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TWO SYNTHETIC ANTIGENS RELATED TO *Streptococcus pneumoniae* TYPE 3 CAPSULAR POLYSACCHARIDE

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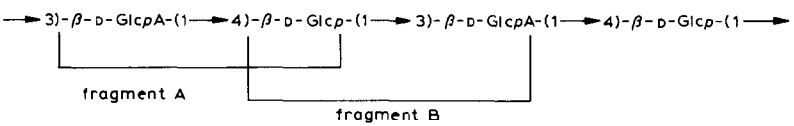
ABSTRACT

The synthesis of the allyl β-glycosides (**8** and **20**, respectively) of β-D-Glc*p*A-(1→4)-D-Glc*p* and β-D-Glc*p*-(1→3)-D-Glc*p*A (overlapping disaccharide fragments *A* and *B*) in the linear chain of the capsular polysaccharide (S3) from *Streptococcus pneumoniae* type 3 is described. Oxidation of allyl 2,3,6,2′,3′,4′-hexa-*O*-acetyl-β-cellobioside with chromic acid and saponification of the product gave **8**. The synthesis of **20** involved glycosylation of methyl 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranuronate or its 3-*O*-trityl derivative and subsequent furanose → pyranose transformation. The derivatives **8** and **20** were each copolymerised with acrylamide. In serological tests (enzyme immunoassay and passive hemagglutination), the resulting antigens exhibited the specificity of S3. It was concluded that fragment *A* was a much stronger immunodeterminant than fragment *B*.

INTRODUCTION

Streptococcus pneumoniae type 3 belongs to the group of clinically important pneumococci[1] and, among the pneumococcal polysacchrides, the structure of its capsular polysaccharide (S3) was one of the first to be reported[2]. The linear chain of S3 is composed (**1**) of cellobiouronic acid repeating-units connected through (1→3)-β-linkages.

In studies with artificial antigens of the neoglycoprotein type, Goebel[3] demonstrated the importance of the cellobiouronic acid moiety (fragment *A* in **1**) for the immunological properties of S3. The immunochemical role of pseudolaminaribiouronic acid (fragment *B* in **1**), which can be also regarded as a



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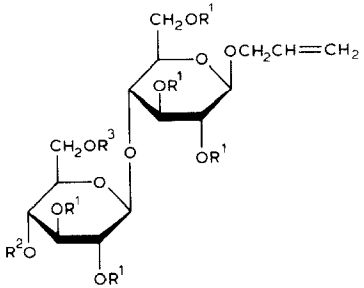
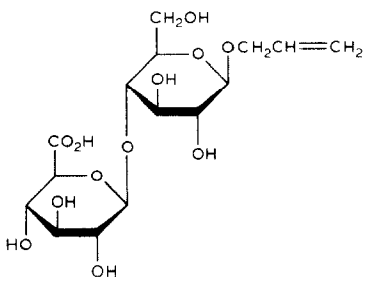
repeating unit of S3, remains unclear. In order to define the immunodeterminants of S3, we have synthesised two artificial antigens, each containing one of the disaccharide fragments *A* and *B* which are overlapped in S3. The synthesis involved copolymerisation of disaccharide derivatives with a non-carbohydrate monomer, an approach[4] applied for the synthesis of antigens that did not contain an immunogenic protein carrier and possessed the group specificity B, D_1, and E of *Salmonella*[5,6].

RESULTS AND DISCUSSION

The first one of the two desired carbohydrate monomers, allyl β-cellobiosiduronic acid (**8**), was synthesised from cellobiose. Helferich condensation of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-cellobiosyl bromide with allyl alcohol afforded allyl hepta-*O*-acetyl-β-cellobioside (**2**, 72% after chromatography) which, with methanolic sodium methoxide, gave allyl β-cellobioside (**3**). The ^{13}C-n.m.r. spectrum (see Table I) of **3** was interpreted on the basis of data[7] for methyl β-cellobioside and by comparison with the spectrum of allyl β-D-glucopyranoside[8].





8

2 $R^1 = R^2 = R^3 = Ac$
3 $R^1 = R^2 = R^3 = H$
4 $R^1 = Ac, R^2, R^3 =$ PhCH<
5 $R^1 = Ac, R^2 = R^3 = H$
6 $R^1 = R^2 = Ac, R^3 =$ Tr
7 $R^1 = R^2 = Ac, R^3 = H$

Treatment of **3** with α,α-dibromotoluene in boiling pyridine followed by acetylation gave crystalline allyl 2,3,6,2′,3′-penta-*O*-acetyl-4′,6′-*O*-benzylidene-β-cellobioside (**4**, 73%). Hydrolysis of **4** with hot, dilute acetic acid gave allyl 2,3,6,2′,3′-penta-*O*-acetyl-β-cellobioside (**5**). The position of the *O*-acetyl groups in **5** followed from ^{13}C-n.m.r. data (see Table I).

Reaction of **5** with trityl chloride in boiling pyridine followed by acetylation gave the 6′-*O*-trityl derivative **6** in high yield, the structure of which was confirmed by the ^{1}H-n.m.r. spectrum. The well-resolved signals for the *O*-acetyl groups were assigned on the basis of a comparison with the data for a similar derivative of

TABLE I

^{13}C-N M R DATA[a]

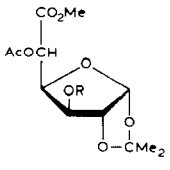
Compound	*Chemical shifts (p.p.m.)*														
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*	*OCH_2*	*CH=*	*$-CH_2$*
2[b]	99.1	70 95	72.6	76.5	71.65	61.9	99.7	71.65	72.7	68.4	72.3	62.4	69.4	116.8	134.3
2[c]	99.6	71.9	73.1	76.6	72.8	62.1	100.8	72.15	72.9	68.2	71.9	61.85	70.0	117.5	133.6
3[b]	102.3	73.6	75.4	80.7	75.3	61.05	103.4	73.8	77.1	70.55	77.0	61.5	69.35	116.7	135.2
5[c]	99.35	71.9	73.55	76.6	72.75	62.1	100.6	72.0	76.05	69.1	75.95	62.4	70.0	117.6	135.5
7[c]	99.6	71.95	73.5	76.55	72.9	62.35	100.7	72.2	73.0	68.75	74.7	61.4	70.1	117.55	133.6
8	102.3	74.05	75.5	80.1	75.4	61.2	103.5	73.9	76.3	72.3	75.8	173.4	71.8	119.85	134.6
21	103.55	74.2	75.9	80.3	75.6	61.5	103.55	74.1	76.5	72.5	75.9	—			
[c,d]	99.7	71.5	73.0	68.7	72.0	62.15							70.0	117.5	133.5
[e]	102.5	74.3	77.05	70.9	77.05	62.1							71.7	119.8	134.7
13	106.1	83.6	81.2[g]	81.0[g]	69.0	174.9	102.5	73.9	77.2	70.8	76.8	61.95			
15[f]	97.3(β) 93.7(α)		84.95(β) 82.7(α)		75.7(β)	173.0 172.2	104.1	74.7	77.35	70.9	76.9	62.1			
20	102.3	73.7	84.9	72.0	75.6	172.0	103.9	74.7	77.3	70.8	76.8	62.0	71.2	120.3	134.6
22	103.65	73.9	85.0	73.0	76.3	174.2	103.9	74.8	77.3	70.9	76.9	62.0			

[a]Solutions in D_2O; other resonances: CH_3CO 169.0–170.6, CH_3CO 20.2–20.9, $COOCH_3$ 54.1–54.4, $C(CH_3)_2$ 114.5, $C(CH_3)_2$ 26.5 and 26.95 p.p.m. [b]Solutions in Me_2SO-d_6 at 80°. [c]Solutions in $CDCl_3$. [d]Allyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside. [e]Allyl β-D-glucopyranoside. [f]The C-4, C-2, and C-5α resonances, 71.95 and 71.2 p.p.m. [g]Mutual assignments are uncertain.

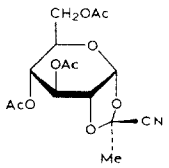
methyl β-cellobioside[9]. Detritylation of **6** was accomplished by methanolysis in the presence of pyridinium perchlorate[10]. That no acetyl migration had occurred in this reaction was established by ^{13}C-n.m.r. spectroscopy.

Oxidation at position 6′ in **7** was effected with chromic acid in acetone[11,12] and saponification of the product afforded allyl β-cellobiosiduronic acid (**8**, 80%). Differences in the ^{13}C-n.m.r. spectra of **8** and **3** reflected[13] the presence of a carboxyl group at position 6′. Compared to **3**, the C-6′ signal was shifted down-field from 61.5 to 173.4 p.p.m., whereas the signals for C-4′ and C-5′ were shifted down-field (1.75 p.p.m.) and up-field (1.2 p.p.m.), respectively. The presence of a free carboxyl group was also indicated by the electrophoretic mobility of **8**.

The second desired carbohydrate monomer (**20**), containing a (1→3)-linkage was obtained by glycosylation of the glucofuranuronic acid derivative **9**, with HO-3 unsubstituted, easily obtained in 3 steps from D-glucofuranurono-6,3-lactone[14].



9 R = H
10 R = Tr



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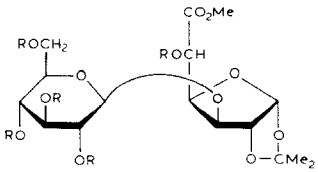
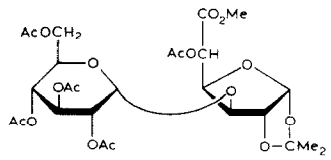


12 R = Ac
13 R = H



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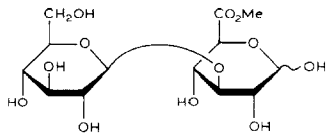

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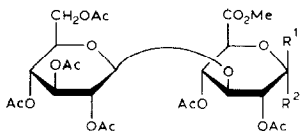
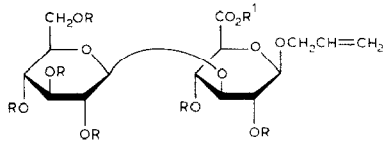


16 R^1 = H, R^2 = OAc
17 R^1 = OAc, R^2 = H
18 R^1 = H, R^2 = Br



19 R^1 = Me, R = Ac
20 R = R^1 = H

Reaction of **9** with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide in acetonitrile in the presence of mercury(II) cyanide gave the isomeric disaccharide derivatives **12** and **14**; the required β-isomer **12** preponderated. The use of a high-vacuum technique in the glycosylation procedure to remove traces of moisture from the reagents and solvents improved the yield of **12** from 13 to 47%. Glycosylation of **9** in acetonitrile in the presence of mercury(II) cyanide and bromide (2:1), or

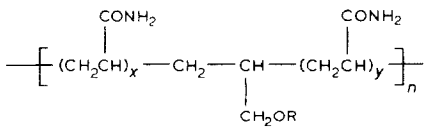
benzene–nitromethane in the presence of mercury(II) cyanide, or dichloromethane using silver triflate and 1,1,3,3-tetramethylurea, or nitromethane in the presence of silver triflate and collidine, did not yield better results.

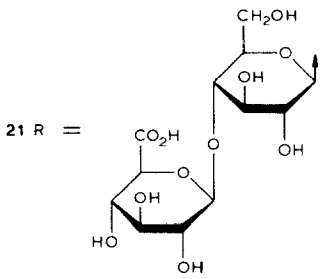
Condensation of the 3-*O*-trityl derivative **10** with 3,4,6-tri-*O*-acetyl-1,2-*O*-[1-(*exo*-cyano)ethylidene]-α-D-glucopyranose (**11**, prepared by a modified procedure[15]) under standard conditions[16] afforded the isomeric disaccharide derivatives **12** (30%) and **14** (13%). The structures of **12** and **14** were confirmed by the ^{1}H-n.m.r. data and the configurations of the interglycosidic bonds wee indicated by the $J_{1',2'}$ values [7.7 Hz for **12** (*i.e.* β) and 3.7 Hz for **14** (*i.e.* α)].

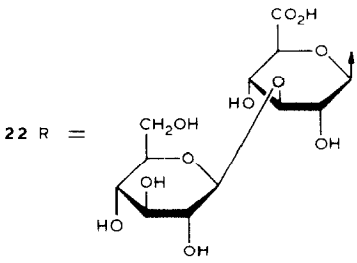
The glucofuranuronic acid residue in **12** was transformed into the pyranose form by the sequence *O*-deacetylation (→ **13**) and mild acid hydrolysis to give methyl pseudolaminaribiouronate (**15**). The ^{13}C-n.m.r. spectrum of **15** indicated an αβ-ratio of ~1:1 and confirmed the glucuronic acid residue to be pyranoid[13] (see Table I). Treatment of **15** with acetic anhydride–pyridine yielded an αβ-mixture of acetates (**16α** and **17β**) in the ratio ~2:1 (^{1}H-n.m.r. data); **16α** could be isolated by crystallisation. Treatment of the mixture **16** + **17** with hydrogen bromide in glacial acetic acid gave the relatively stable glycosyl bromide **18** (82% after column chromatography), the structure of which was confirmed by the ^{1}H-n.m.r. spectrum. Reaction of **18** with allyl alcohol in the presence of mercury(II) cyanide yielded 88% of crystalline methyl [allyl 2,4-di-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosid]uronate (**19**). The β configuration of the glycosidic bond formed was indicated by the $J_{1,2}$ value of 7.7 Hz. Two-step saponification (methanolic sodium methoxide, aqueous ethanolic alkali) of **19** removed the blocking groups and gave amorphous allyl β-pseudolaminaribiosiduronic acid (**20**). The structure of **20** was ascertained by comparison of its ^{13}C-n.m.r. data (Table I) with those for methyl β-D-glucopyranoside and methyl (methyl β-D-glucopyranosid)uronate[13].

The carbohydrate monomers **8** and **20** were converted into the polymers **21** and **22** by copolymerisation with acrylamide in water and promotion of the radical process with ammonium persulphate and *N,N,N',N'*-tetramethylethylenediamine. The copolymers **21** and **22** were isolated by elution from a column of Sephadex G-50 with pyridine–acetate buffer (pH 5), and ultrafiltration studies (Amicon Diaflo XM 100 and XM 300 membranes) indicated molecular masses of >100 kilodaltons.

When the ratio of allyl glycoside to acrylamide was 2:1, copolymers containing 27% of carbohydrate determinants were obtained, and ratios of 1:1 and 1:2 copolymers with 13% (**21a**) and 3% (**21b**) of determinants, respectively. Methanolysis of the copolymers and acetylation of the products gave (g.l.c.) the acetylated methyl glycosides of glucose and glucuronic acid in the molar ratio 1:1. The structures of **21** and **22** were also confirmed by the ^{13}C-n.m.r. data (see Table I). The resonances associated with the carbohydrate moieties of **21** and **22** practically coincided with those of the respective monomers **8** and **20**. A slight shift of the C-1 signal in the spectra of **21** and **22** was due to the change in the aglycon







after polymerisation. The spectra of copolymers **21** and **22** also contained ^{13}C signals for the non-carbohydrate linear chain, $CONH_2$ (181.6 and 180.6 p.p.m.), CH (43.5–42.7 p.p.m.), and CH_2 (37.1–35.4 p.p.m.).

The serological specificity of the copolymers **21, 21a,** and **21b** containing 27, 13, and 3% of determinant groups, respectively, was studied by enzyme immunoassay (e.i.a.) with 15 antisera to various pneumococcal types (see Experimental) and by passive hemagglutination. In e.i.a., S3 exhibited a clear-cut reaction with homologous anti-pneumococcal type 3 serum, as well as a weak cross-reaction with antiserum to type 8. Cross reactions between the pneumococcal type 3 and type 8 capsular polysaccharides have been repeatedly noted[2,17]. Copolymer **21** reacted specifically with antiserum to type 3 with an intensity comparable to that of S3. The reaction with other antipneumococcal sera was either not observed or very weak. The behaviour of **21a** was similar to that of **21** but the intensity of reaction was somewhat lower, whereas the reactions of **21b** were all weak. A single reactive serum was observed after decreasing the sensitising concentration from 100 to 10 and 2 μg/mL: only antiserum to type 3 reacted with **21** whereas, with antigen **22**, this reaction could not be ascertained.

The copolymers **21** and **22** can attach to the surface of formalin-treated sheep erythrocytes. Passive hemagglutination end-point titres of rabbit anti-Pn3 serum were 1:2000–1:4000 against **21**, 1:128–1:256 against **22**, and 1:8000 against S3. The results of the serological tests seemed to be associated with the different contributions of the fragments *A* and *B* in the structure (**1**) of S3 to the interaction with antiserum. The properties of the synthetic antigens indicate that the sequence β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→ (*A* in **1**) is a substantially stronger immunodeterminant than the sequence β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→ (*B* in **1**).

EXPERIMENTAL

T.l.c. was performed on "Silufol" plates (Czechoslovakia) with chloroform–acetone mixtures 8:2 (*A*), 9:1 (*B*), and 94:6 (*C*), benzene–acetone 8:2 (*D*), chloroform–ethanol 8:2 (*E*), 9:1 (*F*), and 97:3 (*G*), and benzene–ether 1:1 (*H*). Descending p.c. was performed on Filtrak FN 11 paper (GDR) with ethyl acetate–acetic acid–formic acid–water, 18:3:1:4 (*I*). P.e. was carried out in 0.025M pyridine–acetate buffer (pH 4.5) at 28 V/cm. Allyl glycoside derivatives were detected with aqueous 1% potassium permanganate. G.l.c. was performed with a Pye–Unicam 104 (Model 64) chromatograph with a flame-ionisation detector and a stainless-steel column (150 × 0.4 cm), packed with 3% of OV-1 on Diatomite CQ (100–120 mesh) at 200° and a nitrogen flow-rate of 30 mL/min. ^{1}H- and ^{13}C-n.m.r. spectra, optical rotations, column chromatography, and melting points were performed as described previously[5]. Acrylamide (4 times recrystallised) was used for copolymerisation. Allyl β-D-glucopyranoside and allyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside were prepared as described by Lee and Lee[8].

The capsular polysaccharide (S3) of *S. pneumoniae* type 3 was isolated from the culture medium by removing the bacteria by centrifugation, followed by precipitation of the antigen with acetone, and deproteinisation with phenol[18]. The rabbit antisera to pneumococcal types (or serogroups) (Danish nomenclature) (anti-Pn) 1–4, 6–9, 12, 14, 15, 18, 19, 23, and 25, prepared[19] in the I. I. Mechnikov Institute for Vaccines and Sera, were used in serological tests. E.i.a. was performed by using Linbro EIA microtiter plates and sheep anti-rabbit immunoglobulin horseradish peroxidase conjugate[20].

Passive hemagglutination. — Formalin-treated sheep erythrocytes were freed from conservation agent by thorough washing with saline and then resuspended in solutions of antigen (1 mg/mL). Mixtures were incubated at 37° for 90 min with periodic shaking, and then the suspensions were washed free from unattached antigen with saline containing 0.2% of normal rabbit serum. Two-fold dilutions of antiserum were mixed with equal volumes of 0.015% suspensions of sensitised erythrocytes. After incubation for 2 h at 37°, the highest serum dilution showing visible agglutination was recorded as the end-point of titration.

Allyl 2,3,6,2′,3′,4′,6′-hepta-O-acetyl-β-cellobioside (**2**). — A suspension of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-cellobiosyl bromide[21] (6.6 g) and mercury(II) cyanide (2.38 g) in allyl alcohol (30 mL, freshly distilled from CaO) was stirred at 50–60° for 0.5 h until the dissolution of glycosyl bromide was complete. T.l.c. (solvent *A*) then revealed a main component with R_F 0.63 and a contaminant with R_F 0.23. The mixture was concentrated *in vacuo,* and a solution of the residue in chloroform was washed with M potassium iodide containing a small amount of potassium hydrogencarbonate and then with water, dried ($MgSO_4$), and concentrated. Column chromatography (chloroform–acetone, 9:1) gave **2** (4.5 g, 72%), m.p. 186–188° (from ethanol), $[\alpha]_D^{20}$ −23° (*c* 4.8, chloroform) (Found: C, 51.40; H, 5.94. $C_{29}H_{40}O_{18}$ calc.: C, 51.47; H, 5.95%).

Allyl β-cellobioside (**3**). — A suspension of **2** (4 g) in methanolic 0.1M sodium methoxide (30 mL) was stirred at room temperature for 1–1.5 h, neutralised with KU-2 (H^+) resin, and concentrated to give **3** (1.9 g, 85%), m.p. 134–136° (from 2-propanol–ethanol), $[\alpha]_D^{20}$ −18° (*c* 1.8, methanol), R_{GlcA} 1.6 (solvent *I*) (Found: C, 46.81; H, 6.69. $C_{15}H_{26}O_{11}$ calc.: C, 47.11; H, 6.85%).

*Allyl 2,3,6,2′,3′-penta-*O*-acetyl-4′,6′-*O*-benzylidene-β-cellobioside* (**4**). — A solution of **3** (2 g) in dry pyridine (45 mL) was boiled for 1 h with freshly distilled α,α-dibromotoluene (1 mL, 1.2 equiv.) and then cooled, and acetic anhydride (10 mL) was added. The mixture was kept for 12 h at room temperature and then poured into ice–water, the precipitate was collected, and a solution in chloroform was washed with cold, saturated, aqueous potassium hydrogencarbonate and then water, dried ($MgSO_4$), and concentrated. Column chromatography (chloroform) of the crude syrup gave **4** (2.6 g, 73%), m.p. 234–236° (from ethanol), $[\alpha]_D^{20}$ −43° (*c* 1, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 2.00–2.14 (15 H, 5 Ac), 4.36 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.53 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 5.64 (s, 1 H, PhC*H*), 5.60–6.10 (m, 1 H, CH_2=C*H*CH_2O), 7.27–7.55 (m, 5 H, Ph) (Found: C, 56.61; H, 5.72. $C_{32}H_{40}O_{16}$ calc.: C, 56.47; H, 5.92%).

*Allyl 2,3,6,2′,3′-penta-*O*-acetyl-β-cellobioside* (**5**). — A suspension of **4** (2.5 g) in aqueous 50% acetic acid (60 mL) was heated at 100° until dissolution was complete, and then concentrated . Ethanol and toluene were evaporated from the residue, which contained a main component with R_F 0.4 (t.l.c., solvent *F*) and a slow-moving contaminant. Column chromatography (ethanol gradient, 0→11% in chloroform) of the residue gave **5** (1.94 g, 87%), m.p. 194–196° (from toluene), $[\alpha]_D^{23}$ −37° (*c* 2.6, chloroform) (Found: C, 51.09; H, 5.98. $C_{25}H_{36}O_{16}$ calc.: C, 50.67; H, 6.12%).

*Allyl 2,3,6,2′,3′,4′-hexa-*O*-acetyl-6′-*O*-trityl-β-cellobioside* (**6**). — To a solution of **5** (1.68 g) in dry pyridine (40 mL) was added chlorotriphenylmethane (1.85 g), and the mixture was boiled for 3 h. T.l.c. (solvent *G*) then revealed the tritylated derivative with R_F 0.4 but no **5**. Acetic anhydride (5 mL) was added to the cooled mixture which, after 12 h, was concentrated. Toluene and heptane were repeatedly evaporated from the residue, which was then subjected to column chromatography (acetone gradient, 0→4% in chloroform) to yield **6** as a foam (2.16 g, 87%), $[\alpha]_D^{20}$ +3.4° (*c* 4.5, chloroform), R_F 0.63 (solvent *F*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.73 (s, 3 H, Ac-4′), 1.83 (s, 3 H, Ac-3), 1.95 (s, 3 H, Ac-3′), 2.03 (s, 6 H, Ac-2,2′), 2.10 (s, 3 H, Ac-6), 4.15 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.52 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1′), 5.60–6.10 (m, 1 H, CH_2=C*H*CH_2O), 7.20–7.50 (m, 15 H, Ph_3C) (Found: C, 63.29; H, 6.15. $C_{46}H_{52}O_{17}$ calc.: C, 63.01; H, 5.98%).

*Allyl 2,3,6,2′,3′,4′-hexa-*O*-acetyl-β-cellobioside* (**7**). — To a solution of **6** (2 g) in dry nitromethane (30 mL) and dry methanol (10 mL) was added pyridinium perchlorate (1.27 g, dried over phosphorus pentaoxide *in vacuo* for 2 h at 100°), and the mixture was heated for 5 h at 60–65°. T.l.c. (solvent *F*) then revealed a product with R_F 0.5 but no **6**. The mixture was concentrated, a solution of the residue in chloroform was filtered through a short column of silica gel and concen-

trated, and the residue was extracted thrice with hot hexane to remove triphenylmethanol. Compound **7** was obtained as an amorphous powder (1.2 g, 83%), $[\alpha]_D^{20}$ −23° (*c* 2.4, chloroform) (Found: C, 50.76; H, 5.91. $C_{27}H_{38}O_{17}$ calc.: C, 51.04; H, 6.03%).

Allyl β-cellobiosiduronic acid (**8**). — A mixture of chromium trioxide (1 g), conc. sulfuric acid (0.5 mL), and water (1.5 mL) was added dropwise to a solution of **7** (270 mg) in acetone (5 mL), and the mixture was stirred for 2 h at 20°. The excess of the reagent was decomposed with ethanol (2 mL), the mixture was neutralised with saturated aqueous potassium hydrogencarbonate, filtered, stirred with KU-2 (H^+) resin, and extracted with chloroform. The extract was filtered through a bed of silica gel, and the combined filtrate and washings (ethanol) was concentrated to yield a syrup (260 mg), with R_F 0.3 (t.l.c., solvent *A*), which was dissolved in aqueous 90% ethanol (8 mL) containing 3% of potassium hydroxide. After 1.5 h, the mixture was neutralised with KU-2 (H^+) resin, filtered through active carbon on a Celite bed, concentrated, and then lyophilised to give **8** (135 mg, 80%) as a chromatographically (p.c., R_{GlcA} 1.3, solvent *I*) and electrophoretically homogeneous (M_{GlcA} 0.7) amorphous powder. This material was purified by elution from a column of Dowex 1-X8 (AcO^-) resin (100–200 mesh) with water and then with aqueous 10% acetic acid. The pure uronic acid **8**, eluted with acetic acid, had $[\alpha]_D^{20}$ −35.5° (*c* 1, water) (Found: C, 45.11; H, 6.10. $C_{15}H_{24}O_{12}$ calc.: C, 45.46; H, 6.08%).

Methyl 5-O-acetyl-1,2-O-isopropylidene-α-D-glucofuranuronate (**9**). — A solution of 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone[22] (400 mg) in dry methanol (5 mL) was stirred with dry Amberlite IR-4B (HO^-) resin for 30–35 min. T.l.c. (solvent *C*) then revealed a product with R_F 0.3. After further stirring, t.l.c. revealed starting lactone (R_F 0.07) and an increasing amount of a contaminant with R_F 0.45 (probably a product of acetyl migration). The resin was collected, and washed with methanol, and the combined filtrate and washings were concentrated. A solution of the residue in dry benzene was treated with active carbon and concentrated, and the residue was crystallised from the minimum amount of toluene to give **9** (250 mg, 56%), m.p. 108–109° (from dry ether–light petroleum), $[\alpha]_D^{22}$ −12° (*c* 1, methanol); lit.[14] m.p. 105–106°, $[\alpha]_D^{20}$ −14° (*c* 6.3, methanol). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.32 and 1.50 (2 s, each 3 H, CMe_2), 3.34 (d, 1 H, $J_{3,OH}$ 5.5 Hz, OH), 3.83 (s, 3 H, COOMe), 4.26 (dd, 1 H, $J_{3,4}$ 2.7, $J_{3,OH}$ 5.5 Hz, H-3), 4.40 (dd, 1 H, $J_{3,4}$ 2.7, $J_{4,5}$ 7.2 Hz, H-4), 4.56 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-2), 5.27 (d, 1 H, $J_{4,5}$ 7.2 Hz, H-5), 5.97 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1).

Methyl 5-O-acetyl-1,2-O-isopropylidene-3-O-trityl-α-D-glucofuranuronate (**10**). — To a solution of **9** (290 mg, 1 mmol) and 2,4,6-collidine (135 μL, ~1.02 mmol) in dichloromethane (7 mL) was added triphenylmethylium perchlorate (346 mg, 1.01 mmol), and the mixture was stirred at room temperature. After 1 h, t.l.c. (solvent *D*) revealed **9** (R_F 0.16). More collidine (63 μL) and tritylium perchlorate (173 mg) were then added, followed, after 40 min, by pyridine (0.4 mL). The mixture was diluted with chloroform (50 mL), washed with water (2 × 50 mL), and

concentrated, and the residue was subjected to column chromatography (ether gradient, 0→30% in benzene) to give **10** (450 mg, 85%), m.p. 134–135° (from dry ether–hexane), $[\alpha]_D^{21}$ −48° (*c* 2, chloroform), R_F 0.6 (solvent *D*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.04 and 1.33 (2 s, each 3 H, CMe_2), 1.92 (s, 3 H, Ac), 3.84 (s, 3 H, COOMe), 3.91 (d, 2 H, $J_{1,2} \approx J_{3,4}$ = 3.5 Hz, H-2,3), 4.35 (dd, 1 H, $J_{3,4}$ 3.5, $J_{4,5}$ 9.4 Hz, H-4), 5.42 (d, 1 H, $J_{4,5}$ 9.4 Hz, H-5), 5.91 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 7.25–7.45 (m, 15 H, Ph_3C) (Found: C, 70.19; H, 6.25. $C_{31}H_{32}O_8$ calc.: C, 69.91; H, 6.05%).

3,4,6-Tri-O-*acetyl-1,2*-O-[*(1*-exo-*cyano)ethylidene*]-α-D-*glucopyranose* (**11**). — To a solution of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (1.17 g, 3 mmol) in acetonitrile (10 mL) were added trimethylsilyl cyanide (1.2 mL, 9 mmol) and then dry stannous chloride (90 mg, ~0.45 equiv.), and the mixture was stirred under argon at room temperature. After 12 h, t.l.c. (solvent *H*) revealed the maximum content of the *exo*-isomer **11** (R_F 0.5) together with the *endo*-isomer (R_F 0.45) and starting material, R_F 0.4. The mixture was diluted with ethyl acetate (100 mL), washed with water (5 × 25 mL), and concentrated, and benzene was evaporated several times from the residue, which was then subjected to column chromatography (ether gradient, 0→60% in benzene) to give **11** (360 mg), an *exo,endo*-mixture (194 mg), and *endo*-isomer (80 mg). Compound **11** had m.p. 74–77° (from ethanol), $[\alpha]_D^{20}$ +14° (*c* 2, chloroform); lit.[23] m.p. 77–78°, $[\alpha]_D^{20}$ +13.6° (*c* 2.9, chloroform). The *endo*-isomer had m.p. 73–74° (from ether–pentane), $[\alpha]_D^{20}$ +110° (*c* 4, chloroform); lit.[23] m.p. 74–75°, $[\alpha]_D^{20}$ +108° (*c* 1.8, chloroform).

Methyl 5-O-*acetyl-1,2*-O-*isopropylidene-3*-O-*(2,3,4,6-tetra*-O-*acetyl*-β- *and* -α-D-*glucopyranosyl)*-α-D-*glucofuranuronate* (**12** *and* **14**). — (*a*) Reagents and solvents were dried by a vacuum technique. Separate solutions of **9** (330 mg, 1.14 mmol) plus mercury(II) cyanide (1 g, 4 mmol), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.64 g, 4 mmol), in benzene (twice distilled from CaH_2) were freeze-dried and the procedure was repeated. Acetonitrile (3.5 and 6 mL, twice distilled from CaH_2) was distilled separately onto **9** and the glucosyl bromide. The solution of the glucosyl bromide was then added dropwise during 40 min with stirring under argon to the solution of **9**. After 12 h, the mixture was diluted with chloroform (100 mL), washed with M potassium iodide (2 × 50 mL) containing a small amount of potassium hydrogencarbonate and then with water (2 × 50 mL), dried, and concentrated. Crystallisation of the residue from toluene gave **12** (150 mg). Column chromatography (acetone gradient, 0→20% in benzene) of the residue obtained from the mother liquor gave **14** (150 mg, 21%) and more **12** (180 mg, total yield 47%).

Compound **12** had m.p. 204–206° (from toluene), $[\alpha]_D^{20}$ −31.5° (*c* 2, chloroform), R_F 0.42 (solvent *D*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.34 and 1.50 (2 s, each 3 H, CMe_2), 2.00, 2.03, 2.04, and 2.09 (4 s, 15 H, 5 Ac), 3.71 (ddd, 1 H, $J_{4',5'}$ 9.5, $J_{5',6'a}$ 4.5, $J_{5',6'b}$ 2 Hz, H-5'), 3.80 (s, 3 H, COOMe), 4.07 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6'b}$ 2 Hz, H-6'b), 4.28 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6'a}$ 4.5 Hz, H-6'a), 4.41 (d, 1 H, $J_{3,4}$ 3.2 Hz, H-3), 4.49 (dd, 1 H, $J_{3,4}$ 3.2, $J_{4,5}$ 9 Hz, H-4) overlapped with the signal at 4.50 (d, 1 H, $J_{1,2}$ 3.3 Hz, H-2), 4.70 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1'), 4.88 (asym. t, 1

H, $J_{1',2'} \approx J_{2',3'} = 9$ Hz, H-2'), 5.07 (t, 1 H, $J_{3',4'} \approx J_{4',5'} = 9$ Hz, H-4'), 5.10 (d, 1 H, $J_{4,5}$ 9 Hz, H-5), 5.21 (t, 1 H, $J_{2',3'} \approx J_{3',4'} = 9$ Hz, H-3'), 5.89 (d, 1 H, $J_{1,2}$ 3.3 Hz, H-1) (Found: C, 50.33; H, 5.97. $C_{26}H_{36}O_{17}$ calc.: C, 50.32; H, 5.84%).

Compound **14** had m.p. 158–160° (from ether), $[\alpha]_D^{22}$ +73° (*c* 1.1, chloroform), R_F 0.54. ^{1}H-N.m.r. data ($CDCl_3$): δ 1.33 and 1.50 (2 s, each 3 H, CMe_2), 2.00, 2.06, 2.09, and 2.11 (4 s, 15 H, 5 Ac), 3.82 (s, 3 H, COOMe), 4.10 (ddd, 1 H, $J_{4',5'}$ 9.5, $J_{5',6'a}$ 6, $J_{5',6'b}$ 2.5 Hz, H-5'), 4.15 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6'b}$ 2.5 Hz, H-6'b), 4.26 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6'a}$ 6 Hz, H-6'a), 4.30 (d, 1 H, $J_{3,4}$ 3.1 Hz, H-3), 4.43 (dd, 1 H, $J_{3,4}$ 3.1, $J_{4,5}$ 9.2 Hz, H-4), 4.63 (d, 1 H, $J_{1,2}$ 3.3 Hz, H-2), 4.86 (d, 1 H, $J_{4,5}$ 9.2 Hz, H-5), 4.92 (dd, 1 H, $J_{1',2'}$ 3.7, $J_{2',3'}$ 10 Hz, H-2'), 5.01 (t, 1 H, $J_{3',4'} \approx J_{4',5'} = 9.5$ Hz, H-4'), 5.03 (d, 1 H, $J_{1',2'}$ 3.7 Hz, H-1'), 5.10 (t, 1 H, $J_{2',3'} \approx J_{3',4'} = 9.5$ Hz, H-3'), 6.03 (d, 1 H, $J_{1,2}$ 3.3 Hz, H-1) (Found: C, 50.21; H, 5.85%).

(*b*) In one limb of a fork-shaped tube (⅄) was placed a solution of **10** (266 mg, 0.5 mmol) and **11** (178 mg, 0.5 mmol) in dry benzene (2 mL), and in the other limb a solution of triphenylmethylium perchlorate (17 mg, 0.05 mmol) in nitromethane (0.2 mL). The tube was connected to a vacuum line (10^{-3} mmHg) and the solutions were freeze-dried. Benzene (2 mL, twice distilled from CaH_2) was distilled into the first limb, and lyophilisation was repeated. Dichloromethane (2 mL, twice distilled from CaH_2) was then distilled into the tube, and the solutions were mixed and kept under vacuum for 48 h at room temperature in the dark. The yellow mixture was then treated with 1:1 methanol–pyridine (0.5 mL), and the decolorised solution was diluted with chloroform (50 mL), washed with water (3 × 50 mL), and concentrated. T.l.c. (solvent *D*) of the residue revealed **12** and **14**, and a contaminant (R_F 0.64) with chromatographic behavior indistinguishable from that of 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone. Column chromatography (acetone gradient, 0→25% in benzene) of the residue gave **14** (42 mg, 13%), m.p. 157–160° (from ether), $[\alpha]_D^{20}$ +69° (*c* 1, chloroform), and **12** (103 mg, 30%), m.p. 203–204° (from toluene), $[\alpha]_D^{22}$ −34° (*c* 1, chloroform).

*Methyl 3-*O*-β-*D*-glucopyranosyl-*D*-glucopyranuronate* (**15**). — To a suspension of **12** (405 mg) in dry methanol (10 mL) was added methanolic M sodium methoxide (0.2 mL). The mixture was stirred for 3 h at room temperature. T.l.c. (solvent *E*) then showed the absence of **12** (R_F 0.76) and the formation of a product with R_F 0.27. The mixture was neutralised with KU-2 (H^+) resin and concentrated to give syrupy methyl 3-*O*-β-D-glucopyranosyl-1,2-*O*-isopropylidene-α-D-glucofuranuronate (**13**) in quantitative yield, $[\alpha]_D^{20}$ −8.5° (*c* 2.2, methanol); for ^{13}C-n.m.r. data, see Table I.

To a solution of **13** in water (2 mL) was added trifluoroacetic acid (3 mL). After 1.5 h, the mixture was concentrated, and methanol and toluene were evaporated from the residue, p.c. (solvent *I*) of which revealed a main component with R_F 0.6 and a contaminant with R_F 0.2. P.e. of the mixture revealed a minor amount of acidic component which was removed by using a column of Dowex 1-X8 (AcO^-) resin and elution first with water and then with aqueous 5% acetic acid. The neutral

disaccharide **15** (178 mg, 73.5%), eluted by water, had $[\alpha]_D^{20}$ +35° (*c* 2.7, methanol). A correct elemental analysis was not obtained for this compound, but its purity was demonstrated by chromatography. From the ratios of the integrated intensities of the signals for C-1 or C-3 (see Table I), it was concluded that **15** was a ~1:1 $\alpha\beta$-mixture.

On treatment with diazomethane in methanol, the acidic component (40 mg), $[\alpha]_D^{20}$ +28° (*c* 2, methanol), eluted from the column, was transformed into **15** (p.c.).

*Methyl 1,2,4-tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-α- and -β-*D*-glucopyranuronate* (**16** *and* **17**). — Disaccharide **15** (174 mg) was treated conventionally with acetic anhydride in pyridine (20°, 12 h), and the reaction mixture was concentrated. Toluene and heptane were evaporated several times from the residue, which was then subjected to column chromatography (benzene–acetone, 8:2) to yield a mixture (200 mg, 65%) of **16** and **17**, R_F 0.3 (solvent *D*) (Found: C, 49.00; H, 5.51. $C_{27}H_{36}O_{19}$ calc.: C, 48.78; H, 5.46%). In the ^{1}H-n.m.r. spectrum of the mixture, the signals for H-1 at δ 6.31 ($J_{1,2}$ 3.8 Hz) and 5.67 ($J_{1,2}$ 7.8 Hz) indicated an $\alpha\beta$-ratio of ~2:1.

Slow crystallisation of the mixture from ethanol–benzene at room temperature gave **16** (55 mg) as long needles, m.p. 219–221°, $[\alpha]_D^{23}$ +15° (*c* 2.1, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.99, 2.00, 2.03, 2.07, 2.11, 2.12, and 2.21 (7 s, each 3 H, 7 Ac), 3.75 (m, 1 H, H-5′), 3.76 (s, 3 H, COOMe), 4.11 (dd, 1 H, $J_{6'a,6'b}$ 12, $J_{5,6'b}$ 2.5 Hz, H-6′b), 4.13 (t, 1 H, $J_{2,3} \approx J_{3,4}$ = 9 Hz, H-3), 4.38 (dd, 1 H, $J_{6'a,6'b}$ 12, $J_{5',6'a}$ 4 Hz, H-6′a), 4.39 (d, 1 H, $J_{4,5}$ 10 Hz, H-5), 4.66 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.92 (dd, 1 H, $J_{1',2'}$ 8, $J_{2',3'}$ 9 Hz, H-2′), 5.11 (dd, 1 H, $J_{1,2}$ 3.8, $J_{2,3}$ 9 Hz, H-2), 5.05–5.20 (m, 3 H, H-3,4,4′), 6.32 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1).

*Methyl [2,4-di-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-α-*D*-glucopyranosyl bromide]uronate* (**18**). — A solution of **16** + **17** (250 mg) in dichloromethane (1 mL) and acetic acid (5 mL) was treated with 32% hydrogen bromide in glacial acetic acid (13 mL) for 40 min at 0° and then for 2.5 h at room temperature. The mixture was poured onto ice and extracted with chloroform, the extract was washed with cold, saturated, aqueous sodium hydrogencarbonate and water, dried ($MgSO_4$), and concentrated. The residue was then eluted from silica gel with dry benzene–acetone (8:2) to give **18** (210 mg, 81.5%), m.p. 212–214° (from dichloromethane–hexane), $[\alpha]_D^{23}$ +93° (*c* 0.75, dichloromethane), R_F 0.45 (solvent *D*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.97, 1.99, 2.02, 2.06, 2.09, and 2.19 (6 s, each 3 H, 6 Ac), 3.75 (ddd, 1 H, $J_{4',5'}$ 9.7, $J_{5',6'a}$ 4.4, $J_{5',6'b}$ 2.4 Hz, H-5′), 3.76 (s, 3 H, COOMe), 4.12 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6'b}$ 2.4 Hz, H-6′b), 4.22 (t, 1 H, $J_{2,3} \approx J_{3,4}$ = 9 Hz, H-3), 4.47 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6'a}$ 4.4 Hz, H-6′a), 4.53 (d, 1 H, $J_{4,5}$ 10.4 Hz, H-5), 4.71 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.85 (dd, 1 H, $J_{2,3}$ 9.5, $J_{1,2}$ 4 Hz, H-2), 4.90 (dd, 1 H, $J_{1',2'}$ 8, $J_{2',3'}$ 9.4 Hz, H-2′), 5.07 (t, 1 H, $J_{2',3'} \approx J_{3',4'}$ = 9 Hz, H-3′), 5.16 (dd, 1 H, $J_{3,4}$ 9, $J_{4,5}$ 10.5 Hz, H-4), 5.175 (dd, 1 H, $J_{3',4'}$ 8.6, $J_{4',5'}$ 9.7 Hz, H-4′), 6.54 (d, 1 H, $J_{1,2}$ 4 Hz, H-1) (Found: C, 43.64; H, 4.94; Br, 11.51. $C_{25}H_{33}BrO_{17}$ calc.: C, 43.81; H, 4.85; Br, 11.65%).

*Methyl [allyl 2,4-di-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-*

β-D-glucopyranosid]uronate (**19**). — A suspension of **18** (280 mg, 0.415 mmol) and mercury(II) cyanide (110 mg, 0.415 mmol) in allyl alcohol (15 mL, freshly distilled from CaO) was stirred for 0.5 h at 60–70°, and then kept for 12 h at room temperature. The mixture was diluted with chloroform, washed with M potassium iodide containing a small amount of potassium hydrogencarbonate and then with water, dried ($MgSO_4$), and concentrated. The residue was eluted from silica gel with chloroform–acetone (9:1) to give **19** (240 mg, 88%), m.p. 216–218° (from ethanol), $[\alpha]_D^{23}$ −51° (*c* 1.6, chloroform), R_F 0.42 (solvent *D*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.98, 2.01, 2.02, 2.09, and 2.14 (5 s, 18 H, 6 Ac), 3.68 (ddd, 1 H, $J_{4',5'}$ 9.4, $J_{5',6'a}$ 4.2, $J_{5',6'b}$ 2.4 Hz, H-5'), 3.77 (s, 3 H, COOMe), 3.95 (t, 1 H, $J_{2,3} \approx J_{3,4}$ = 9.5 Hz, H-3), 4.09 (m, 2 H, CH_2=CHCH_2O), 4.48 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.61 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.91 (t, 1 H, $J_{2',3'} \approx J_{3',4'}$ = 9 Hz, H-3'), 5.20 and 5.25 (2 m, 2 H, CH_2=CHCH_2O), 5.81 (m, 1 H, CH_2=C*H*CH_2O) (Found: C, 50.89; H, 5.64. $C_{28}H_{38}O_{18}$ calc.: C, 50.75; H, 5.78%).

Allyl 3-O-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid (**20**). — To a suspension of **19** (170 mg) in methanol (3 mL) was added methanolic M sodium methoxide (0.2 mL). The mixture was stirred at room temperature until homogeneous. After 1–1.5 h, aqueous 2% potassium hydroxide (1 mL) was added, and the mixture was stirred for 1 h, neutralised with KU-2 (H^+) resin, filtered, concentrated to a small volume, and applied to a column of Dowex 1-X8 (AcO^-) resin. The column was eluted first with water and then with aqueous 12% acetic acid. The latter fraction was concentrated and freeze-dried to give **20** (70 mg, 70%) as an amorphous solid, $[\alpha]_D^{20}$ −37° (*c* 1, water), R_{GlcA} 1.1 (solvent *I*), M_{GlcA} 0.55; for ^{13}C-n.m.r. data, see Table I. A satisfactory elemental analysis was not obtained for this compound, but p.c. and p.e. data demonstrated its purity.

Copolymerisation of **8** *and* **20** *with acrylamide*. — A solution of **8** (50 mg) and acrylamide (25 mg) in distilled water (0.7 mL) was deaerated and then treated with *N,N,N',N'*-tetramethylethylenediamine (2 μL) and ammonium persulphate (1 mg). The mixture was kept for 2 h at room temperature, diluted with water (1 mL), applied to a column (V_0 45 mL) of Sephadex G-50, and eluted with pyridine–acetate buffer (pH 5). Fractions (5 mL) were analysed by using a sugar analyser. Fractions 9–14 were combined and concentrated and water was evaporated several times from the residue. Lyophilisation then yielded copolymer **21** (40 mg), $[\alpha]_D^{20}$ −8° (*c* 0.5, water).

Copolymerisation of **8** and acrylamide in weight ratios 1:1 and 1:2 gave copolymers **21a** and **21b**.

Copolymer **22**, $[\alpha]_D^{20}$ −9° (*c* 2.5, water), was obtained from **20** by a similar procedure.

The carbohydrate content of the copolymers was determined after methanolysis (M HCl, 100°, 1 h) and acetylation. The resulting acetylated methyl glycosides were analysed by g.l.c., using *myo*-inositol hexa-acetate as internal standard.

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