FX-100 instrument, tetramethylsilane being the internal standard in both cases. Optical rotations were recorded with a Union Giken PM-201 automatic digital polarimeter. Liquid chromatography was performed with a Toyo Soda HLC-802 UR unit equipped with an RI detector; the flow rate of the eluent (0.1M sodium chloride) was 1.2 mL/min at 40° for a column of G2000SW + G3000SW. T.l.c. was conducted on precoated silica gel plates (Merck GF-254), and column chromatography on silica gel (Merck Kieselgel 60).

Growth conditions. — A strain of *Epidermophyton floccosum*[7] was grown for 4 days at 28° in 100 Erlenmeyer flasks, each containing 200 mL of Sabouraud medium (4% of D-glucose, 1% of polypeptone, and 0.5% of yeast extract). The mycelia were harvested by filtration and washed five times with distilled water.

Isolation of S-Iawe. — Dried, whole cells were treated with M sodium hydroxide for 5 h at 100°. After filtration, the filtrate was dialyzed against distilled water, and then poured into absolute ethanol. The precipitate was centrifuged off and dissolved in water, and Fehling solution was added. The precipitate was centrifuged off after 5 h, washed with water, and solubilized by adding 5% hydrogen chloride in methanol. The solution was concentrated to a small volume under diminished pressure and poured into ethanol. The precipitate was centrifuged off and dissolved in water. The solution was dialyzed against distilled water and lyophilized. The yield of S-Iawe based on the weight of dried mycelia was 4.5%, $[\alpha]_D^{24}$ +194.2° (*c* 0.25, water); ν_{max}^{KBr} 810 cm^{-1}; average mol. wt. ~30 000, as determined by the Park–Johnson method[8].

Methylation analysis. — S-Iawe (50 mg) was dissolved in dimethyl sulfoxide (5 mL) under a nitrogen atmosphere. The solution was treated[9] with methylsulfinyl carbanion (2 mL) for 4 h at room temperature, and then with methyl iodide (2 mL) for 1 h at 20°. The reaction mixture was extracted with chloroform. After evaporation, the residue was methylated[10] twice with methyl iodide in *N*,*N*-dimethylformamide (5 mL) in the presence of silver oxide (0.5 g). The methylated product was methanolyzed with 5% hydrogen chloride in methanol (2 mL) in a sealed ampoule for 5 h.

The resulting methyl *O*-methylmannosides were analyzed by g.l.c. at 170°, in a Shimadzu GC-6A gas chromatograph equipped with a hydrogen-flame, ionization detector and a glass column packed with 10% DEGS-Chromosorb W. The relative retention times (T_M, relative to methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside) were: methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (T_M, 1.00), methyl 2,3,4-tri-*O*-methyl-α-D-mannopyranoside (T_M, 2.36), and methyl 3,4-di-*O*-methyl-α-D-mannopyranoside (T_M, 6.15).

Acetolysis. — S-Iawe (50 mg) was suspended in acetic anhydride (50 mL), acetic acid (50 mL), and sulfuric acid (4 mL), and the acetolysis[11] was carried out for 3 days at room temperature. The solution was extracted with chloroform, and the organic layer was washed with water and dried (sodium sulfate). After removal of the solvent, the resulting syrup was chromatographed on silica gel in 20:1 (v/v) benzene–acetone as a developing solvent. The resulting mannobiose peracetate was dissolved in 50% methanol, and triethylamine (few drops) was added. After being kept for one day, the solution was evaporated, and the residue methylated with methylsulfinyl carbanion (2 mL) and methyl iodide (2 mL) in dimethyl sulfoxide (6 mL). The per-*O*-methylmannobiose was methanolyzed, and analyzed by g.l.c. The relative retention times were: methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (T_M, 1.00) and methyl 3,4,6-tri-*O*-methyl-α-D-mannopyranoside (T_M, 2.34).

Periodate oxidation. — S-Iawe (50 mg) was added to a solution of 0.02M sodium periodate (25 mL). The oxidation was carried out in the dark at 10°. Aliquots (5 mL) were removed from the solution at timed intervals for estimation of the iodate content[12]. The formic acid liberated was titrated with 10mM sodium hydroxide after addition of 1,2-ethanediol. The oxidation was complete after 48 h.

Methyl 3-O-benzyl-4,6-O-benzylidene-2-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**4**). — A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide[13] (**3**, 11 g, 27 mmol) in nitromethane (36 mL) was added to a mixture of methyl 3-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside[3] (**2**, 7.85 g, 21 mmol), mercuric cyanide (7 g), and molecular sieve 4 Å (1 g) in the same solvent (10 mL). After being stirred for 3 h at 40°, the mixture was cooled and washed successively with saturated aqueous sodium hydrogencarbonate, saturated aqueous sodium chloride, and water, dried (sodium sulfate) ,and evaporated to a syrup that contained, as shown by t.l.c. in 4:1 (v/v), benzene–acetone, a major product (R_F 0.65) and a hydrolysis product of **3** (R_F 0.32). The residue was chromatographed on a column of silica gel. The product, eluted with 4:1 (v/v) benzene–acetone, crystallized from ethanol (yield 10.4 g, 70%), colorless needles, m.p. 132–132.5°, $[\alpha]_D^{24}$ +50.3° (*c* 0.92, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 7.50–7.26 (m, 10 H, arom.), 5.66 (s, 1 H, PhC*H*), 5.12 (s, 1 H, H-1′), 4.70 (s, 1 H, H-1), 3.36 (s, 3 H, OMe), 2.08 (s, 6 H, 2 OAc), 2.04, and 1.98 (s, each 3 H, OAc).

Anal. Calc. for $C_{35}H_{42}O_{15}\cdot 0.5\ H_2O$: C, 59.06; H, 6.08. Found: C, 59.08; H, 5.82.

Methyl 3-O-benzyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**5**). — Compound **4** (1.6 g) was treated with 50% acetic acid for 2 h

at 90°. The solution was evaporated at a temperature <40° to give a colorless powder, which crystallized from diethyl ether (yield 1.33 g, 95%), m.p. 51–53°, $[\alpha]_D^{24}$ +31.4° (*c* 1.05, chloroform); t.l.c. (4:1, v/v, benzene–acetone): R_F 0.21; ^{1}H-n.m.r. ($CDCl_3$): δ 7.32 (m, 5 H, arom.), 4.90 (s, 1 H, H-1'), 4.80 (s, 1 H, H-1), 4.63 (s, 2 H, PhCH_2), 3.36 (s, 3 H, OMe), 2.09 (s, 6 H, 2 OAc), 2.04, and 1.97 (s, each 3 H, OAc).

Anal. Calc. for $C_{28}H_{38}O_{15}\cdot 1.5\ H_2O$: C, 52.40; H, 6.39. Found: C, 52.44, H, 6.22.

Methyl 3-O-benzyl-2,6-di-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**6**). — Compound **5** (0.62 g, 1 mmol) was condensed with **3** (0.41 g, 1 mmol) as described for the synthesis of **4**. The dried solution was evaporated *in vacuo* to a syrup, which was chromatographed on a column of silica gel with 4:1 (v/v) chloroform–acetone as eluent. Compound **6** was obtained as colorless prisms (0.55 g, 60%), m.p. 67–68°, $[\alpha]_D^{24}$ +47.7° (*c* 0.55, chloroform), t.l.c. (4:1, v/v, chloroform–acetone): R_F 0.54; ^{1}H-n.m.r. ($CDCl_3$): δ 7.37–7.24 (m, 5 H, arom.), 4.91 (s, 2 H, H-1', H-1''), 4.78 (s, 1 H, H-1), 4.63 (s, 2 H, PhCH_2), 3.40 (s, 3 H, OMe), 2.44 (br.s, 1 H, D_2O exchangeable), 2.11 (s, 12 H, 4 OAc), 2.05 (s, 9 H, OAc), and 1.96 (s, 3 H, OAc).

Anal. Calc. for $C_{42}H_{55}O_{24}\cdot 1.5\ H_2O$: C, 51.95; H, 5.98. Found: C, 52.00; H, 6.01.

Methyl 2,6-di-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**7**). — To a solution of **6** (100 mg) in abs. tetrahydrofuran (4 mL) was added platinum oxide (50 mg). The mixture was stirred under hydrogen and then filtered through Celite. Evaporation of the solution gave **7** as a syrup in 95% yield, $[\alpha]_D^{24}$ +76.3° (*c* 0.4, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.00, 4.93, 4.78 (s, each 1 H, anomeric H), 3.40 (s, 3 H, OMe), 2.15, 2.13 (s, each 3 H, OAc), 2.12 (s, 6 H, 2 OAc), 2.05 (s, 6 H, 2 OAc), 1.97, and 1.96 (s, each 3 H, OAc).

Methyl 2,6-di-O-α-D-mannopyranosyl-α-D-mannopyranoside (**8**). — Compound **7** (86 mg) was *O*-deacetylated with triethylamine (0.15 mL) in 50% aqueous methanol (4 mL) and, after removal of the solvents, the residue was crystallized from ethanol, (m.p. 207–208, $[\alpha]_D^{21}$ +76.6° (*c* 0.72, water); t.l.c. (4:1, chloroform–methanol): R_F 0.22; ^{1}H-n.m.r. (D_2O): δ 5.03 (s, 1 H), 4.94 (s, 2 H) (anomeric H), and 3.36 (s, 3 H, OMe).

Anal. Calc. for $C_{19}H_{33}O_{16}$: C, 44.10; H, 6.43. Found: C, 44.32; H, 6.31.

Methyl 3-O-benzyl-2,4,6-tri-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (**9**). — Compound **5** (1 g, 1.63 mmol) was condensed with twice the molar amount of **3** (1.34 g, 3.26 mmol), as described for the synthesis of **4**. After being stirred for 24 h at 40°, the mixture was cooled and washed successively with saturated aqueous sodium hydrogencarbonate, saturated aqueous sodium chloride, and water, dried (sodium sulfate), and evaporated to a syrup which contained (t.l.c.) a major (R_F 0.61) and a minor (R_F 0.54) component, and some slower-moving components. The syrup was chromatographed on silica gel with 10:1 (v/v) chloroform–acetone as the developing solvent. The tetrasaccharide **9** was obtained as colorless prisms (0.92 g, 85%), m.p. 75–76°, $[\alpha]_D^{21}$ +34.1° (*c* 0.5, chloroform);

^{1}H-n.m.r. ($CDCl_3$): δ 7.28 (m, 5 H, arom.), 4.96, 4.78, 4.64, 4.57 (s, each 1 H, anomeric H), 4.73 (s, 2 H, PhCH_2), 3.41 (s, 3 H, OMe), 2.16 (s, 3 H, OAc), 2.12 (s, 9 H, 3 OAc), 2.10, 2.07 (s, each 3 H, OAc), 2.06 (s, 6 H, 2 OAc), 2.00 (s, 6 H, 2 OAc), 1.98, and 1.96 (s, each 3 H, OAc).

Anal. Calc. for $C_{56}H_{73}O_{33}$: C, 52.79; H, 5.78. Found: C, 52.84; H, 5.81.

Methyl 2,4,6-tri-O-(*2,3,4,6-tetra*-O-*acetyl*-α-D-*mannopyranosyl*)-α-D-*mannopyranoside* (**10**). — To a solution of **9** (76.6 mg) in abs. tetrahydrofuran (3 mL) was added platinum oxide (40 mg). After the usual processing, the solution was evaporated to give **10** as a syrup in 92% yield, $[\alpha]_D^{21}$ +60.2° (*c* 0.42, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.22, 5.06, 4.97, 4.82 (s, each 1 H, anomeric H), 3.40 (s, 3 H, OMe), 2.14 (s, 9 H, 3 OAc), 2.12 (s, 3 H, OAc), 2.09, 2.04 (s, each 9 H, 3 OAc), 1.99, and 1.96 (s, each 3 H, OAc).

Anal. Calc. for $C_{49}H_{67}O_{33}$: C, 49.77; H, 5.76. Found: C, 49.97; H, 5.72.

Methyl 2,4,6-tri-O-α-D-*mannopyranosyl*-α-D-*mannopyranoside* (**11**). — Compound **10** (37.2 mg) was *O*-deacetylated with triethylamine (0.3 mL). The solution was evaporated to give a white powder (20.3 mg, yield 95%), $[\alpha]_D^{21}$ +77.4° (*c* 0.53, water); t.l.c. (4:1, v/v, chloroform–methanol): R_F 0.12; ^{1}H-n.m.r. (D_2O): δ 5.29 (d, 1 H, *J* 1.5 Hz), 5.08 (d, 1 H, *J* 1.0 Hz), 5.03 (s, 2 H) (anomeric H), and 3.46 (s, 3 H, OMe).

Anal. Calc. for $C_{25}H_{44}O_{21}$: C, 44.12; H, 6.52. Found: C, 44.19; H, 6.48.

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