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Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides

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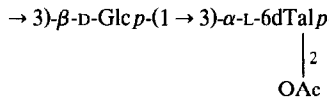
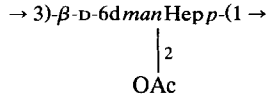
ABSTRACT

The pathogenic bacterium *Pseudomonas pseudomallei* strain 57576 produces two partially *O*-acetylated O-antigenic polysaccharides (PS-I and PS-II). Methylation analysis and ^{1}H and ^{13}C NMR spectroscopy, including NOE experiments, showed PS-I to have the structure



and PS-II to have the structure



where 6d*man*Hep is the unusual higher sugar 6-deoxy-D-*manno*-heptose. PS-II is produced also by *P. pseudomallei* strains 100 and 110, and PS-I and *O*-deacetylated PS-II by strain 97.

INTRODUCTION

Pseudomonas pseudomallei is a highly pathogenic micro-organism which causes melioidosis, and serological studies[1] have revealed at least two O-serovars. The structures of the O-antigens of *P. pseudomallei* have not been investigated hitherto and we report now the structures of two O-antigenic polysaccharides of strain 57576, which are characteristic also for several other strains.

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RESULTS AND DISCUSSION

A moderately virulent strain 57576, two highly virulent strains 100 and 110, and a weakly virulent strain of *P. pseudomallei* were studied. The O-antigenic polysaccharides were isolated by mild hydrolysis with dilute acetic acid of the lipopolysaccharides extracted from dry bacterial cells by the phenol–water procedure[2], followed by gel-permeation chromatography on Sephadex G-50.

The polysaccharide fraction (PS), obtained from strain 57576, had $[\alpha]_D$ −61° (*c* 0.5, water). The ^{13}C NMR spectrum of PS (Fig. 1) contained, together with the major signals, several minor signals, thus indicating the absence of a regular structure, most probably owing to non-stoichiometric *O*-acetylation (there were signals for OAc groups at 21.5–21.7 and 174.6–174.7 ppm). PS was *O*-deacetylated with aqueous ammonia to give material (DPS) whose ^{13}C NMR spectrum (Fig. 2) was typical for a regular polysaccharide and contained signals for C-1 at 97.9, 102.6 and 102.8 ppm, $HOCH_2$ groups at 59.5 and 62.1 ppm, CH_3C at 16.9 ppm, CCH_2C at 34.9 ppm, and other resonances in the region 69.0–83.3 ppm. The ^1H NMR spectrum of DPS (Fig. 3) contained, inter alia, signals for H-1 at δ 4.65 (d, $J_{1,2}$ 7.9 Hz), 4.80 (s), and 5.26 (s); CH_3C at δ 1.23 (d, $J_{5,6}$ 6.7 Hz); and CCH_2C at δ 1.74 (m) and 2.10 (m).

These data showed that DPS contained three different monosaccharide residues, including two deoxy sugars or one dideoxy sugar. The total number (19) of the ^{13}C signals indicated that one of the constituent monosaccharides was a heptose or its deoxy derivative.

Hydrolysis of DPS with trifluoroacetic acid gave three monosaccharides, two of which were identified as glucose and 6-deoxytalose by comparison with authentic

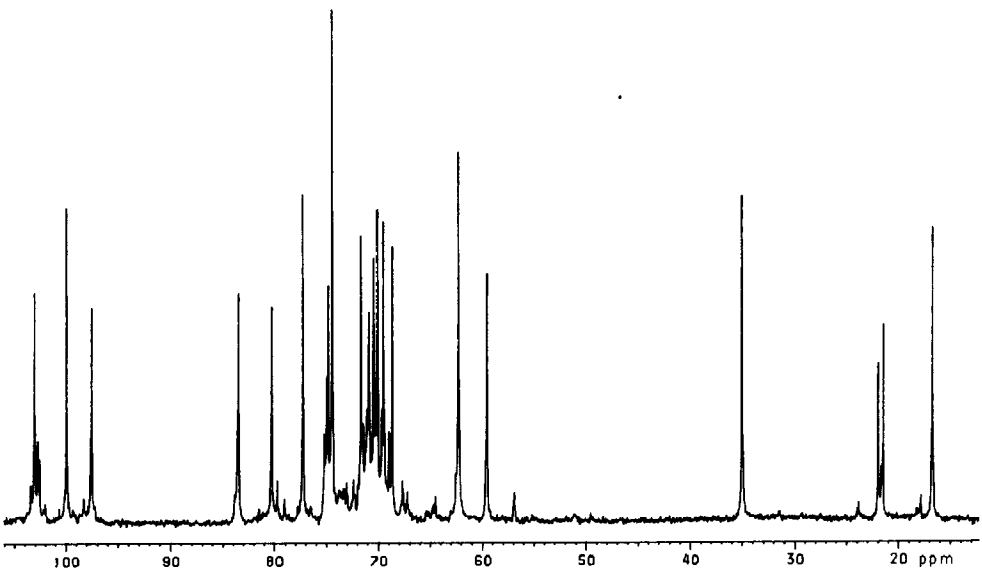

Fig. 1. ^{13}C NMR spectrum of PS.

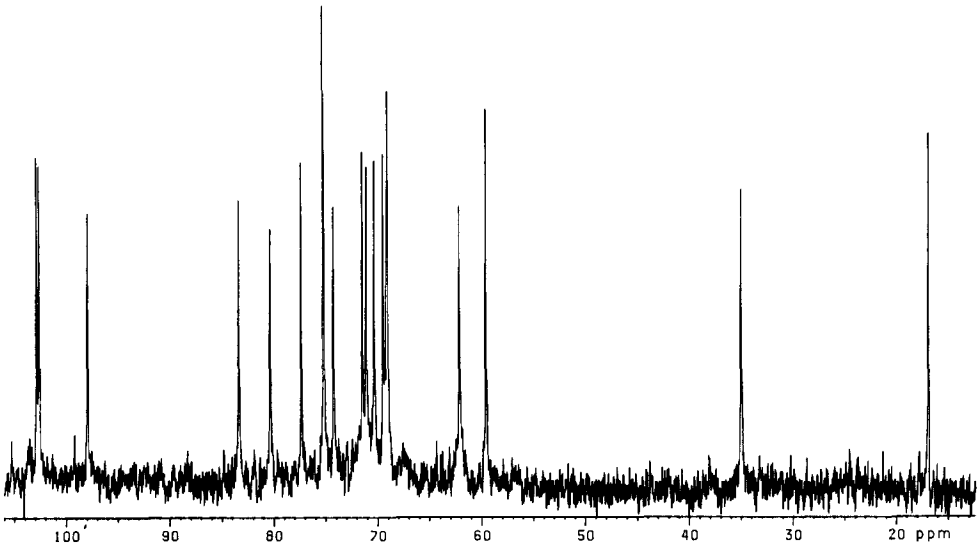

Fig. 2. ^{13}C NMR spectrum of *O*-deacetylated PS (DPS).

samples using PC, a sugar analyser, and GLC of the alditol acetates. In GLC–MS, the alditol acetate derived from the third monosaccharide gave a mass spectrum identical to that described[3] for a 2-deoxyheptitol hexa-acetate and, hence, this sugar was a 2- or 6-deoxyheptose.

Each monosaccharide was isolated by preparative PC. The ^1H NMR spectrum of 6-deoxytalose (Table I) was identical to that described[4] and confirmed the *talo* configuration. As judged by the multiplicities and J values[5], determined from the ^1H NMR spectrum (Table I), the second deoxy sugar was 6-deoxy-*manno*-heptose (6d*man*Hep). The $[\alpha]_D$ values (see Experimental) indicated the glucose and 6-deoxy-*manno*-heptose to be D and the 6-deoxytalose to be L.

Methylation analysis[3,6] of DPS gave (GLC–MS) the alditol acetates of 2,4,6-tri-*O*-methylglucose, 6-deoxy-2,4-di-*O*-methyltalose, and 6-deoxy-2,4,7-tri-*O*-methyl-*manno*-heptose. The mass spectrum of the last-named alditol acetate was identical to that described[7] for the product obtained on methylation analysis of the O-specific polysaccharide from *Yersinia (Pasteurella) pseudotuberculosis* group IIA. The above data showed that each sugar residue in DPS was pyranosidic and 3-substituted.

The ^1H NMR spectrum of DPS (Table I) was assigned on the basis of sequential, selective spin-decoupling and 2D homonuclear shift-correlated (COSY) and one-step relayed-coherence-transfer shift-correlated (COSYRCT) spectroscopy. The relatively highfield positions (δ 4.65 and 4.80) of the signals for H-1 of the Glc and 6d*man*Hep and the $J_{1,2}$ value of 7.9 Hz for the former proved[8] that each residue was β. In contrast, the position (δ 5.26) of the third H-1 signal indicated[8] the 6dTal to be α. These conclusions were confirmed[9] by the $J_{C\text{-}1,H\text{-}1}$ values determined from the gated-decoupling spectrum of DPS. Two of these values, for the signals at 97.9 and 102.6 ppm, were relatively small (< 165 Hz) and

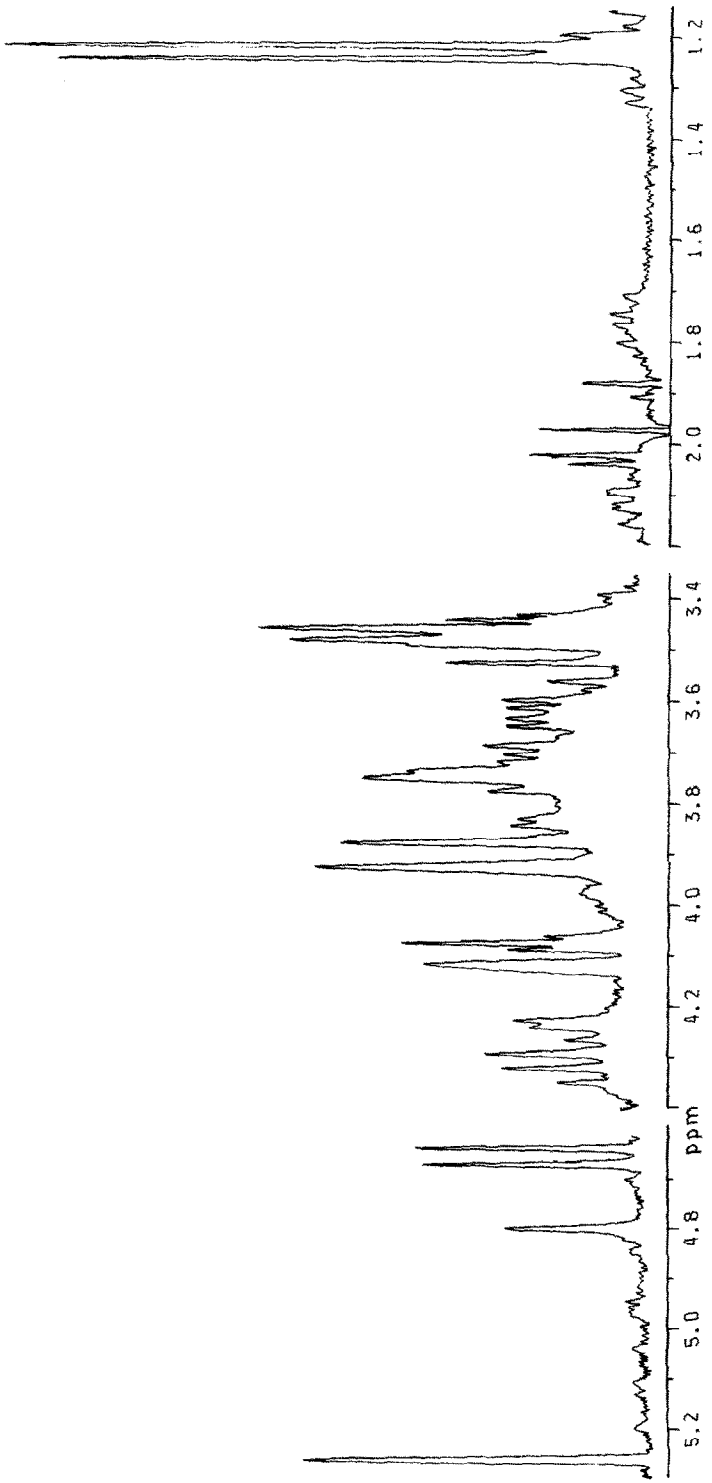

Fig. 3. ^{1}H NMR spectrum of *O*-deacetylated PS (DPS).

TABLE I

^{1}H NMR data (δ in ppm, J in Hz)

	H-1	H-2	H-3	H-4	H-5	H-6	H-7
*6-Deoxy-*L*-talose*							
*α-6dTal*p							
δ	5.23 (d)	3.83 (dd)	3.93 (t)	3.76 (dd)	4.19 (dq)	1.26 (d, 3 H)	
	$J_{1,2}$ 2.3	$J_{2,3}$ 3.5	$J_{3,4}$ 3.5	$J_{4,5}$ 1.6	$J_{5,6}$ 6.9		
*β-6dTal*p							
δ	4.79 (d)	3.87 (dd)	3.77 (t)		3.66–3.75 (m)	1.29 (d, 3 H)	
	$J_{1,2}$ 1.5	$J_{2,3}$ 3.4	$J_{3,4}$ 3.4		$J_{5,6}$ 6.8		
*α-6dTal*f							
δ	5.25 (d)	3.99 (dd)	4.23 (dd)	3.75 (dd)	3.87 (dq)	1.25 (d, 3 H)	
	$J_{1,2}$ 2.0	$J_{2,3}$ 5.0	$J_{3,4}$ 6.6	$J_{4,5}$ 1.6	$J_{5,6}$ 6.6		
*β-6dTal*f							
δ	5.37 (m)	4.08–4.11 (m)		3.88 (m)	nf [a]	1.24 (d, 3 H)	
						$J_{5,6}$ 6.6	
*6-Deoxy-*D*-manno-heptose*							
*α-6d*man*Hep*p							
δ	5.09 (d)	3.89 (dd)	3.77 (dd)	3.49 (t)	3.81 (dt)	2.04 (ddd)	3.60–3.77 (m, 2 H)
						1.67 (ddd)	
	$J_{1,2}$ 2	$J_{2,3}$ 3.6	$J_{3,4}$ 9.6	$J_{4,5}$ 9.6	$J_{5,6a}$ 2.8	$J_{5,6b}$ 9.5	$J_{6a,7}$ 7.2
					$J_{6a,6b}$ 14.7	$J_{6b,7}$ 3.1	
*β-6d*man*Hep*p							
δ	4.81 (d)	4.91 (dd)	3.57 (dd)	3.49 (t)	3.33 (dt)	2.07 (ddd)	3.60–3.77 (m, 2 H)
						1.73 (ddd)	
	$J_{1,2}$ 1.1	$J_{2,3}$ 3.5	$J_{3,4}$ 9.1	$J_{4,5}$ 9.5	$J_{5,6a}$ 3.0	$J_{5,6b}$ 9.5	$J_{6a,7}$ 7.2
					$J_{6a,6b}$ 14.7	$J_{6b,7}$ 3.1	
Polysaccharide 1							
*→ 3)-β-*D*-Glc*p*-(1 →*							
δ	4.65 (d)	3.48 (dd)	3.65 (t)	3.46 (m)	3.46 (m)	3.90 (d, 2 H)	
	$J_{1,2}$ 7.9	$J_{2,3}$ 9.5	$J_{3,4}$ 9.5				
*→ 3)-α-*L*-6dTal*p*-(1 →*							
δ	5.26 (s)	4.11 (bs)	4.07 (t)	3.92 (bs)	4.31 (q)	1.23 (d, 3 H)	
		$J_{2,3}$ 3.2	$J_{3,4}$ 3.2	$J_{4,5} < 2$	$J_{5,6}$ 6.7		
Polysaccharide 2							
*→ 3)-β-*D*-man*Hep*p-(1 →*							
δ	4.80 (s)	4.23 (d)	3.83 (dd)	3.60 (t)	3.46 (m)	2.10 (m)	3.74 (m, 2 H)
						1.74 (m)	
	$J_{1,2} < 2$	$J_{2,3}$ 3.5	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5			

[a] Not found.

the third, for the signal at 102.8 ppm, was relatively large (170 Hz) and consistent with the presence of two β- and one α-pyranosidic residues.

In NOE experiments on DPS, pre-irradiation of Glc H-1 at 4.65 ppm affected Glc H-2,3,5 and caused a significant (> 3%) NOE on 6dTal H-3. On pre-irradiation of 6dTal H-1 at 5.26 ppm, NOEs on 6dTal H-2 and Glc H-3 were observed. Pre-irradiation of 6d*man*Hep H-1 at 4.80 ppm caused NOEs only on 6d*man*Hep H-2,3,5. These data proved that DPS was a mixture of two polysaccharides with the structures **1** and **2**.

→ 3)-β-D-Glc*p*-(1 → 3)-α-L-6dTal*p*-(1 →

1

→ 3)-β-D-6d*man*Hep*p*-(1 →

2

The ^{13}C NMR spectrum of DPS was assigned tentatively on the basis of the data for the monosaccharides (Table II) and glycosylation effects[10]. Comparison of the spectra of DPS and PS showed that the significant β-effect of *O*-acetylation[11] was on the signal for 6dTal C-1, which was shifted from 102.8 to 99.8 ppm, reflecting the presence of an OAc group at position 2 of the 6dTal unit. The signal for 6d*man*Hep C-2 was shifted from 69.1 to 70.4 ppm due to the α-effect of *O*-acetylation[11] and indicated the second OAc group to be at position 2 of the 6d*man*Hep. Therefore, each of the polysaccharides from strain 57576 was partially *O*-acetylated and had the structures **3** and **4**.

→ 3)-β-D-Glc*p*-(1 → 3)-α-L-6dTal*p*-(1 →

|2

OAc

3

→ 3)-β-D-6d*man*Hep*p*-(1 →

|2

OAc

4

As judged by the relative intensities of the major and minor ^{13}C signals for PS, the degree of *O*-acetylation was ~ 80% in each polysaccharide and varied from 50 to 80% from batch to batch of bacterial cells, depending on the growth conditions and, in particular, the duration of growth.

It is of interest that strain 57576 produced two different O-specific polysaccharides. Not only can the degree of *O*-acetylation vary, but also the relative content of the polysaccharides **3** and **4** can vary (from 0.7 to 1.3) from batch to batch of bacterial cells. Other examples of the simultaneous production of two polysaccharides by Gram-negative bacteria are *P. cepacia*[12] and *Serratia marcescens*[13].

Each polysaccharide from strain 57576 contained a relatively rare deoxy sugar. 6-Deoxy-L-talose occurs in O-antigens of *P. fluorescens*[4,14] and in several other

TABLE II

^{13}C NMR data [a] (δ in ppm)

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7
6-Deoxy-L-talose							
α-6dTal*p*	95.4	71.2	66.3	73.0	67.7	16.5	
β-6dTal*p*	94.7	72.0	69.6	71.8	72.0	16.4	
α-6dTal*f*	101.6	76.3	72.0	88.6	69.6	18.7	
β-6dTal*f*	97.0	71.8	71.8	87.0	68.0	19.2	
*6-Deoxy-*D-manno-*heptose*							
α-6d*man*Hep*p*	95.4	72.0	71.5	71.9	70.5	34.6	59.7
β-6d*man*Hep*p*	95.0	72.5	74.3	71.5	74.1	34.6	59.4
Polysaccharide 1 [b]							
→3)-β-D-Glc*p*-(1→	102.6	75.1	83.3	69.4	77.3	62.1	
→3)-α-L-6dTal*p*-(1→	102.8	71.4	75.1	71.0	69.0	16.7	
Polysaccharide 3 [b]							
→3)-β-D-Glc*p*-(1→	103.0	74.8	83.4	69.5	77.2	62.2	
→3)-α-L-6dTal*p*-(1→	99.9	74.4	71.6	70.0	68.8	16.7	
Polysaccharide 2 [b]							
→3)-β-D-6d*man*Hep*p*-(1→	97.9	69.1	80.3	70.3	74.2	34.9	59.5
Polysaccharide 4 [b]							
→3)-β-D-6d*man*Hep*p*-(1→	97.5	70.8	80.2	70.4	74.4	34.9	59.5

[a] Additional signals: δ 21.5–21.7 (CH_3CO) and 174.6–174.7 (CH_3CO). [b] Assignments for the polysaccharides are tentative.

bacterial polysaccharides[15]. To our knowledge, 6-deoxy-D-*manno*-heptose has been discovered hitherto only in the O-antigens of *Yersinia (Pasteurella) pseudotuberculosis*[7,16].

Polysaccharide **4** from *P. pseudomallei* strain 57576 is the first heptan to be found in nature. ^{13}C NMR spectroscopy showed that the O-specific polysaccharide obtained from strain 100 also had the structure **4**, as did that from strain 110, but the latter was contaminated with another polysaccharide (or polysaccharides) of unknown structure that contained galactose and rhamnose. Strain 97 gave polysaccharides **3** and **2** (i.e., *O*-deacetylated **4**). As with strain 110, a carbohydrate impurity was detected in strain 97 by ^{13}C NMR spectroscopy.

An immunochemical study of the O-antigens of *P. pseudomallei* should reveal their role in serological specificity and virulence.

EXPERIMENTAL

General methods.—Optical rotations were measured with a Jasco DIP 360 polarimeter on aqueous solutions at 25°.

Ascending PC was carried out on FN-11 paper, using 1-butanol–pyridine–water (6:4:3). GLC was performed with a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector and a glass capillary Ultra 1 column (0.2 mm × 25

m). GLC–MS was performed on a Varian MAT 311 instrument. Gel-permeation chromatography was performed on a column (3.5 × 70 cm) of Sephadex G-50 in a pyridine–acetic acid buffer (pH 5.5) and monitored by the phenol–H_2SO_4 method[17]. Sugar analysis with a Technicon Autoanalyzer II was performed as described[18].

The ^1H and ^{13}C NMR spectra were recorded with Bruker WM-250 and AM-300 instruments, respectively, for solutions in D_2O at 30° for monosaccharides and 60° for polysaccharides (internal acetone, ^1H δ 2.23 and ^{13}C δ 30.45). Sequential, selective spin-decoupling and 1D NOE experiments were performed as described[19]. The 2D homonuclear shift-correlated (COSY) and one-step relayed-coherence-transfer shift-correlated (COSYRCT) spectra were obtained under the conditions described[20].

Bacterial strains, growth, and isolation of lipopolysaccharides and polysaccharides. —*P. pseudomallei* strains 57576 and 100 (Dalat) were isolated in Vietnam; strain 110 (Pierce), isolated in Australia, was kindly provided by Dr. L.R. Ashdown (Laboratory of Pathology, Townsville); and the place where strain 97 was isolated is unknown.

Bacteria was grown on a two-phase medium containing meat-peptone agar with added glycerol under a thin layer of Difco nutrient broth for 24 or 48 h at 37° and pH 6.8. Cells were centrifuged at 10000 *g*, washed with cold 0.15 M NaCl, killed with acetone (3 vol), and dried with 4–5 vol of acetone.

Dried cells were extracted by the phenol–water procedure[2], the extract was dialysed without separation of the layers and then centrifuged at 15000 *g*, and the nucleic acids were removed by precipitation with streptomycin[21]. The supernatant solution was dialysed, then concentrated using an Amicon XM-100 membrane, the lipopolysaccharides were precipitated with EtOH (10 vol) and dissolved in water, and the solution was freeze-dried.

The lipopolysaccharides were degraded with aq 1% HOAc for 3–4 h at 100°, the lipid precipitate was removed by centrifugation, and the polysaccharides were isolated by gel-permeation chromatography on Sephadex G-50.

O-Deacetylation.—PS was treated with aq 10% ammonia for 2 h at 50°, the solution was concentrated, and a solution of the residue in water was freeze-dried to give the *O*-deacetylated polysaccharide (DPS).

Methylation analysis.—The Hakomori conditions[6] were used as described[20].

Acid hydrolysis.—DPS (40 mg) was treated with 2 M CF_3COOH for 2 h at 100° and the hydrolysate was concentrated. Preparative PC of the residue gave D-glucose, $[\alpha]_D$ +47° (*c* 0.8) [lit.[21] +52.2° (water)], 6-deoxy-L-talose, $[\alpha]_D$ −8.7° (*c* 0.6) [lit.[22] −18.9 ± 2° (water)], and 6-deoxy-D-*manno*-heptose, $[\alpha]_D$ +24° (*c* 0.5) [lit.[7] +30 ±5° (water)].

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