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STRUCTURAL STUDIES OF THE O-ANTIGEN FROM *Vibrio cholerae* O:2

LENNART KENNE, BENGT LINDBERG, ELKE SCHWEDA,
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

BJÖRN GUSTAFSSON*, AND TORD HOLME
Department of Bacteriology, Karolinska Institute, S-104 01 Stockholm (Sweden)

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ABSTRACT

The structure of the O-antigen from *Vibrio cholerae* O:2 has been investigated, mainly by methylation analysis, specific degradations, and n.m.r. spectroscopy, and concluded to involve the trisaccharide repeating-unit

→4)-β-D-Qui*p*NAc-(1→4)-Sug*p*-(2→4)-β-D-Gal*p*-(1→,

in which QuiNAc is 2-acetamido-2,6-dideoxyglucose and Sug is tentatively assigned as 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-nonulosonic acid. The acetamidino group is basic and, consequently, the polysaccharide is neutral. When this group is transformed into an *N*-acetyl group, by treatment with aqueous triethylamine, the polysaccharide becomes acidic.

INTRODUCTION

The species *Vibrio cholerae* is divided into several serogroups on the basis of their O-antigens. In addition to serogroup O-1, which causes Asiatic cholera, at least 72 serogroups, collectively known as non-O:1 *V. cholerae,* occur. Structural studies of the O-antigens from *V. cholerae* have been performed on lipopolysaccharides (LPS) from serogroup O:1[1] and O:21[2], each of which contains unusual components in the repeating unit. We now report structural studies of the O-antigen from *V. cholerae* O:2.

RESULTS AND DISCUSSION

The LPS from *Vibrio cholerae* O:2, isolated by conventional methods, was contaminated by RNA and a glucan in different amounts from batch to batch. The

*Also Department of Vaccine Production, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden.

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RNA was hydrolysed by treatment with ribonuclease and the product was delipidated by treatment with aqueous acetic acid. When the resulting material was fractionated on a column of Sephadex G-50, the glucan was eluted with the void volume, followed by the O-specific polysaccharide (PSI) and fractions containing low-molecular-weight material.

Methylation analysis and ^{1}H- and ^{13}C-n.m.r. spectroscopy indicated that the glucan was probably a glycogen, but it was not investigated further. The PSI, which was contaminated by the glucan, on hydrolysis yielded 2-amino-2,6-dideoxy-D-glucose, D-glucose, D-galactose, and L-*glycero*-D-*manno*-heptose in the relative proportions 1.3:2.2:1.0:0.2. The determination of the absolute configurations of the sugars is discussed below. The corresponding values for the low-molecular-weight fraction were 0.6:17.0:1.0:2.6, indicating glucose and heptose to be the main components of the lipopolysaccharide core.

Methylation analysis of PSI demonstrated that the D-galactose and 2-amino-2,6-dideoxy-D-glucose residues were 4-linked and the n.m.r. evidence indicated them to be pyranosidic.

Treatment of PSI with aqueous triethylamine gave PSII (see below), and methanolysis of PSII and fractionation of the product by gel-permeation chromatography yielded a disaccharide derivative (**1**) and a mixture of monosaccharide methyl glycosides. The latter compounds were acetylated and the products were fractionated on a column of silica gel, yielding fully acetylated methyl α-D-glucopyranoside, methyl 2-amino-2,6-dideoxy-α-D-glucopyranoside, and methyl L-*glycero*-α-D-*manno*-heptopyranoside as the main components. The last two compounds were identified from their optical rotations and ^{1}H-n.m.r. spectra (Table I), which were fully assigned. The signals of H-1/4 in the heptopyranoside are similar to those for acetylated methyl D-*glycero*-α-D-*manno*-heptopyranoside[2] but those of H-5/7 differ as expected. The galactose was shown to be D (see below).

The ^{1}H-n.m.r. spectrum of PSI (Fig. 1) contained, *inter alia*, signals for methyl groups at δ 1.19 (J 6.3 Hz), 1.40 (J 6.3 Hz), and 2.29, for NAc groups at δ 2.00 and 2.05, and for a methylene group at δ 1.75 (^2J 12.4 Hz, ^3J 12.4 Hz, 1 H) and 2.85 (^3J 4.0 Hz, 1 H). The low-field region contained signals for anomeric protons at δ 4.62 (J 8.0 Hz) and 4.51 (J 8.0 Hz). Smaller signals (0.15 H and 0.30 H) in this region most probably derive from the core sugars and indicate that the polysaccharide contains on average six repeating-units.

The ^{13}C-n.m.r. spectrum of PSI (Fig. 2) contained, *inter alia*, signals for methyl groups at δ 17.44 and 17.77, for NAc methyl groups at δ 22.85 and 23.27, for a methylene group at δ 35.78 (t in an off-resonance spectrum), for three carbons carrying nitrogen at δ 51.75, 54.46, and 56.54, for a $-CH_2OH$ group at δ 61.74, for three anomeric carbons at δ 101.00 ($J_{C,H}$ 160 Hz), 102.75 (s), and 104.11 ($J_{C,H}$ 160 Hz), and for three carbonyl carbons at δ 172.37, 174.37, and 175.34. It further contained signals at δ 19.64 and 168.41.

The last two signals, in conjunction with the signal at δ 2.29 in the ^{1}H-n.m.r.

TABLE I

^{1}H-N.M.R. DATA FOR FULLY ACETYLATED METHYL D-*glycero*-α-D-*manno*-HEPTOPYRANOSIDE[2] (A), METHYL L-*glycero*-α-D-*manno*-HEPTOPYRANOSIDE (B), AND METHYL 2-AMINO-2,6-DIDEOXY-α-D-GLUCOPYRANOSIDE (C)

Compound	*Chemical shifts (δ)*								
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-7*	*H-7'*	OCH_3
A	4.684	5.198	5.302	5.274	3.969	5.194	4.270	4.439	3.393
B	4.686	5.191	5.22[a]	5.25[a]	3.998	5.224	4.180	4.254	3.327
C[b]	4.588	3.751	4.785	5.095	4.239	1.129			3.318
	Coupling constants (Hz)								
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{6,7}$	$J_{6,7'}$	$J_{7,7'}$	
A	1.7	3.4	9.8	9.2	2.9	3.4	6.5	12.1	
B	1.7	3.2	>8	9.8	2.0	7.5	5.8	11.2	
C[b]	3.7	9.8	9.8	10.8	6.1				

[a]Not first-order signals. [b]δ 5.670 ($J_{2,NH}$ 9.3 Hz, NH).

spectrum, indicate that PSI contains an acetamidino group. Such a group has been observed in the O-antigen of *Pseudomonas aeruginosa* O:13, which contains 2-acetamido-2,6-dideoxy-L-galactopyranosyl residues[3]. Treatment of PSI with aqueous triethylamine gave PSII which contained three NAc groups, as evident from its ^{1}H- and ^{13}C-n.m.r. spectra. PSI was neutral, but PSII was acidic and could be purified further by chromatography on a column of Trisacryl M DEAE, thus affording almost complete separation from the neutral glycan. A hydrolysate of PSII contained 2-amino-2,6-dideoxy-D-glucose, D-glucose, D-galactose, and L-

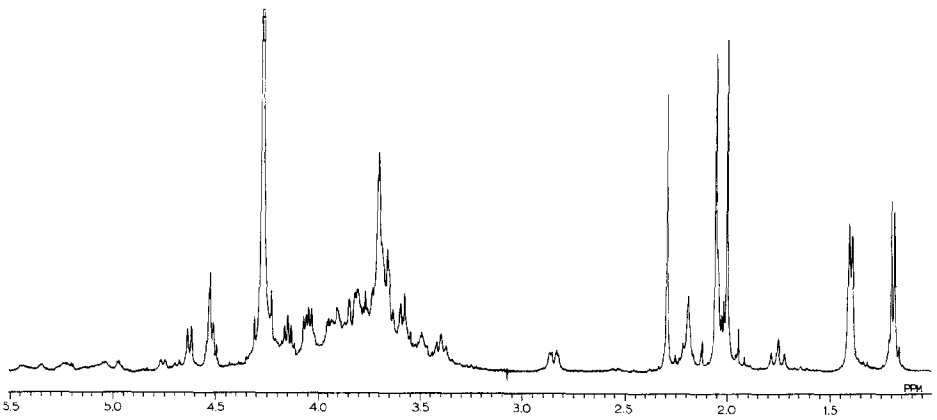


Fig. 1. 400-MHz ^{1}H-n.m.r. spectrum of *Vibrio cholerae* O:2 O-polysaccharide (PSI).

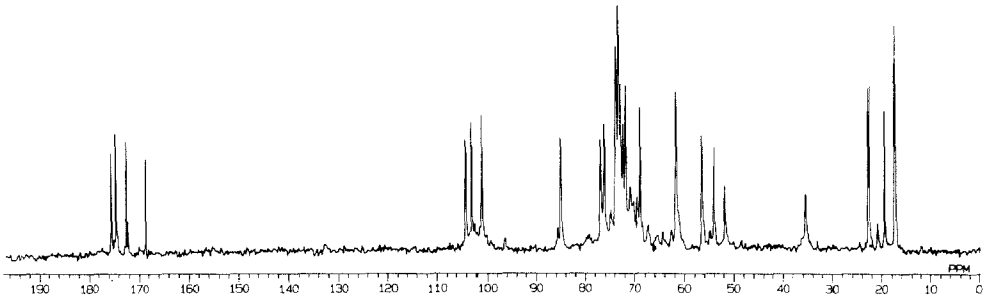


Fig. 2. ^{13}C-N.m.r. spectrum of *Vibrio cholerae* O:2 O-polysaccharide (PSI).

glycero-D-*manno*-heptose in the relative proportions 2.0:1.9:1.0:0.5. PSII contained 3.8% of N (*cf.* 5.4% for PSI).

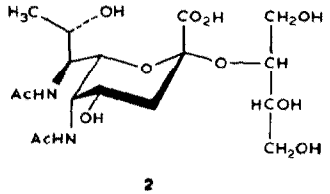
The above results indicate that PSI is composed of trisaccharide repeating-units containing 2-amino-2,6-dideoxy-β-D-glucose, β-D-galactose, and a third sugar. The last component should be a ketose with one carboxyl group, one methyl group, one methylene group, and two carbon atoms carrying nitrogen. Two sugars fulfilling these requirements, namely, 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid[4–6] and the corresponding D-*glycero*-L-*galacto* isomer[3], have been found in bacterial lipopolysaccharides by Knirel *et al.*, and it seemed possible that the sugar in the *V. cholerae* O:2 O-antigen belonged to this class of compounds.

The disaccharide derivative (**1**), obtained on methanolysis of PSII, was composed of the esterified keto sugar and galactose. The only sugar detected in a hydrolysate was galactose, which was shown to be D by the method of Gerwig *et al.*[7]. Methylation analysis confirmed that the galactosyl residue was 4-linked. Smith degradation (periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions) of saponified **1** gave a glycoside **2**, $[\alpha]_D$ −11° (water), in which the acidic ketose residue was linked to O-2 in D-threitol.

Smith degradation of PSII gave a glycoside (**3**) in which a 2-acetamido-2,6-dideoxy-β-D-glucopyranosyl group was linked to the acidic ketose of **2**.

All signals in the ^{1}H-n.m.r. spectra of PSI and PSII could be fully assigned (Table II) by using 2D-n.m.r. spectroscopy (COSY and relayed COSY). The signals given by the sugar residues, but not by the D-threitol moieties, in **2** and **3** could also be fully assigned (Table II). The spectra confirm the assumption that the acidic ketose is a 3-deoxynonulosonic acid in which C-9 is a methyl carbon. Comparison of the spectra of PSI and PSII further demonstrates that the acetamidino group is linked to N-5 of this sugar. On transforming the amidino group into an *N*-acetyl group, the signal of H-5 is shifted from δ 3.82 to 4.35, and a signal in the ^{13}C-n.m.r. spectrum from δ 51.75 to 45.88. Similar shifts were obtained when the acetamidino group in the *P. aeruginosa* O:13 O-antigen was transformed into an *N*-acetyl group[3].

The chemical shifts of the signals for H-7,8 of the nonulosonic acid residue



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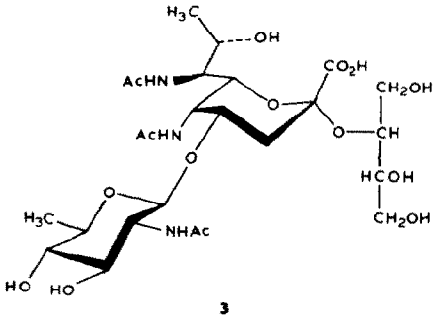

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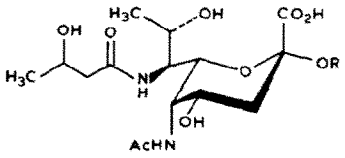
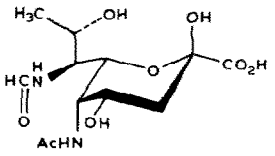


4a R = CH_2CH_2OH

4b R = →4)-D-FucNAc



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are similar to those observed for *N*-acylated 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid residues[4,6], indicating that the second *N*-acetyl group in the sugar is located on C-7 and not on C-8. This conclusion is confirmed by the large downfield shift (0.91 p.p.m.) of the signal for H-8 on acetylation of **2**. The third sugar in PSI is consequently a 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxynonulosonic acid.

Comparison of the spectra of **2**, in which the ulosonic acid is unsubstituted, and **3**, in which it is substituted by a 2-acetamido-2,6-dideoxy-β-D-glucopyranosyl residue, shows that the signals of H-6/9 are virtually unaffected by substitution but that those of H-3/5 are shifted downfield. This is the expected result when the amino sugar is linked to O-4 of the ulosonic acid.

The ^1H chemical shift data and the coupling constants of the nonulosonic acid residue in PSI, PSII, **2, 3** (the structures of the last two are anticipated in the formulas), and two derivatives (**4**[6] and **5**[4]) of 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid are given in Table II. The coupling constants of H-3*e*, H-3*a*, H-4, H-5, and H-6 in PSI, PSII, and **2** demonstrate that the 5-substituent is axial and those on C-4 and C-6 are equatorial, as in **6** or its mirror image.

Partial structures **7** and **8** give the most populated conformations of the nonulosonic acids with H-5 equatorial and H-6 axial, in which the C-6,7 fragment has the *erythro* and *threo* configuration, respectively, and in which H-6,7 are *anti* ($J_{6,7}$ 12 Hz). The distance between H-5 and H-8 in **8** is short enough to give an n.O.e. The absence of such an effect in the NOESY spectrum of **2** accords with the *erythro* configuration of the C-6,7 fragment, as in **7**. These data and the similarity of the chemical shifts and coupling constants for the signals of H-6/9 indicate that

TABLE II

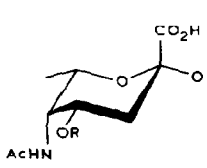
^{1}H-N.M.R. DATA FOR THE 2-ACETAMIDO-2,6-DIDEOXY-β-D-GLUCOPYRANOSYL (A), 5-ACETAMIDO-(OR ACETAMIDINO)-7-ACETAMIDO-3,5,7,9-TETRADEOXY-L-*glycero*-L-*manno*-NONULOSONIC ACID (B), AND β-D-GALACTOPYRANOSYL (C) RESIDUES IN PSI, PSII, **2**, **3**, **4b**, AND **5**

Residue	*Proton*	*Chemical shifts (δ)*					
		PSI	*PSII*	**2**	**3**	**4b**	**5**
A	*H-1*	4.62	4.67		4.68		
	H-2	3.70	3.70		3.64		
	H-3	3.65	3.66		3.48		
	H-4	3.40	3.44		3.20		
	H-5	3.67	3.66		3.48		
	H-6	1.40	1.40		1.36		
	NAc	2.00	2.00		2.00		
B	*H-3*a	1.75	1.78	1.63	1.71	1.61	1.77
	*H-3*e	2.85	2.66	2.52	2.58	2.55	1.89
	H-4	4.05	4.04	3.87	4.02	3.8–3.85	4.14
	H-5	3.82	4.35	4.20	4.31	3.8–3.85	4.22
	H-6	3.82	3.74	3.94	3.95	3.69	4.01
	H-7	4.05	3.95	4.02	4.06	3.91	4.21
	H-8	4.15	4.08	4.08	4.11	4.00	4.10
	H-9	1.19	1.19	1.17	1.19	1.07	1.08
	NAc	2.05	1.98	2.02	2.02		
	NAc	2.29[a]	1.94	1.95	1.96		
C	*H-1*	4.51	4.54				
	H-2	3.60	3.58				
	H-3	3.70	3.66				
	H-4	4.52	4.50				
	H-5	3.70	3.66				
	H-6	3.65	3.5–3.7				
	H-6'	3.72	3.5–3.7				

[a]Signal for the acetamidino group.

	Coupling constants (Hz)				
	PSI	*PSII*	**2**	**4b**	**5**
$J_{3a,3e}$	12.4	12.4	12.7		12.3
$J_{3a,4}$	12.4	12.4	12.7		13.5
$J_{3e,4}$	4.0	4.0	4.8		5.5
$J_{3e,5}$			1.0		
$J_{4,5}$		<4[a]	<4[a]		4.0
$J_{5,6}$		<1[a]	<1[a]	1.5	2.0
$J_{6,7}$	11[a]	11[a]	12[a]	10	10.6
$J_{7,8}$	6[a]	6[a]	6[a]	5.2	3.5
$J_{8,9}$	6.3	6.3	6.3	6	6.6

[a]Calculated from the cross-peaks in the COSY spectrum.



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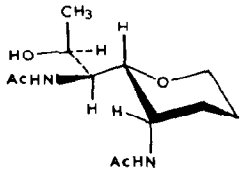

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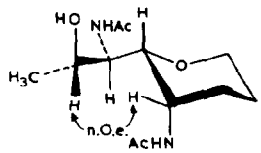

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the nonulosonic residue in each of the derivatives (Table II) has the same relative configuration, with the *erythro* configuration also of the C-7,8 fragment. The chemical shifts of the signal of H-3*e* and H-3*a* are similar for PSI, PSII, **2**, **3**, and **4b**, but differ for **5**, indicating that the former are β-glycosides, with an axial carboxyl group.

The shifts observed for the signals of H-3*a*, H-3*e*, H-4, and H-5 in a 5,7-diacylamino-3,5,7,9-tetradeoxy-L-*glycero*-β-L-*manno*-nonulosonic acid residue on substitution with a 2-acetamido-2-deoxy-β-D-galactopyranosyl group are ~0.1, ~0.1, 0.12, and 0.09 p.p.m., respectively[4]. The corresponding shifts on substitution of the 5,7-diacetamido-3,5,7,9-tetradeoxynonulosonic acid residue in **2** with a 2-acetamido-2-deoxy-β-D-glucopyranosyl group (as in **3**) are 0.08, 0.06, 0.15, and 0.11 p.p.m., respectively. As the substitution shifts depend on the absolute configuration of the sugars around the glycosidic linkage, the similarity between the two sets of shifts strongly indicates that the nonulosonic acid in **2** and **3** also has the L-*glycero*-L-*manno* configuration. The dependence of substitution shifts in β-linked disaccharides upon the absolute configuration of the sugar moieties has been investigated[8].

The $[\alpha]_D$ value [−11° (water)] of **2** is not very accurate because of the small amount of amorphous product available. The corresponding values for **4a**[5] and **5**[4] are −30° and −38°, respectively. The negative value for each component is further evidence that they have the same absolute configuration, namely, L-*glycero*-L-*manno*.

Thus, it is concluded that the *V. cholerae* O:2 O-antigen contains the trisaccharide repeating-unit **9**, in which QuiNAc is 2-acetamido-2,6-dideoxyglucose and Sug is a 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxynonulosonic acid residue, in which the configuration of the ring moiety is that of **6**. Strong evidence has been adduced that this sugar has the L-*glycero*-β-L-*manno* configuration.

→4)-β-D-Quip*N*Ac-(1→4)-Sug*p*-(2→4)-β-D-Gal*p*-(1-

9

The acetamidino group is strongly basic and neutralizes the carboxyl group. When the acetamidino group is transformed into an *N*-acetyl group, the polysaccharide becomes acidic.

The biosynthesis of this class of 3-deoxynonulosonic acids should proceed by

reaction of 2,4-diamino-2,4,6-trideoxyhexose derivatives with enolpyruvate phosphate. Only two such hexoses have been found, namely, those with the D-*gluco* and D-*galacto* configurations[9]. The 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acids with the L-*glycero*-L-*manno* and D-*glycero*-L-*galacto* configuration should derive from 2,4-diamino-2,4,6-trideoxyhexoses with the L-*altro* and D-*gulo* configurations, respectively, which have not yet been detected. Another possibility is that they are formed by modification of a neuraminic acid residue.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or at room temperature by flushing with air. For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates and partially methylated alditol acetates were performed on SE-54 fused-silica capillary columns either isothermally or using a temperature gradient of 150→220° at 2°/min. G.l.c.–m.s. was performed with a Hewlett–Packard 5970 instrument, using the above conditions.

Methylation analyses were performed essentially as previously described[10]. Products were recovered by reversed phase chromatography on Sep-Pak C_{18} cartridges[11]. The sample was diluted with an equal volume of water and applied to the cartridge which was washed with water and acetonitrile–water (15:85), and the sample was then eluted with acetonitrile.

N.m.r. spectra of solutions in D_2O were determined at 70° or 85°, using a JEOL GX-400 instrument. Chemical shifts are reported in p.p.m., using internal 1,4-dioxane (δ 67.40, ^{13}C) and sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00, ^1H) as references. 2D-N.m.r. spectroscopy was performed with standard COSY, relayed COSY, and NOESY pulse sequences, using a 90° mixing pulse. In the NOESY experiment on **2**, a mixing time of 0.6 s was used.

Preparation of LPS and PS. — *V. cholerae* O:2 was cultivated in an aerated, stirred, 12-L fermentor at 37° at a constant pH of 7.2, using a tryptone–yeast extract medium[12,13]. LPS was extracted from the bacteria by the hot phenol–water method[14] and purified by high-speed centrifugation. Sugar analysis of this material revealed 28% of ribose and therefore the LPS was treated with RNAse. A solution of LPS (1.3 g) in a sodium acetate buffer (60 mL, pH 5.0) containing RNAse (20 mg) was dialysed against the buffer for 24 h at 37° and then for 3 days against water. Sugar analysis of the freeze-dried material revealed only 3% of ribose.

LPS (1.1 g) was treated at pH 3.1 with aqueous acetic acid for 2 h at 100°. The precipitate (344 mg) was removed by centrifugation, the supernatant solution was freeze-dried, and the product was fractionated on a column (90 × 3 cm) of Sephadex G-50 which was irrigated with water. A void fraction consisted of a glucan (74 mg). The following fraction was the PSI (94 mg) containing small amounts of the glucan, and then different oligomeric materials were eluted from the column. Elemental analysis of a PS without contaminating glucan showed: C, 41.7; H, 6.6; N, 5.4%.

Treatment of PSI with base. — PSI (20 mg) was treated with aqueous 5% triethylamine for 16 h at 60°, the solution concentrated to dryness, the product dissolved in water, and the pH adjusted to 7. The material was purified on a column (15 × 1 cm) of Trisacryl M DEAE which was washed first with water, and the product (PSII) was eluted with M NaCl and purified further on a column (70 × 2 cm) of Bio-Gel P-2 irrigated with water. Elemental analysis showed: C, 40.7; H, 6.5; N, 3.8%.

Methanolysis of PSII. — A suspension of PSII (20 mg) in methanolic 1.1M hydrogen chloride was kept in a sealed tube for 16 h at 80°. The solution was neutralised with silver carbonate, acetic anhydride (0.1 mL) was added, the solution was kept for 4 h at room temperature and then filtered, the solvent was evaporated, and the product was fractionated on a column (70 × 2 cm) of Bio-Gel P-2 irrigated with water. The fractions were analysed by ^{1}H-n.m.r. spectroscopy. A disaccharide glycoside (**1**) and a mixture of monosaccharide glycosides were obtained.

The latter products were treated with pyridine (0.5 mL) and acetic anhydride (0.5 mL) for 1 h at 100°. After the usual work-up, the products were fractionated on a column of silica gel, using chloroform–acetone–light petroleum (10:3:1). The amorphous acetates of methyl 2-acetamido-2,6-dideoxy-α-D-glucopyranoside and methyl L-*glycero*-α-D-*manno*-heptopyranoside had $[\alpha]_D$ +125° (*c* 0.4, chloroform) and $[\alpha]_D$ +20° (*c* 0.3, chloroform), respectively.

Smith degradation of PSII. — A solution of PSII (6.8 mg) in 0.14M acetate buffer (pH 6, 6.8 mL) was treated in the dark with sodium metaperiodate (59 mg) for 56 h at 5°. The excess of periodate was reduced with ethylene glycol (0.1 mL), the solution was concentrated to 2 mL, and the product was reduced with sodium borohydride (10 mg) in water (2 mL) for 4 h. The mixture was neutralised with acetic acid, and the product was purified on a column (35 × 2 cm) of Bio-Gel P-2 (irrigated with water) and then hydrolysed with 0.5M trifluoroacetic acid for 16 h at 25°. The hydrolysate was diluted with water (3 mL) and freeze-dried, and the product was purified on a column (70 × 2 cm) of Bio-Gel P-2, irrigated with water, yielding disaccharide **2** (1.3 mg).

Smith degradation of disaccharide **1**. — A solution of **1** (9 mg) in buffer (2 mL) was treated with sodium metaperiodate (17.2 mg) under the same conditions as described above. In order to avoid reduction of the carboxyl group, the solution was adjusted to pH 10 before the addition of the borohydride. The product (4.3 mg) was obtained after hydrolysis and purification as described above.

Absolute configuration of galactose. — Disaccharide **1** (1 mg) was hydrolysed with 2M trifluoroacetic acid (2 mL) for 1 h at 120°. The galactose in the hydrolysate was proved to be D by using the method devised by Gerwig *et al.*[7].

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