Carbohydrate Research, 204 (1990) 1–9
Elsevier Science Publishers B.V., Amsterdam

G.l.c.–m.s. of partially methylated and acetylated derivatives of L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptopyranoses and -heptitols*

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(Received August 26th, 1989; accepted for publication, December 8th, 1989)

ABSTRACT

Methylated or acetylated heptopyranose derivatives were prepared variously from L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptopyranose, *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→3)-L-*glycero*-D-*manno*-heptopyranose, *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→7)-L-*glycero*-D-*manno*-heptopyranose, and *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→7)-*O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→3)-L-*glycero*-D-*manno*-heptopyranose, which are structural elements of the heptose region of enterobacterial lipopolysaccharides. Each derivative was investigated by g.l.c. and g.l.c.–m.s., and the retention times and fragmentation patterns were used to identify partial structures of the heptose region of the core oligosaccharide of bacterial LPS.

INTRODUCTION

3-Deoxy-D-*manno*-2-octulosonic acid (Kdo) and heptose, reported as L-*glycero*-D-*manno*- (**1**) and D-*glycero*-D-*manno*-heptose (**2**), are common constituents of many bacterial lipopolysaccharides (LPS)[1,2]. Usually, these sugars are constituents of the so-called inner core region, but, in *Vibrio cholerae* O:21, D-*glycero*-D-*manno*-heptose has been reported[3] as part of the O-specific chain. Some LPS lack heptose, such as those from enterobacterial Re mutants or bacteria remote from the Enterobacteriaceae[2].

Several investigations of the structure of the heptose part of the inner core region from different LPS have been published. One of the best known structures is that from *Salmonella minnesota* R60 (Ra-core)[1], where the trisaccharide *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→7)-*O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→3)-L-*glycero*-D-*manno*-heptopyranose (**7**) was identified. This constituent carries phosphate and 2-aminoethyl diphosphate substituents. The trisaccharide **7** has been suggested to be present in LPS of other bacteria[1,4], and the partial structure *O*-L-*glycero*-α-D-*manno*-

* Presented at EUROCARB V, the Vth European Carbohydrate Symposium, Prague, Czechoslovakia, August 21–25, 1989.
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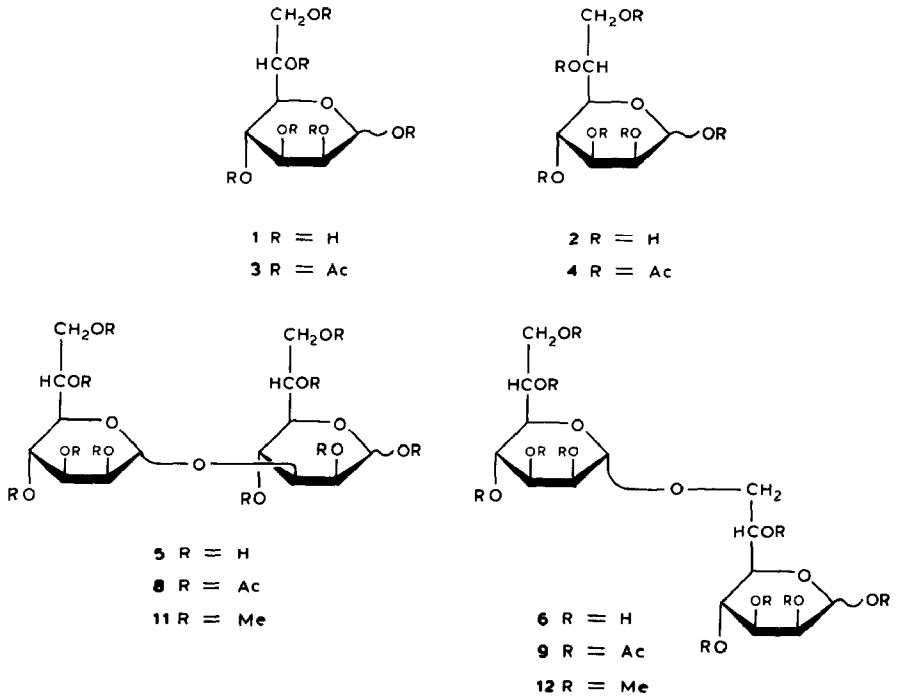
0008-6215/90/$ 03.50

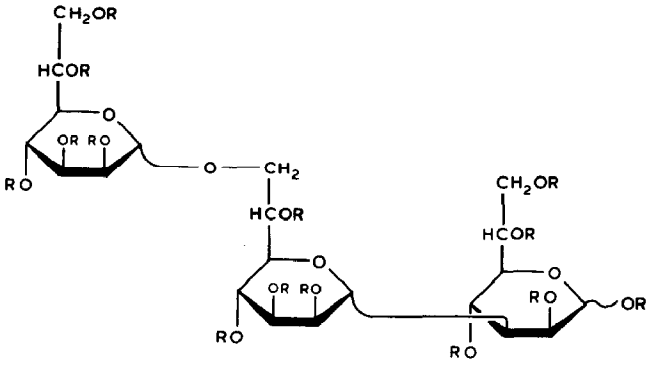
heptopyranosyl-(1→3)-L-*glycero*-D-*manno*-heptopyranose (**5**) was found in the rough mutants Rc and Rd_1 of *S. minnesota*. Structures different from the heptose region of *S. minnesota* and *Escherichia coli* strains were identified in other bacterial species, *e.g.*, in *V. ordalii*[5], where four heptose residues were found, in *Aeromonas salmonicida*[6], in which **5** is substituted by a third L-*glycero*-D-*manno*-heptose at position 2 of the second heptose in the main chain, and in *Proteus mirabilis*[7], where the disaccharide *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→4)-L-*glycero*-D-*manno*-heptopyranose was detected.

Analysis of the structure of the heptose region is difficult since it is substituted with (pyro)phosphate groups to various extents and at different positions, and bound to the Kdo region. The heptose-Kdo and inter-heptose linkages are rather stable, whereas the Kdo linkages are acid-labile. Therefore, methylation analysis requires special conditions. Following investigations[1,8] on the Kdo region by g.l.c.–m.s. and n.m.r. spectroscopy, systematic studies of the heptose region have been started. Compounds **1**, **2**, **5**, *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→7)-L-*glycero*-D-*manno*-heptopyranose (**6**), and **7** were used for acetylation, methylation, and methylation analysis, in order to provide standards for the methylation analysis of natural LPS, and the g.l.c. and g.l.c.–m.s. data are now reported.

EXPERIMENTAL

The syntheses of L-*glycero*-D-*manno*-heptopyranose (**1**), D-*glycero*-D-*manno*-heptopyranose (**2**), *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→3)-L-*glycero*-D-*manno*-





7 R = H
10 R = Ac
13 R = Me

TABLE I

Relative retention times (*T*) of derivatives in g.l.c.

Compound	T[a]	*Compound*	T[a]
3	1.76	**18**	0.26
4	1.81	**19**	0.21
8	3.92/3.95	**20**	0.39
9	3.96	**21**	0.61
10	5.32/5.42	**22**	0.71
11	2.88	**23**	1.03
12	2.93	**24**	1.11
13	4.44	**27**	2.78
16	2.05	**28**	2.87
17	1.95	**30**	4.55

[a] Relative to α-D-glucopyranose penta-acetate, *T* 1.00.

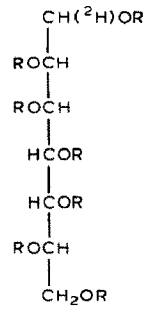
heptopyranose (**5**), *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→7)-L-*glycero*-D-*manno*-heptopyranose (**6**), and *O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→7)-*O*-L-*glycero*-α-D-*manno*-heptopyranosyl-(1→3)-L-*glycero*-D-*manno*-heptopyranose (**7**) have been described[9,10].

Reduction with NaB^2H_4 was done conventionally, as was acetylation with pyridine–acetic anhydride (1:1, 30 min, 100°). Methylation was performed according to the modified[11] Hakomori[12] procedure, followed by purification on SEP-PAK C_{18} cartridges[13]. Reduced and methylated oligosaccharides were hydrolysed in 2M trifluoroacetic acid (2 h, 120°). Methylated LPS was methanolysed in methanolic M HCl for 16 h at 85°.

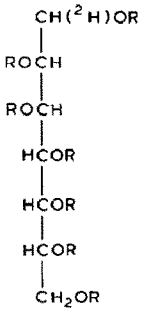
Derivatisation of **1**, **2**, *and* **5**–**7** *for g.l.c.–m.s.* — All *T* values are relative to that of α-D-glucopyranose penta-acetate and are shown in Table I. Acetylation of the heptoses **1** and **2** gave the hexa-acetates **3** and **4**, respectively. Saponification (methanolic 0.25M sodium methoxide, 15 min, room temperature) of **3** and **4** followed by reduction

(NaB^2H_4) gave the (1-^{2}H)heptitols **14** and **15**, respectively, which, after acetylation or methylation, yielded **16–19**. All derivatives were identified by e.i.- and c.i.(ammonia)-m.s. (data not shown). Acetylation of the disaccharides **5** and **6** gave the deca-acetates **8** and **9**, respectively, and likewise the trisaccharide **7** gave the tetradeca-acetate **10**, which were identified by g.l.c. and g.l.c.–m.s. Methylation of **8–10** gave **11–13**. Compounds **5–7** were reduced with NaB^2H_4 to give the corresponding (1-^{2}H)alditol derivatives **25**, **26**, and **29** that were methylated to afford **27**, **28**, and **30**, respectively, which were identified by g.l.c. and g.l.c.–m.s. Hydrolysis, reduction with NaB^2H_4, and acetylation gave the heptitol derivatives **20** and **22** from **27**, and **21** and **22** from **28**. Likewise, **13** gave **22–24**.

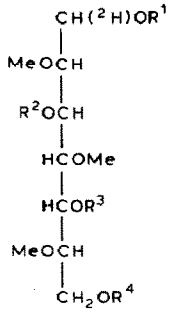
G.l.c. was performed with a Varian 3700 gas chromatograph equipped with a flame-ionisation detector and a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded SE-54 (0.2 μm; Weeke, Mühlheim); the carrier gas was H_2 (1.5 bar). Temperature programmes: 140° for 3 min, then 3°.min^{-1} → 250° for methylated and acetylated heptopyranoses and partially methylated and acetylated heptitols; 150° for 5 min, then 5°.min^{-1} → 300° for reduced and methylated oligosaccharides. G.l.c.–m.s. was carried out on a Hewlett–Packard instrument (Model 5985) equipped with an SE-54 column and an HP-1000 data system. E.i.-mass spectra were recorded at 70 eV, and c.i.-mass spectra were obtained with ammonia as reactant gas. The ion-source temperature was 200°.



14 R = H
16 R = Ac
18 R = Me



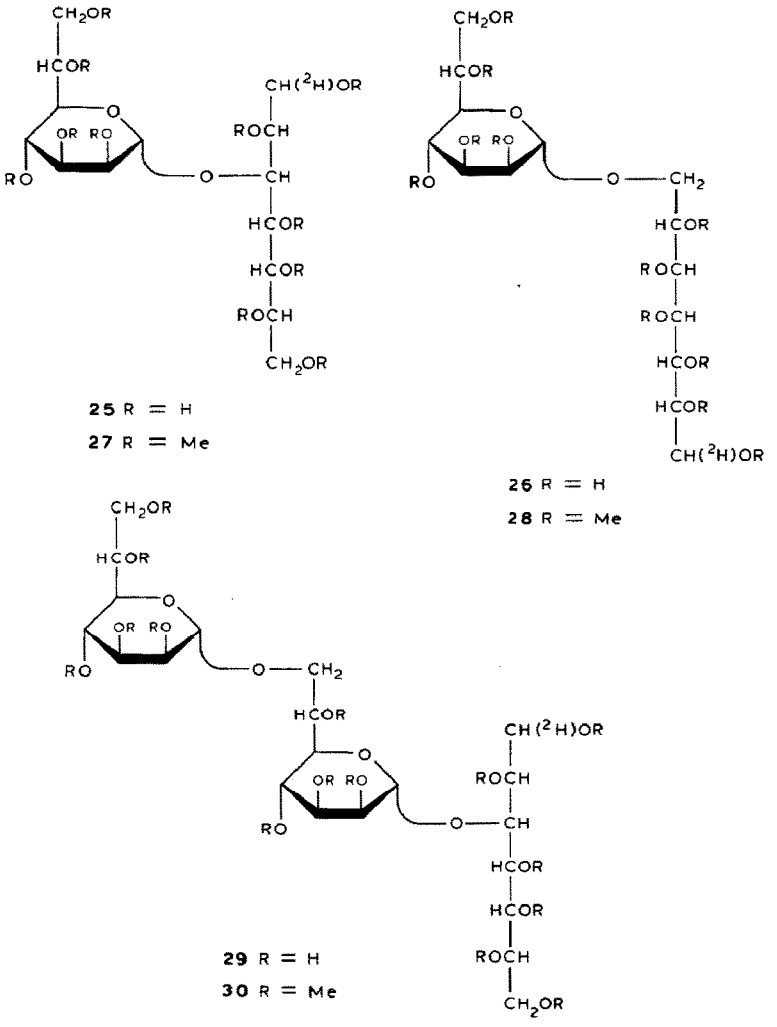
15 R = H
17 R = Ac
19 R = Me



20 R^2 = Ac; R^1, R^3, R^4 = Me
21 R^4 = Ac; R^1, R^2, R^3 = Me
22 R^1, R^3 = Ac; R^2, R^4 = Me
23 R^1, R^2, R^3 = Ac; R^4 = Me
24 R^1, R^3, R^4 = Ac; R^2 = Me

RESULTS AND DISCUSSION

G.l.c. and g.l.c.–m.s. of the disaccharide derivatives **8**, **9**, **11**, *and* **12**, *and the trisaccharide derivatives* **10** *and* **13**. — The e.i.-m.s. data for **8–10** are summarised in Table II. Most of the prominent peaks were derived from the ion at *m/z* 403, which was the base peak in the spectrum of **9**. The loss of two acetates and a ketene gave the ion at *m/z* 241 (base peak in the spectra of **8** and **10**), from which the daughter ions at *m/z* 199



(−42) and 181 (−60) were derived. The ion at *m/z* 139 may originate from that at *m/z* 199 (−60) and/or from the ion at *m/z* 181 (−42). After elimination of a ketene (−42), an ion at *m/z* 97 was obtained. The peak at *m/z* 157 was derived from that at *m/z* 199 (−42) and led to the appearance of ions at *m/z* 97 (−60) and *m/z* 115 (−42). All fragments appeared in the e.i.-mass spectra of **8**–**10**, with different intensities. Compounds **8** and **9** were identified by their base peaks (ions at *m/z* 241 for **8** and *m/z* 403 for **9**). The e.i.-mass spectra of **8** and **10** differed in that several fragments of **10** had lower intensities than those of **8**. In c.i. (ammonia)-m.s., only the mol. wt. of **8** and **9** could be determined ($[M + 18]^+$, 840). Direct inlet probe-c.i.-(ammonia)-m.s. (d.i.p.-m.s.) of **10** revealed only fragment 32 (*m/z* 763) as the highest mass; therefore, determination of the mol. wt. was not possible.

The e.i.-m.s. data of **11**–**13** are summarised in Table III. Besides the ions at *m/z* 73,

TABLE II

E.i.-m.s. data for **8–10**

Compound	*Mol. wt.*	m/z *(% of base peak)*
8	822	45 (6.6/9.2), 69 (14.2/7.4), 71 (12.7/3.0), 73 (21.2/21.6), 81 (17.1/10.6), 85 (19.1/11.0), 97 (45.8/30.1), 99 (75.8/5.4), 101 (6.4/3.8), 103 (31.4/19.0), 110 (23.0/14.5), 111 (50.5/18.7), 115 (40.1/25.8), 127 (13.3/8.2), 139 (81.1/53.9), 145 (16.9/15.6), 152 (11.6/10.7), 153 (47.2/15.7), 157 (13.7/11.0), 181 (35.1/24.8), 199 (14.4/11.5), 241 (100.0/100.0), 242 (12.0/11.1), 403 (28.3/24.9).
9	822	45 (8.4), 69 (10.3), 73 (10.4), 81 (11.4), 85 (12.9), 97 (38.2), 103 (21.8), 110 (14.7), 111 (29.5), 115 (33.5), 139 (64.5), 145 (13.9), 152 (13.1), 153 (17.5), 157 (13.9), 181 (31.6), 199 (14.6), 227 (12.2), 241 (69.2), 403 (100.0).
10	1182	45 (25.7/11.7), 97 (48.6/30.6), 103 (24.8/20.7), 110 (22.0/12.6), 111 (27.5/17.1), 115 (22.9/14.4), 127 (19.3/15.3), 139 (87.2/79.3), 153 (29.4/18.0), 181 (33.0/22.5), 199 (22.9/23.4), 241 (100.0/100.0), 403 (48.6/37.8)

TABLE III

E.i.-m.s. data for **11–13**

Compound	*Mol. wt.*	m/z *(% of base peak)*
11	542	45 (16.6), 59 (15.8), 71 (13.8), 75 (38.1), 88 (53.1), 89 (37.6), 101 (100.0), 111 (17.5), 145 (65.7), 175 (15.7), 199 (22.0), 263 (23.8), 323 (10.1)
12	542	45 (15.3), 59 (12.0), 71 (11.6), 73 (13.6), 75 (100.0), 88 (97.1), 89 (30.8), 101 (96.6), 111 (10.6), 145 (7.6), 199 (19.3), 263 (27.4), 323 (7.2)
13	790	45 (15.5), 59 (13.0), 71 (16.0), 73 (11.4), 75 (57.6), 88 (61.9), 89 (40.2), 101 (100.0), 111 (19.4), 145 (34.8), 175 (13.5), 199 (25.6), 263 (18.7), 323 (2.2)

TABLE IV

E.i.-m.s. data for **27**, **28**, and **30**

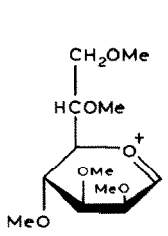
Compound	*Mol. wt.*	m/z *(% of base peak)*
27	559	45 (21.7), 59 (15.5), 71 (10.3), 75 (22.1), 88 (39.3), 89 (38.2), 90 (10.6), 101 (100.0), 102 (7.2), 111 (12.9), 128 (13.4), 145 (20.0), 146 (6.2), 199 (23.6), 231 (6.1), 280 (11.5), 340 (3.9)
28	559	45 (13.8), 59 (9.0), 71 (22.7), 75 (31.6), 88 (38.2), 89 (23.6), 90 (20.7), 101 (100.0), 102 (14.5), 111 (17.0), 128 (3.6), 145 (6.8), 146 (15.5), 199 (23.7), 231 (7.1), 263 (10.3), 280 (21.8)
30	807	59 (12.9), 71 (13.9), 75 (30.7), 88 (51.5), 89 (45.5), 90 (9.7), 101 (100.0), 102 (6.0), 111 (13.3), 128 (14.3), 145 (19.9), 199 (32.1), 231 (8.9), 263 (19.2), 280 (20.0), 340 (6.1)

75, 88, and 101 (base peak in the spectra for **11** and **13**; ions H_2, J_1^2, H_1^2, and F_1^2, see ref. 14), an A-series[14] was present, starting with **31** (*m/z* 263), which resulted in the daughter ions at *m/z* 231 (−32) and 199 (−64). The e.i.-mass spectra of **11–13** contained a J_1-fragment[15] at *m/z* 323 (263 + 60, small intensity for **13**). Differentiation of **11** and **12** was possible by the intensities of ions at *m/z* 145 (65.7% for **11**, 7.6% for **12**) and 88 (53.1% for **11**, 97.1% for **12**). The mol. wts. of **11–13** were verified by c.i.(ammonia)-m.s., where ions at *m/z* 560 [$(M + 18)^+$, for **11** and **12**] and 808 (for **13**) were identified.

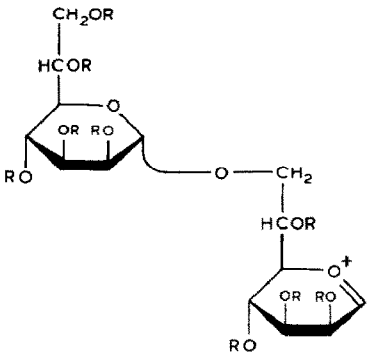
G.l.c. and g.l.c.–m.s. of the disaccharide-alditols **27** *and* **28**, *and the trisaccharide-alditol* **30**. — Table IV summarises the e.i.-m.s. data. Typical for **27** and **28** were ions at *m/z* 280 (from the alditol chain) and 263 (**31**). The ion **31** afforded daughter ions at *m/z* 231 (−32) and 199 (−64), which were also intense, whereas the ion at *m/z* 280 gave peaks of low intensity at *m/z* 248 (−32) and 184 (−96). The expected J_1-fragment (*m/z* 340) could not be identified for **28**. A poor fragmentation of the alditol chain was found for **27** and **28**. The mass spectra of these two derivatives were very similar; however, the missing J_1-fragment in e.i.-m.s. of **28** could allow differentiation.

The ion at *m/z* 280 and its J_1 fragment at *m/z* 340 were present in the mass spectrum of **30**. Furthermore, **31** was present (*m/z* 263), and its fragments at *m/z* 231 and 199. The ion **33** at *m/z* 511 gave the daughter ions at *m/z* 479 (−32) and *m/z* 415 (−96). The molecular weights of **27**, **28**, and **30** were confirmed by c.i.(ammonia)-m.s., by the appearance of the ion for $(M + 18)^+$.

Methylation analysis of **13**, **27**, *and* **28**. — The relevant e.i.-m.s. data are summarised in Table V. The fragmentation of compounds **20–24** was essentially the same as described[15] for partially methylated hexitol acetates.



31



32 R = Ac

33 R = Me

Thus, the identification of **1** and **2** as constituents of LPS is possible by g.l.c. and g.l.c.–m.s. Reduced or non-reduced, methylated or acetylated derivatives are suitable for such studies. Furthermore, the results show that the disaccharides **5** and **6** and the trisaccharide **7** can be distinguished by g.l.c.–m.s., in the methylated or acetylated form. The structure **12** was identified as a component of natural LPS from *S. minnesota* R 345

TABLE V

E.i.-m.s. data for **20–24**

Compound	*Mol. wt.*	m/z *(% of base peak)*
20	339	45 (17.8), 46 (4.9), 75 (35.6), 88 (16.7), 89 (34.9), 90 (74.4), 101 (100.0), 114 (17.8), 129 (10.0), 145 (13.3), 158 (22.2), 174 (8.9), 206 (34.4), 250 (26.7).
21	339	45 (22.4), 46 (19.8), 75 (14.4), 88 (17.8), 90 (34.4), 101 (100.0), 102 (22.9), 117 (68.9), 131 (14.0), 134 (14.0), 146 (37.3), 161 (21.1), 178 (14.4), 205 (4.0), 222 (2.7), 249 (2.4), 279 (< 1)
22	367	45 (17.0), 59 (24.8), 75 (37.0), 88 (22.4), 89 (100.0), 101 (75.9), 102 (81.0), 113 (18.9), 118 (98.1), 128 (17.9), 162 (24.7), 205 (25.1)
23	395	45 (11.1), 59 (13.5), 89 (32.8), 100 (10.5), 101 (28.6), 118 (100.0), 128 (18.8), 202 (12.0), 205 (9.2), 234 (12.7)
24	395	45 (9.7), 75 (14.8), 85 (13.8), 88 (21.3), 101 (48.8), 102 (100.0), 117 (86.9), 118 (80.1), 127 (19.0), 143 (18.8), 159 (14.5), 162 (38.3), 233 (15.5), 277 (8.6)

by g.l.c. and g.l.c.–m.s. after methylation, methanolysis, and methylation, in comparison with the synthetic compound.

ACKNOWLEDGMENTS

We thank Veronika Susott for expert technical assistance and Hermann Moll for help with g.l.c.–m.s. The work was supported by grants from the Bundesministerium für Forschung und Technologie (01ZR8604) and the Polish Academy of Sciences (No. CPBP 01.13.2.12).

REFERENCES

1 H. Brade, L. Brade, and E. T. Rietschel, *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg., Abt. 1: Orig., Reihe A*, 268 (1988) 151–179.
2 H. Mayer, R. N. Tharanathan, and J. Weckesser, *Methods. Microbiol.*, 18 (1985) 157–207.
3 A. A. Ansari, L. Kenne, B. Lindberg, B. Gustafsson, and T. Holme, *Carbohydr. Res.*, 150 (1986) 213–219.
4 E. Romanowska, A. Gamian, and J. Dabrowski, *Eur. J. Biochem.*, 161 (1986) 557–564.
5 J. H. Banoub and H. J. Hodder, *Can. J. Biochem. Cell. Biol.*, 63 (1985) 1199–1205.
6 D. H. Shaw, M. J. Squires, E. E. Ishiguro, and T. J. Trust, *Eur. J. Biochem.*, 161 (1986) 309–313.
7 J. Radziejewska-Lebrecht, U. R. Bhat, H. Brade, and H. Mayer, *Eur. J. Biochem.*, 172 (1988) 535–541.
8 O. Holst, E. Röhrscheidt-Andrzejewski, H. P. Cordes, and H. Brade, *Carbohydr. Res.*, 188 (1989) 212–218.
9 K. Dziewiszek and A. Zamojski, *Carbohydr. Res.*, 150 (1986) 163–171.
10 K. Dziewiszek, A. Banaszek, and A. Zamojski, *Tetrahedron Lett.*, 28 (1987) 1569–1572.
11 A. Tacken, E. T. Rietschel, and H. Brade, *Carbohydr. Res.*, 149 (1986) 279–291.
12 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
13 T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
14 N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
15 J. Lönngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.