*Carbohydrate Research*, 149 (1986) 263–277
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

# G.L.C.–M.S. OF PARTIALLY METHYLATED AND ACETYLATED DERIVATIVES OF 3-DEOXYOCTITOLS*

ANGELIKA TACKEN, HELMUT BRADE**,
*Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Parkallee 1-40, D-2061 Borstel (Federal Republic of Germany)*

FRANK M. UNGER,
*Sandoz-Forschungsinstitut, Brunnerstr. 59, A-1235 Vienna (Austria)*

AND DANIEL CHARON
*Centre d'Etudes Pharmaceutiques, Equipe No. 55 du C.N.R.S., F-92290 Châtenay-Malabry (France)*
(Received September 18th, 1985; accepted for publication, November 1st, 1985)

ABSTRACT

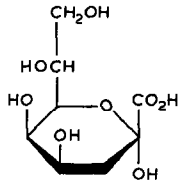
Partially methylated and acetylated 3-deoxyoctitols were prepared from derivatives of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), and identified as the D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* isomers by g.l.c.–m.s. Mono- and oligosaccharide derivatives of KDO were subjected in sequence to methylation, carboxyl-reduction, hydrolysis, carbonyl-reduction, and acetylation to yield 1,2,6-tri-*O*-acetyl-3-deoxyoctitol derivatives. Carboxyl-reduction and then methylation gave the series of 2,6-di-*O*-acetyl derivatives. Oligosaccharides with KDO at the reducing end, *e.g.*, β-D-ribofuranosyl-(1→7)-KDO, α-L-*glycero*-D-*manno*-heptopyranosyl-(1→5)-KDO, and α-KDO*p*-(2→4)-KDO, yielded, after carbonyl-reduction, methylation, carboxyl-reduction, hydrolysis, and acetylation, the 1,7-, 1,5-, and 1,4-di-*O*-acetyl derivatives, whereas remethylation after carboxyl-reduction gave the 7-, 5-, and 4-*O*-acetyl derivatives of 3-deoxyoctitol. General rules for the fragmentation of 3-deoxyoctitols during e.i.-m.s. were established.

## INTRODUCTION

3-Deoxy-D-*manno*-2-octulosonic acid (KDO, **1**), first detected in 1963 (ref. 1), is a common constituent of bacterial lipopolysaccharides (LPS), and is also found in some acidic capsular polysaccharides of *Escherichia coli*[2,3] and *Neisseria meningitidis*[4]. Due to its different functional groups (carboxyl, keto, deoxy, and hydroxyl), the synthetic and analytical chemistry of KDO is difficult[5].

*Presented at the Third European Symposium on Carbohydrates, Grenoble, September 16–20, 1985.
**To whom correspondence should be addressed.

0008-6215/86/$ 03.50 © 1986 Elsevier Science Publishers B.V.



1

We have been interested in the structural elucidation of the inner core region of bacterial LPS, which, in various Gram-negative bacteria, is made up of KDO and neutral sugars[6–8]. We have characterised certain structural elements of this region, *i.e.*, an α-(2→4)-linked KDO disaccharide as a common constituent of enterobacterial LPS[9–11], and one KDO residue[12] that was not substituted at O-7 and O-8, but was substituted at O-5 by a heptosyl residue, in *Salmonella minnesota* rough mutants of the chemotypes $Rd_1P^-$, $Rd_2P^-$, and $RcP^-$. The latter results were obtained by g.l.c.–m.s. of carbonyl-reduced and methylated derivatives of complex oligosaccharides (up to a tetrasacharide) with KDO at the reducing end, which were obtained from LPS and Smith-degraded LPS after mild hydrolysis with acid. However, this method could not be applied to higher oligosaccharides, and, moreover, it did not allow the determination of the linkages between several KDO residues, as they occur in enterobacterial LPS. Therefore, conventional methylation analysis[13–16] is not yet applicable to bacterial LPS. Apparently, there is only one report[7] describing the methylation analysis of LPS-derived KDO oligomers in the LPS of *E. coli*, but we could not reproduce the procedure. Recently, Albersheim and associates[17] reported on the occurrence of KDO in plant cell-walls, and they applied methylation analysis to the reducing KDO moiety of a rhamnosyl-KDO disaccharide. However, their methodology cannot be adapted for the analysis of complex oligosaccharides containing more than one KDO residue. We now describe a modified procedure for methylation analysis which was developed using synthetic and natural KDO-containing oligosaccharides. The application of this procedure in the elucidation of the structure of the inner core region in bacterial LPS is reported in a following paper.

## EXPERIMENTAL

*Reference compounds.* — Ammonium 3-deoxy-α-D-*manno*-2-octulopyranosonate[5], methyl (methyl 4,5,7,8-tetra-*O*-acetyl-3-deoxy-α-D-*manno*-2-octulopyranosid)onate[18] (**2**), disodium [methyl 3-deoxy-4-*O*-(3-deoxy-β-D-*manno*-2-octulopyranosyl)onate-β-D-*manno*-2-octulopyranosid]onate[19] (**9**), trisodium [methyl 3-deoxy-4,7-di-*O*-(3-deoxy-β-D-*manno*-2-octulopyranosyl)onate-β-D-*manno*-2-octulopyranosid]onate[19] (**15**), and sodium [methyl 3-deoxy-7-*O*-(β-D-ribofuranosyl)-β-D-*manno*-2-octulopyranosid]onate[20] (**19**) were synthesised as described in the

literature. Dimethyl [3-deoxy-4-*O*-(3-deoxy-4,5,7,8-tetra-*O*-methyl-α-D-*manno*-2-octulopyranosyl)onate-2,5,6,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*]octonate (**28**) and methyl [3-deoxy-5-*O*-(2,3,4,6,7-penta-*O*-methyl-α-L-*glycero*-D-*manno*-heptopyranosyl)-2,4,6,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*]octonate (**33**) were obtained[10] from the LPS of *S. minnesota* rough mutants (chemotypes Re and $Rd_2P^-$, respectively).

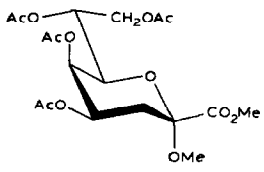
*Methylation analysis.* — Methylation was performed according to Hakomori[13], with the following modifications. To a solution of the dry sample in $Me_2SO$ (1 vol.) was added potassium methylsulfinylmethanide (3.3M, 2 vol.). The sample was stirred (room temperature, 4 h) and sonicated for 15 min every hour. After freezing (−20°), methyl iodide (4 vol.) was added and stirring was continued for 16 h. The methylated sample was purified by reversed-phase chromatography on silica $C_{18}$ cartridges (SEP-PAK, Waters)[14]. Reductions with sodium borohydride or sodium borodeuteride were performed in water at room temperature for 1 h and carboxyl-reduction[21] of KDO methyl ester derivatives in 1:1 methanol–water [or ($^2H_3$)methanol–$^2H_2O$] at 0° for 16 h. Excess of borohydride was destroyed at 0° by adding AG 50W-X8 ($H^+$) resin (Bio-Rad), and the boric acid was removed conventionally as trimethyl borate. Acetylation was performed in pyridine–acetic anhydride (1:1) at 100° for 30 min.

*Acid-catalysed cleavage of glycosidic linkages.* — Methyl ketosides of methylated KDO were hydrolysed in acetic acid (0.03M; 100°, 1 h) and other ketosidic linkages in 0.1M trifluoroacetic acid at 100° for 1 h. Glycosidic linkages of neutral sugars were methanolysed with methanolic 0.5M HCl at 85° for 16 h.

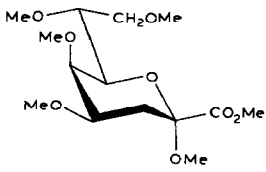
*G.l.c.–m.s.* — G.l.c. was performed with a Varian 3700 gas chromatograph equipped with a flame-ionisation detector and a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded SE-54 (0.2 μm) (Weeke, Mühlheim); the carrier gas was $H_2$. Temperature programmes: monosaccharides, 140° for 3 min, 3°/min → 230°; oligosaccharides, 150° for 5 min, 5°/min → 300°. 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 the reactant gas. The ion-source temperature was 200°.

*Partially methylated and acetylated derivatives of 3-deoxyoctitols.* — Reduction of the keto group of KDO derivatives was not stereospecific, and gave mixtures of D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* derivatives which were not differentiated. Most of the reduction steps were performed with $NaBH_4$ and $NaB^2H_4$ in parallel.

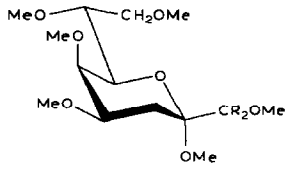
Compound **2** was carboxyl-reduced, hydrolysed, carbonyl-reduced, and then methylated to give 3-deoxy-1,2,4,5,6,7,8-hepta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**6**), or acetylated to give 1,2,4,5,6,7,8-hepta-*O*-acetyl-3-deoxy-D-*glycero*-D-*talo*/*galacto*-octitol (**5**). Methylation of **2** yielded **3**, from which 1,2,6-tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**7**) was obtained after carboxyl-reduction, hydrolysis, carbonyl-reduction, and acetylation. Com-



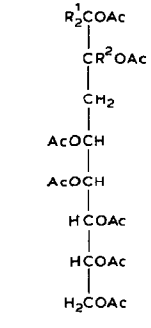
2



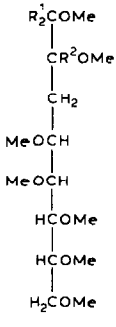
3



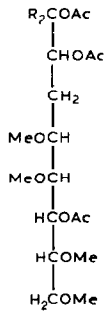
4a R = H
4b R = D



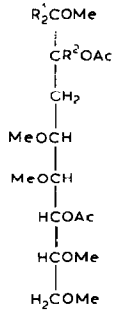
5a $R^1$ = H, $R^2$ = H
5b $R^1$ = D, $R^2$ = D



6a $R^1$ = D, $R^2$ = H
6b $R^1$ = D, $R^2$ = D

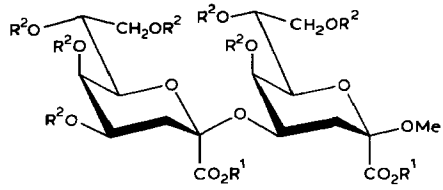


7a R = H
7b R = D

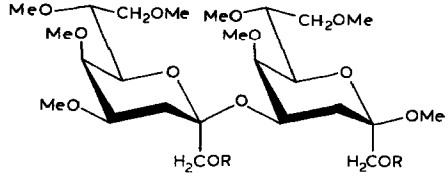


8a $R^1$ = H, $R^2$ = H
8b $R^1$ = D, $R^2$ = H
8c $R^1$ = D, $R^2$ = D

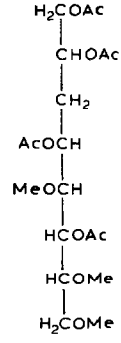
pound **4**, prepared from **3** by carboxyl-reduction, was hydrolysed, carbonyl-reduced, and acetylated to yield 2,6-di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**8**).



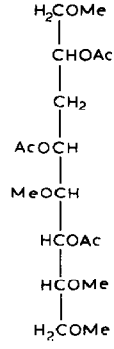
9 $R^1$ = Na, $R^2$ = H
10 $R^1$ = Me, $R^2$ = Me



11 R = H
12 R = Me

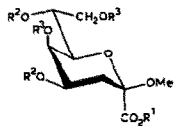


13

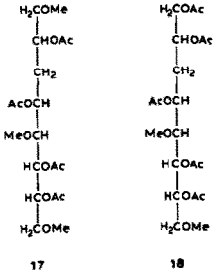


14

The KDO disaccharide **9** was methylated to give **10**, which, after carboxyl-reduction, yielded **11**. Hydrolysis of **11**, followed by carbonyl-reduction and acetylation, gave 1,2,4,6-tetra-*O*-acetyl-3-deoxy-5,7,8-tri-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**13**) and **7a**. Likewise, **12** (derived from **11** by methylation) yielded 2,4,6-tri-*O*-acetyl-3-deoxy-1,5,7,8-tetra-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**14**) and **8a**.



15 $R^1$ = Na, $R^2$ = sodium 3-deoxy-β-D-*manno*-2-octulopyranosylonate, $R^3$ = H
16 $R^1$ = Me, $R^2$ = methyl 3-deoxy-4,5,7,8-tetra-O-methyl-β-D-*manno*-2-octulopyranosylonate, $R^3$ = Me



17 18

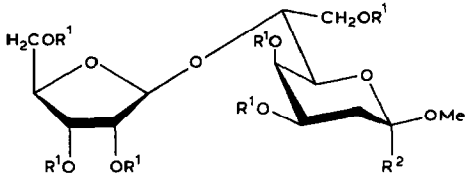
Methylation of trisaccharide **15** gave **16**, which was carboxyl-reduced, hydrolysed, carbonyl-reduced, and acetylated to give 2,4,6,7-tetra-*O*-acetyl-3-deoxy-1,5,8-tri-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**17**) and **8a**, or carboxyl-reduced, methylated, hydrolysed, carbonyl-reduced, and acetylated to give 1,2,4,6,7-penta-*O*-acetyl-3-deoxy-5,8-di-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**18**) and **7a**.

Methylation of disaccharide **19** gave **20**, which was carboxyl-reduced to give **21**. Methanolysis of **21** (85°, 4 h), followed by carbonyl-reduction and acetylation, yielded 1,2,6,7-tetra-*O*-acetyl-3-deoxy-4,5,8-tri-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**23**). Likewise, **22** (derived from **21** by methylation) gave 2,6,7-tri-*O*-acetyl-3-deoxy-1,4,5,8-tetra-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**24**). Selective hydrolysis of **19** (0.1M acetate buffer, pH 4.4; 100°, 30 min), carbonyl-reduction, and methylation gave **25**. Carboxyl-reduction of **25**, followed by methylation (→**26**), methanolysis, and acetylation, gave 7-*O*-acetyl-3-deoxy-1,2,4,5,6,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**27**).

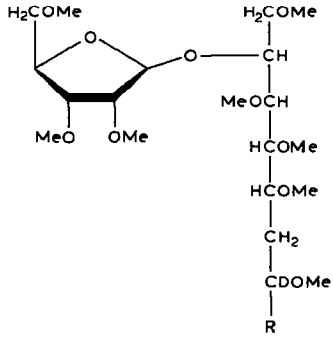
Carboxyl-reduction of **28** gave **29**, which was hydrolysed, carbonyl-reduced, and acetylated, to give 1,4-di-*O*-acetyl-3-deoxy-2,5,6,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**32**) and **8a**. Likewise, **30** (obtained after methylation of **29**) yielded 4-*O*-acetyl-3-deoxy-1,2,5,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**31**) and **7a**.

The methylated pseudodisaccharide **33** yielded **34** after carboxyl-reduction. Methanolysis (85°, 16 h) of **34** followed by acetylation gave 1,5-di-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**36**). Methylation of **34** gave **35** which, after methanolysis and acetylation, yielded 5-*O*-acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**37**) and 2,3,4,6,7-penta-*O*-methyl-L-*glycero*-D-*manno*-heptopyranoside.

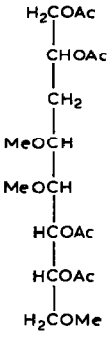
Ammonium KDO (**1**) was carbonyl-reduced and methylated to give methyl



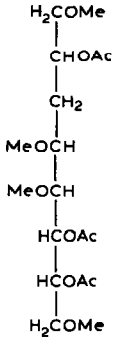
**19** $R^1 = H, R^2 = CO_2Na$
**20** $R^1 = Me, R^2 = CO_2Me$
**21** $R^1 = Me, R^2 = CH_2OH$
**22** $R^1 = Me, R^2 = CH_2OMe$



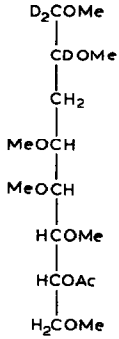
**25** R = $CO_2Me$
**26** R = $CD_2OMe$



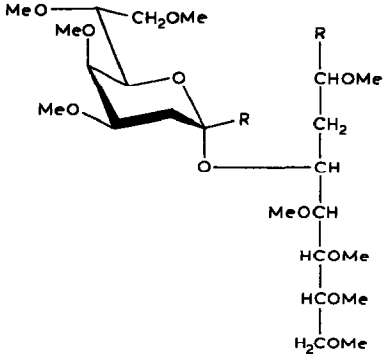
**23**



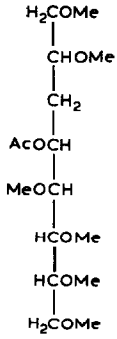
**24**



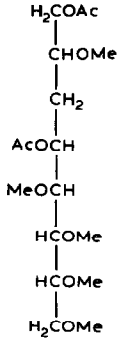
**27**



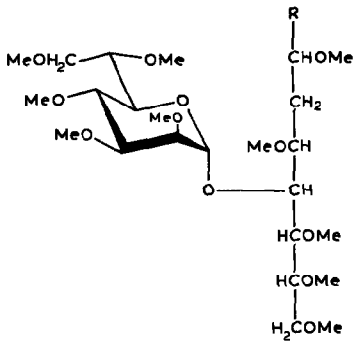
**28** R = $CO_2Me$
**29** R = $CH_2OH$
**30** R = $CH_2OMe$



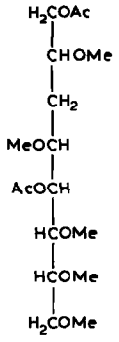
**31**



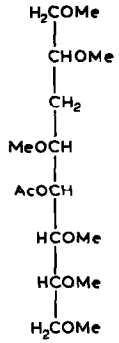
**32**



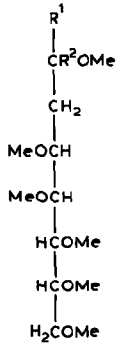
33 R = $CO_2Me$
34 R = $CH_2OH$
35 R = $CH_2OMe$



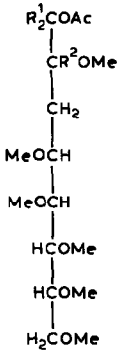
36



37



38a $R^1$ = $CO_2Me$, $R^2$ = H
38b $R^1$ = $CO_2Me$, $R^2$ = D
39a $R^1$ = $CH_2OH$, $R^2$ = D
39b $R^1$ = $CD_2OD$, $R^2$ = H



40a $R^1$ = D, $R^2$ = H
40b $R^1$ = H, $R^2$ = D

3-deoxy-2,4,5,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octonate (**38**) which, after carboxyl-reduction, yielded **39**. Acetylation of **39** gave 1-*O*-acetyl-3-deoxy-2,4,5,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**40**).

## RESULTS AND DISCUSSION

*Methylation analysis.* — 3-Deoxy-D-*glycero*-D-*talo*/*galacto*-octitol derivatives (3-deoxyoctitol derivatives) having various distributions of acetyl and methyl groups were prepared from synthetic KDO derivatives (see Experimental), and from α-KDO*p*-(2→4)-KDO and α-L-*glycero*-D-*manno*-heptopyranosyl-(1→5)-KDO derived from enterobacterial LPS.

Methylation of pyranosidic KDO derivatives, followed by carboxyl-reduction, methylation, hydrolysis, carbonyl-reduction, and acetylation, gave 2,6-di-*O*-acetyl-3-deoxyoctitol derivatives. When the second methylation step was omitted, the 1,2,6-tri-*O*-acetyl derivatives were obtained. Additional acetyl groups were present, depending on the substitution pattern of the KDO derivatives.

Another series of partially methylated 3-deoxyoctitol acetates was prepared from oligosaccharides having KDO at the reducing end; thus, carbonyl-reduction, methylation, carboxyl-reduction, methylation, hydrolysis, and acetylation gave the 4-*O*- (**31**), 5-*O*- (**37**), and 7-*O*-acetyl (**27**) derivatives of 3-deoxy-hexa-*O*-methyl-octitol. Omission of the second methylation step gave derivatives having an additional acetyl group at C-1.

The reactions used in the methylation analysis have been modified many times depending on the requirements of the particular carbohydrate under investigation[13–17]. We have optimised the procedure for KDO residues in complex oligosaccharides. Carboxyl-reduction was performed at 0°, because sodium borohydride is not stable in methanol–water at room temperature[21]. Also, the hydrolysis conditions had to be modified because KDO is sensitive to acid. Methyl ketosides of methylated KDO were cleaved quantitatively in dilute 0.03M acetic acid (100°, 1 h) without splitting α-heptopyranosyl and β-ribofuranosyl linkages. Experiments with the methylated disaccharide **10** and the trisaccharide **16** showed that acetic acid and even 0.1M acetate buffer (pH 4.4) at 100° randomly cleaved methyl ketosides and the other ketosidic linkages (data not shown). Hydrolysis of KDO-oligosaccharides was carried out in 0.1M trifluoroacetic acid (100°, 1 h). Thus, in the methylation analysis of disaccharide **9**, the 3-deoxyoctitol derivatives **13** and **7a** (after hydrolysis of **11**) or **14** and **8a** (after hydrolysis of **12**) were obtained in the ratio of 1:1. The methylation analysis of trisaccharide **15** yielded **17** and **8a** (methylation after carboxyl-reduction) or **18** and **7a** (hydrolysis of carboxyl-reduced **16**) in the ratio of 1:2. The ratio of 3-deoxyoctitol derivative corresponding to the reducing terminus, to that derived from the non-reducing end changed when more vigorous conditions of hydrolysis were employed, *e.g.*, the yield of **17** and **18**, respectively, was drastically reduced in comparison to those of the compounds (**7a** and **8a**) derived from the non-reducing end after hydrolysis in M instead of 0.1M trifluoroacetic acid. Therefore, it appears that the stability of 3-deoxyoctulose towards acid decreases drastically as the number of unsubstituted hydroxyl groups increases (data not shown). Methanolysis was the most convenient method for cleaving the glycosidic linkages of neutral sugars in such methylated oligosaccharides as **22, 26, 33**, and **35**. This method can be also employed when the methoxycarbonyl group of KDO has not been reduced and yields partially acetylated and methylated derivatives of methyl 3-deoxy-D-*glycero*-D-*talo*/*galacto*-octonate (data not shown).

*Identification of KDO derivatives obtained in methylation analysis.* — All derivatives were analysed by g.l.c.–m.s. and identified by their retention time, fragmentation pattern, and molecular weight determined by c.i.(ammonia)-m.s.

TABLE I

G.L.C. AND C.I.-M.S. DATA FOR METHYLATED AND ACETYLATED DERIVATIVES OF THE 3-DEOXYOCTITOL

| *Compound* | *Systematic name*[a] | *Mol. wt.*[b] | *Retention time*[c] | |
|---|---|---|---|---|
| | | | *Absolute (min)* | *Relative*[d] |
| **6a** | 3-Deoxy-1,2,4,5,6,7,8-hepta-*O*-methyl(1,1-$^2H_2$)octitol | 326 | 6.66/6.87 | 0.76/0.78 |
| **6b** | 3-Deoxy-1,2,4,5,6,7,8-hepta-*O*-methyl(1,1,2-$^2H_3$)octitol | 327 | 6.66/6.87 | 0.76/0.78 |
| **40a** | 1-*O*-Acetyl-3-deoxy-2,4,5,6,7,8-hexa-*O*-methyl(1,1-$^2H_2$)octitol | 354 | 10.65/11.02 | 1.21/1.26 |
| **40b** | 1-*O*-Acetyl-3-deoxy-2,4,5,6,7,8-hexa-*O*-methyl(2-$^2H$)octitol | 353 | 10.65/11.02 | 1.21/1.26 |
| **31** | 4-*O*-Acetyl-3-deoxy-1,2,5,6,7,8-hexa-*O*-methyloctitol | 352 | 9.60/9.73 | 1.09/1.11 |
| **37** | 5-*O*-Acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyloctitol | 352 | 9.51/9.60 | 1.08/1.09 |
| **27** | 7-*O*-Acetyl-3-deoxy-1,2,4,5,6,8-hexa-*O*-methyl(1,1,2-$^2H_3$)octitol | 355 | 8.89/9.03 | 1.02/1.03 |
| **8a** | 2,6-Di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyloctitol | 380 | 13.31/13.54 | 1.53/1.55 |
| **8b** | 2,6-Di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyl(1,1-$^2H_2$)octitol | 382 | 13.31/13.54 | 1.53/1.55 |
| **8c** | 2,6-Di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyl(1,1,2-$^2H_3$)octitol | 383 | 13.31/13.54 | 1.53/1.55 |
| **32** | 1,4-Di-*O*-acetyl-3-deoxy-2,5,6,7,8-penta-*O*-methyloctitol | 380 | 13.63 | 1.56 |
| **36** | 1,5-Di-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctitol | 380 | 13.72 | 1.57 |
| **14** | 2,4,6-Tri-*O*-acetyl-3-deoxy-1,5,7,8-tetra-*O*-methyloctitol | 408 | 15.87/16.15 | 1.82/1.85 |
| **7a** | 1,2,6-Tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyloctitol | 408 | 17.12/17.27 | 1.96/1.97 |
| **7b** | 1,2,6-Tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyl(1,1-$^2H_2$)octitol | 410 | 17.12/17.27 | 1.96/1.97 |
| **24** | 2,6,7-Tri-*O*-acetyl-3-deoxy-1,4,5,8-tetra-*O*-methyloctitol | 408 | 15.99/16.24 | 1.83/1.86 |
| **13** | 1,2,4,6-Tetra-*O*-acetyl-3-deoxy-5,7,8-tri-*O*-methyloctitol | 436 | 19.48/19.72 | 2.23/2.26 |
| **23** | 1,2,6,7-Tetra-*O*-acetyl-3-deoxy-4,5,8-tri-*O*-methyloctitol | 436 | 19.68/19.93 | 2.25/2.28 |
| **17** | 2,4,6,7-Tetra-*O*-acetyl-3-deoxy-1,5,8-tri-*O*-methyloctitol | 436 | 18.49/18.83 | 2.10/2.15 |
| **18** | 1,2,4,6,7-Penta-*O*-acetyl-3-deoxy-5,8-di-*O*-methyloctitol | 464 | 21.88/22.04 | 2.50/2.52 |
| **5a** | 1,2,4,5,6,7,8-Hepta-*O*-acetyl-3-deoxyoctitol | 520 | 25.96/26.05 | 2.97/2.98 |
| **5b** | 1,2,4,5,6,7,8-Hepta-*O*-acetyl-3-deoxy(1,1,2-$^2H_3$)octitol | 523 | 25.96/26.05 | 2.97/2.98 |

[a]All compounds were mixtures of the D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* isomers. [b]Determined by c.i.(ammonia)-m.s. on the basis of peaks at *m/z* for $(M + 1)^+$ and $(M + 18)^+$. [c]Using a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded SE-54, a temperature programme of 140° for 3 min and then 3°/min → 220°, and $H_2$ as carrier gas (1.0 bar). [d]Relative to that of methyl (methyl 3-deoxy-4,5,7,8-tetra-*O*-methyl-α-D-*manno*-2-octulopyranosid)onate (**3**) (8.74 min/1.0).

TABLE II

CHARACTERISTIC FRAGMENT IONS OF PARTIALLY METHYLATED AND ACETYLATED DERIVATIVES OF THE 3-DEOXYOCTITOL AFTER G.L.C.–M.S. (E.I., 70 eV)

| *Compound* | *Base peak* (m/z) | *Primary fragment ions* (m/z) *of the moieties* | | | | | | | | | *Characteristic and abundant daughter ions* (m/z)[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *C-1/2* | *C-1/4* | *C-1/5* | *C-1/6* | *C-1/7* | *C-2/8* | *C-5/8* | *C-6/8* | *C-7/8* | |
| **6a** | 117 | 91 | 149 | 193 | [b] | 281 | 279 | 177 | 133 | 89 | 129, 145, 161 |
| **6b** | 101 | 92 | 150 | 194 | — | 282 | — | 177 | 133 | 89 | 118, 130, 145, 162 |
| **40a** | 101 | 119 | 177 | 221 | — | 309 | — | 177 | 133 | 89 | 129, 145 |
| **40b** | 101 | 118 | — | 220 | — | 308 | — | 177 | 133 | 89 | 144, 145 |
| **31** | 45 | 89 | — | 219 | 263 | 307 | 307 | 177 | 133 | 89 | 113, 145, 171 |
| **37** | 115 | 89 | 147 | — | 263 | 307 | — | — | 133 | 89 | 157, 169, 201, 231 |
| **27** | 118 | 92 | 150 | 194 | — | — | — | 205 | 161 | 117 | 129, 142, 145, 174 |
| **8a** | 115 | — | 175 | 219 | — | 335 | — | 205 | — | 89 | — |
| **8b** | 117 | — | 177 | 221 | — | 337 | — | 205 | — | 89 | — |
| **8c** | 118 | — | 178 | — | — | — | — | 205 | — | 89 | — |
| **32** | 45 | 117 | — | 247 | 291 | — | — | — | 133 | 89 | 113, 145, 156 |
| **36** | 117 | 117 | 175 | — | 291 | 335 | — | — | — | 89 | 143, 167, 185, 231 |
| **14** | 205 | — | — | 247 | — | 363 | — | 205 | — | 89 | 113, 145, 155, 173 |
| **7a** | 101 | — | 203 | 247 | — | 363 | 335 | 205 | — | 89 | 113, 143, 173 |
| **7b** | 101 | — | 205 | 249 | — | 365 | — | 205 | — | 89 | 145, 173 |
| **24** | 115 | — | 175 | 219 | — | — | — | 233 | — | 117 | 127, 129, 159 |
| **13** | 205 | — | — | 275 | — | 391 | — | 205 | — | 89 | 141, 173, 183, 201, 215 |
| **23** | 69 | — | 203 | 247 | — | — | — | 233 | — | 117 | 113, 129, 143 |
| **17** | 113 | — | — | 247 | — | — | — | 233 | — | 117 | 113, 129, 143, 155, 173 |
| **18** | 113 | — | — | 275 | — | — | — | 233 | — | 117 | 129, 131, 173, 201 |
| **5a** | 129 | — | 231 | 303 | 375 | 447 | 447 | 289 | 217 | 145 | 170, 187, 201, 273, 345 |
| **5b** | 128 | — | 234 | 306 | 378 | 450 | 448 | 289 | 217 | 145 | 170, 187, 204, 276, 348 |

[a]A maximum of five fragment ions with an intensity of >10% of that of the base peak is listed. [b]Primary fragment ion was not observed.

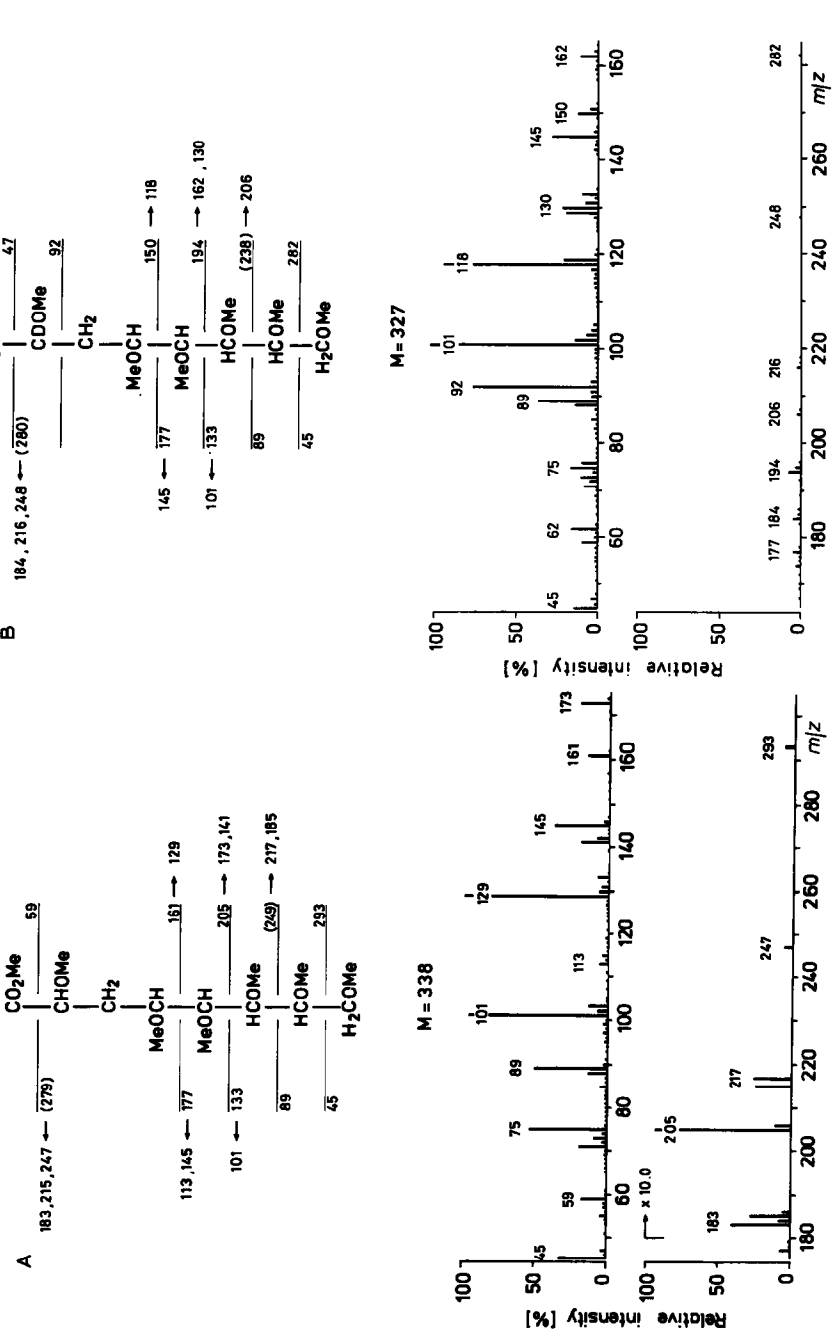



Fig. 1. (continued on following page).

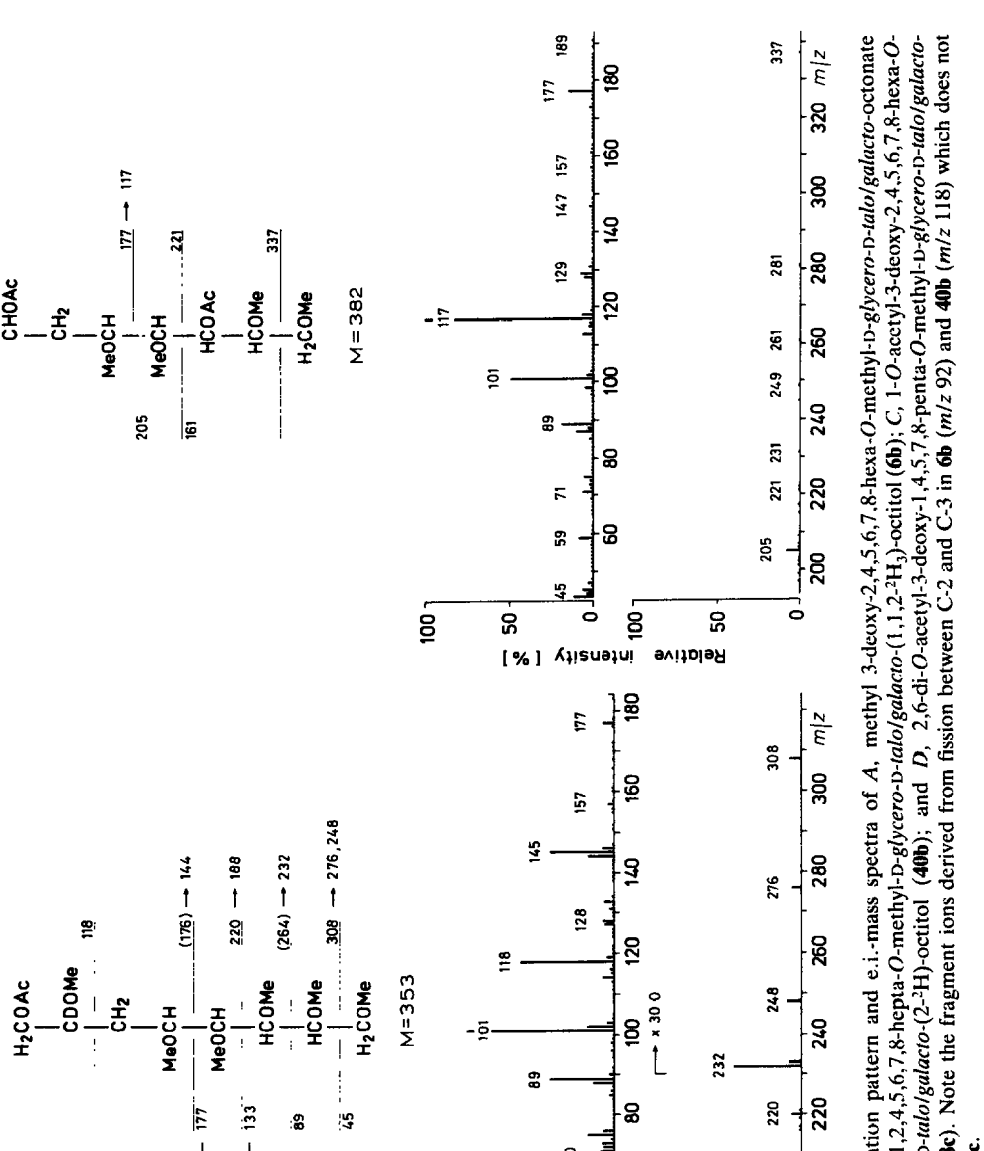



Fig. 1. Fragmentation pattern and e.i.-mass spectra of *A*, methyl 3-deoxy-2,4,5,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octonate (**38a**); *B*, 3-deoxy-1,2,4,5,6,7,8-hepta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-(1,1,2-$^2H_3$)-octitol (**6b**); *C*, 1-*O*-acetyl-3-deoxy-2,4,5,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-(2-$^2$H)-octitol (**40b**); and *D*, 2,6-di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-(1,1-$^2H_2$)-ocitol (**8c**). Note the fragment ions derived from fission between C-2 and C-3 in **6b** (*m*/*z* 92) and **40b** (*m*/*z* 118) which does not occur in **38a** and **8c**.

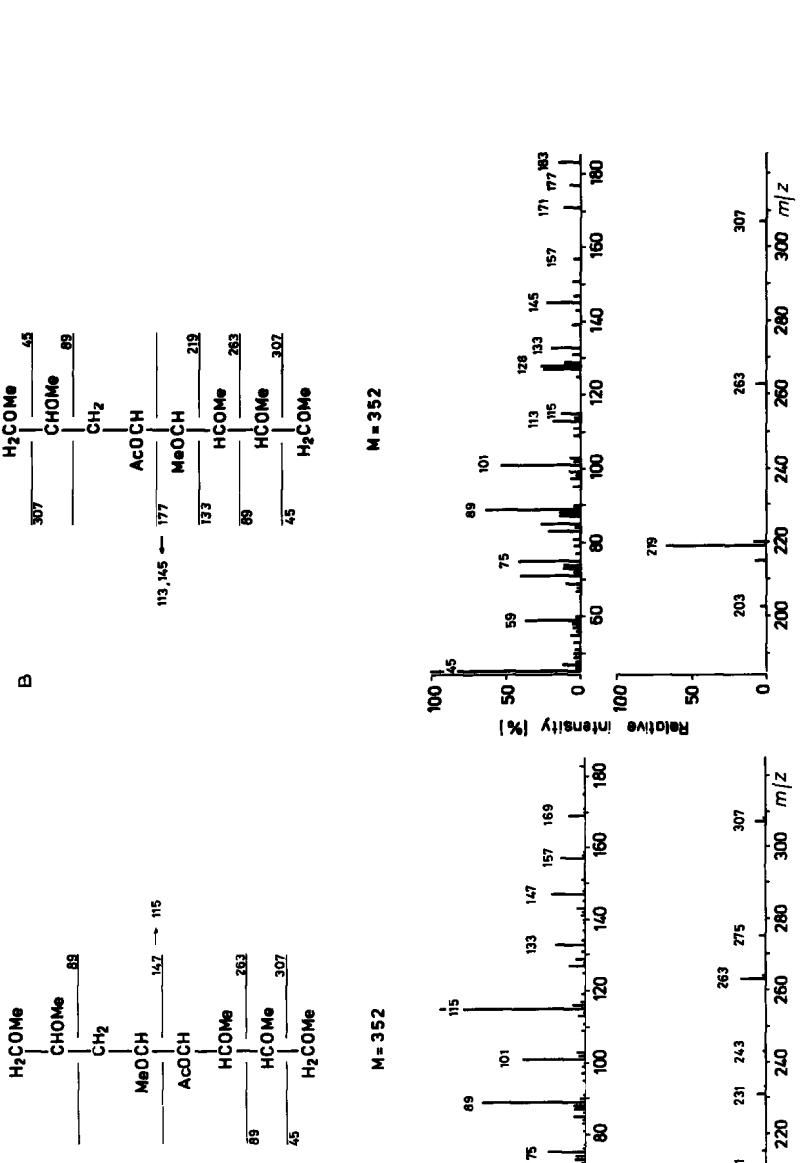



Fig. 2. Fragmentation pattern and e.i.-mass spectra of *A*, 5-*O*-acetyl-3-deoxy-1,2,4,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**37**); and *B*, 4-*O*-acetyl-3-deoxy-1,2,5,6,7,8-hexa-*O*-methyl-D-*glycero*-D-*talo*/*galacto*-octitol (**31**). Note the fission between C-4 and C-5 in **37** (*m*/*z* 147 and 115) which is not significant in **31**.

The data are summarised in Table I. The pairs of D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* isomers could be resolved by g.l.c. on SE-54, except for **32** and **36**. The two values for the absolute and relative retention time, respectively, of each compound in Table I refer to these diastereoisomers. Mono-*O*-acetylated derivatives could be distinguished by their retention time, except the 4- and 5-acetates (**31** and **37**). The corresponding 1,4- and 1,5-diacetates (**32** and **36**) were not clearly separated from each other, but both were separated from the 2,6-diacetate **8**, which was eluted earlier from the column. The triacetates were separated by g.l.c., except the 2,4,6 and 2,6,7 derivatives (**14** and **24**).

On c.i.(ammonia)-m.s., all compounds gave rise to pseudomolecular ion peaks at *m/z* $[M + 1]^+$ and $[M + 18]^+$, the latter having the higher intensity (spectra not shown). The base peak and other characteristic fragment ions obtained on e.i.-m.s. are listed in Table II. As is well known, fission between two methoxyl groups was more prevalent than fission between a methoxyl and an acetoxyl group or between two acetoxyl groups[22,23]. The following rules for the fragmentation of 3-deoxyoctitol derivatives were established. (*1*) Fission between C-2 and C-3 occurred in 3-deoxyoctitols provided that C-2 carried a methoxyl and not an acetoxyl group; this cleavage was not observed in the corresponding methyl octonate derivatives. The presence of an acetoxyl or a methoxyl group at C-1 resulted in a similarly significant fission between C-2 and C-3 (see Fig. 1). (*2*) The fragment comprising C-1/4 (or a sub-fragment thereof) was always observed in high intensity (often as the base peak) when C-4 carried an *O*-methyl group, even if C-5 carried an acetoxyl group (see Fig. 2). This fragmentation behaviour is different from that reported[23] for other partially methylated alditol acetates and seems to be a characteristic feature of 3-deoxyoctitols.

## DISCUSSION

Whereas there are many data in the literature on the general methodology of methylation analysis, few data are available for KDO derivatives and KDO-containing oligosaccharides. The analysis of oligosaccharides having a KDO residue at the reducing end has been described[10,12,17,24,25], but methylation analysis resulting in the formation of partially methylated and acetylated derivatives of 3-deoxyoctitol has been described only twice. Although the procedure given by Prehm *et al.*[7] could not be reproduced in our laboratory, that described by Albersheim and co-workers[17] was reproducible. Since the same g.l.c.–m.s. apparatus (HP 5985) and the same data system (HP-1000) were used, the mass spectra published by the latter group could be compared to those obtained in our study. Thus, the mass spectra of acetylated 3-deoxy-D-*glycero*-D-*talo*/*galacto*-(1,1,2-$^2H_3$)octitol (**5b**) and of the 1,4- and 1,5-diacetates (**32** and **36**) were comparable to those reported by York *et al.*[17]. However, the conditions of hydrolysis and carboxyl-reduction reported were found to be inconvenient for our purposes, and the method of Charon and Szabo[21] was used at 0° and the glycosidic linkages of neutral sugars were methanolysed. G.l.c.–

m.s. of the various 3-deoxyoctitol derivatives allowed general conclusions to be made with regard to the fragmentation of partially methylated and acetylated derivatives of 3-deoxyoctitol: (*1*) fission between C-2 and C-3 occurs (although next to a deoxy group) provided that C-2 carries a methoxyl group; (*2*) a C-1/2 fragment is not observed in the corresponding methyl 3-deoxyoctonate derivatives; and (*3*) a strong fragment ion is obtained from the C-1/4 moiety when C-4 is *O*-methylated, even if C-5 is *O*-acetylated.

The compounds and the procedures described herein represent the basis for the structural elucidation of the KDO-containing inner core region of enterobacterial LPS (see following paper[26]), and may be useful for the methylation analysis of other KDO-containing biopolymers.

ACKNOWLEDGMENTS

We thank P. Kosma, R. Christian, and P. Waldstätten for help with the synthesis of the KDO derivatives, E. T. Rietschel for his interest, and Veronika Susott and Hermann Moll for expert technical assistance.

REFERENCES

1 E. C. HEATH AND M. A. GHALAMBOR, *Biochem. Biophys. Res. Commun.*, 10 (1963) 340–345.
2 P. W. TAYLOR, *Biochem. Biophys. Res. Commun.*, 61 (1974) 148–153.
3 L. ANDERSON AND F. M. UNGER, *ACS Symp. Ser.*, 231 (1983) 171–191.
4 A. K. BHATTACHARJEE, H. J. JENNINGS, AND C. P. KENNY, *Biochem. Biophys. Res. Commun.*, 61 (1974) 489–493.
5 F. M. UNGER, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 323–388.
6 W. DRÖGE, V. LEHMANN, O. LÜDERITZ, AND O. WESTPHAL, *Eur. J. Biochem.*, 14 (1970) 175–184.
7 P. PREHM, S. STIRM, B. JANN, AND K. JANN, *Eur. J. Biochem.*, 56 (1975) 41–55.
8 M. J. OSBORN, *Proc. Natl. Acad. Sci. U.S.A.*, 50 (1963) 499–506.
9 H. BRADE, C. GALANOS, AND O. LÜDERITZ, *Eur. J. Biochem.*, 131 (1983) 201–203.
10 H. BRADE, U. ZÄHRINGER, E. T. RIETSCHEL, R. CHRISTIAN, G. SCHULZ, AND F. M. UNGER, *Carbohydr. Res.*, 134 (1984) 157–166.
11 H. BRADE AND E. T. RIETSCHEL, *Eur. J. Biochem.*, 145 (1984) 231–236.
12 H. BRADE, H. MOLL, AND E. T. RIETSCHEL, *Biomed. Mass. Spectrom.*, 12 (1985) 602–609.
13 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
14 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
15 B. LINDBERG, J. LÖNNGREN, AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185–246.
16 P.-E. JANSSON, L. KENNE, B. LINDBERG, H. LIEDGREN, AND J. LÖNNGREN, *Chem. Commun. Univ. Stockholm*, 8 (1976) 1–75.
17 W. S. YORK, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 138 (1985) 109–126.
18 A. K. BHATTACHARJEE, H. J. JENNINGS, AND C. P. KENNY, *Biochemistry*, 17 (1978) 645–651.
19 L. ANDERSON AND F. M. UNGER, *ACS Symp. Ser.*, 231 (1983) 121–140.
20 A. NESZMELYI, P. KOSMA, R. CHRISTIAN, G. SCHULZ, AND F. M. UNGER, *Carbohydr. Res.*, 139 (1985) 13–22.
21 D. CHARON AND L. SZABO, *Acta Chim. Acad. Sci. Hung.*, 113 (1983) 375–378.
22 N. K. KOCHETKOV AND O. S. CHIZHOV, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
23 J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
24 B. JANN, P. HOFMANN, AND K. JANN, *Carbohydr. Res.*, 120 (1983) 131–141.
25 M. A. SCHMIDT AND K. JANN, *Eur. J. Biochem.*, 131 (1983) 509–517.
26 A. TACKEN, E. T. RIETSCHEL, AND H. BRADE, *Carbohydr. Res.*, 149 (1986) 279–291.