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Note

Application of methylation analysis in the determination of the structure of disaccharides containing 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) associated with the backbone of lipid A

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Endotoxically active lipid A generally contains[1] a (1 → 6)-linked 2-amino-2-deoxy-β-D-glucose (GlcN) disaccharide backbone which has phosphate groups at positions 1 and 4′, and (*R*)-3-hydroxy- and (*R*)-3-acyloxy-acyl residues at positions 2, 3, 2′, and 3′. Methylation analysis of the backbone, which yielded acetylated partially methylated derivatives of 2-acetamido-2-deoxy-D-glucitol (GlcNAc-ol), has proved to be valuable in the analysis of structure and such compounds have been well characterised[2–5].

2,3-Diamino-2,3-dideoxy-D-glucose (GlcN3N), which occurs alone or together with GlcN, has been identified as a constituent of lipid A produced by some 25 species of bacteria from 12 genera or families[6,7]. Moreover, GlcN3N-containing lipid A structures are considered to be potential therapeutic agents either as LPS-inhibitory or immunostimulatory compounds[8], and thus structural investigations are desirable in order to elaborate a clear-cut structure–function relationship between these lipid A's and their biological activities. However, whether GlcN3N is a component of the lipid A backbone or a substituent on its polar head groups remained unclear[6,7] until the first evidence based on serological data indicated a (phosphorylated) GlcN3N-containing disaccharide to be present in *Pseudomonas diminuta* lipid A[9]. The first chemical analysis of GlcN3N-containing lipid A backbone was carried out on *Campylobacter jejuni* LPS from which three disaccharides, namely, 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucitol (**1**), 2-acetamido-2-deoxy-6-*O*-(2,3-diacetamido-2,3-dideoxy-β-D-glucopyranosyl)-D-glucitol (**3**), and 2,3-diacetamido-2,3-dideoxy-6-*O*-(2,3-di-

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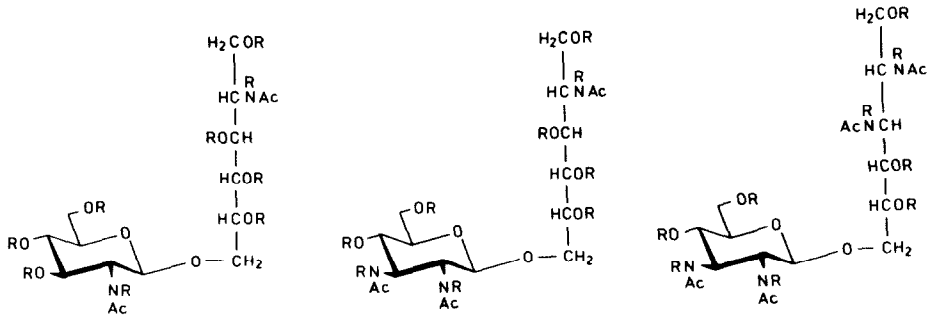


1 R = H
2 R = Me

3 R = H
4 R = Me

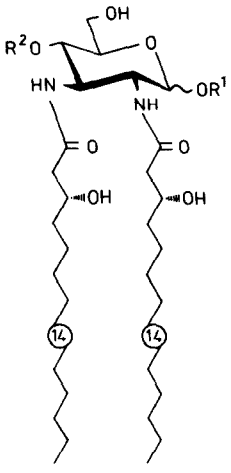
5 R = H
6 R = Me

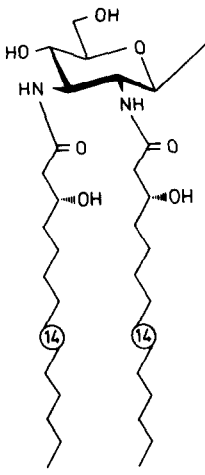
acetamido-2,3-dideoxy-β-D-glucopyranosyl)-D-glucitol (**5**) were prepared from the backbone species[10].

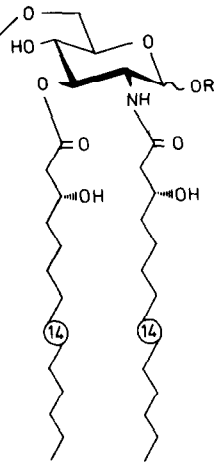
Except for one limited study[11], GLC and GLC–MS data concerning partially methylated 2,3-diacetamido-2,3-dideoxy-D-glucitol (GlcNAc3NAc-ol) acetates are lacking in the literature. We now report on the formation and identification of acetylated partially methylated derivatives of GlcNAc3NAc-ol from LPS, lipid A, and model substances during methylation analysis.

Methylation of **1** (→ **2**) followed by acetolysis, reduction (NaB^2H_4), and acetylation (i.e., methylation analysis) gave (GLC–MS data) 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-[1-^{2}H]glucitol (**12**), 1,6-di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol (**13**), and 6-*O*-acetyl-2,3-dideoxy-1,4,5-tri-*O*-methyl-2,3-di-(*N*-methylacetamido)glucitol (**16**). Likewise, **3** gave **4**, **13**, **16**, and 1,5-di-*O*-acetyl-2,3-dideoxy-4,6-di-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol (**17**), and **5** gave **6**, **17**, and 1,6-di-*O*-acetyl-2,3-dideoxy-4,5-di-*O*-methyl-2,3-di-(*N*-methylacetamido)glucitol (**18**). Compounds **13** and **18** were derived via loss of MeO-1 during acetolysis[5]. The above products reflect the (1 → 6) linkage in **1**, **3**, and **5**.

Methylation analysis of synthetic 2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-α-D-glucopyranosyl phosphate[8] (**8**) yielded **17**, which was also obtained from 2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-D-glucopyranose (**7**) after reduction (NaB^2H_4), methylation, acetolysis, and acetylation. When 2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-D-glucopyranose 4-phosphate (**9**) was subjected to both derivatisation procedures, 1,4,5-tri-*O*-acetyl-2,3-dideoxy-6-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol (**19**) was obtained. When 2-deoxy-6-*O*-{2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxytetradecanamido]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-D-glucopyranose (**10**) was subjected to the chemical degradation pathway for the isolation[10,12] of lipid A







7 $R^1 = R^2 = H$

8 $R^1 = \alpha$-$PO(OH)_2$, $R^2 = H$

9 $R^1 = H$, $R^2 = PO(OH)_2$

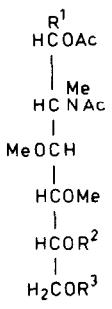
10 $R = H$

11 $R = \alpha$-$PO(OH)_2$

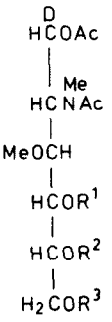
backbone (dephosphorylation, reduction, hydrazinolysis, and *N*-acetylation), **3** was obtained and methylation analysis gave **13** and **17**, as did direct methylation analysis of synthetic 2-deoxy-6-*O*-{2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxytetradecanamido]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-α-D-glucopyranosyl phosphate[8] (**11**).

As confirmed by chemical and ^{31}P-NMR studies[10], the backbone in lipid A of *C. jejuni* comprises three 1,4′-bisphosphorylated β-(1 → 6)-linked D-hexosamine disaccharides, with the polysaccharide moiety in LPS attached at position 6′ and the 1-phosphate substituted by ethanolamine. Also, the 4′-phosphate carries, in non-stoichiometric amounts, a phosphorylethanolamine residue.

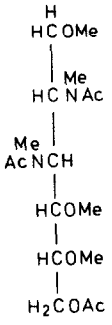
When dephosphorylated lipid A was reduced ($NaBH_4$), and then subjected to methylation analysis, the same derivatives (i.e., **12**, **13**, and **16–18**) were obtained as from **1**, **3**, and **5**. Likewise, dephosphorylated LPS and lipid A yielded **13**, **16**, and **18**. Reduced lipid A also yielded 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-[1-^{2}H]glucitol (**14**) and 1,4,5-tri-*O*-acetyl-2,3-dideoxy-6-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol (**19**). In addition to acetylated partially methylated alditols of neutral sugars, dephosphorylated LPS yielded 1,5,6-tri-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-[1-^{2}H]glucitol (**15**) and 1,5,6-tri-*O*-acetyl-2,3-dideoxy-4-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol (**20**), indicating an acid-labile substituent (Kdo) at position 6′ in each hexosamine of the backbone hybrid of lipid A.



12 R^1 = D, R^2 = Ac, R^3 = Me
13 R^1 = H, R^2 = Me, R^3 = Ac



14 $R^1 = R^2$ = Ac, R^3 = Me
15 R^1 = Me, $R^2 = R^3$ = Ac

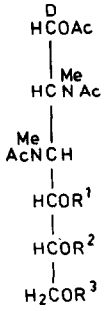


16

17 R^1 = D, R^2 = Ac, R^3 = Me
18 R^1 = H, R^2 = Me, R^3 = Ac

All hexosamine derivatives were analysed by GLC–MS on the basis of retention time, fragmentation pattern, and molecular weight determined by CI(ammonia)-MS. In accordance with published results[2,3,13], GlcNAc-ol derivatives had the following absolute (and relative) retention times: **12** 17.0 min (1.00), **13** 17.35 min (1.02), **14** 19.45 min (1.14), **15** 19.55 min (1.15). The retention times and molecular weights of the GlcNAc3NAc-ol derivatives are presented in Table I. The corresponding 1,5- and 1,6-di-*O*-acetyl derivatives of the partially methylated GlcNAc-ol and GlcNAc3NAc-ol (**12**, **13**, **17**, and **18**) were clearly separable from each other, as were the 1,4,5- and 1,5,6-tri-acetates **14**, **15**, **19**, and **20**.

In CI(ammonia)-MS, each compound yielded pseudomolecular ions at *m/z* $[M + H]^+$ and $[M + NH_4]^+$, the former having the higher intensity (spectra not shown). The EI-mass spectra of the GlcNAc-ol derivatives (**12–15**) agreed with



19 $R^1 = R^2 = Ac$, $R^3 = Me$

20 $R^1 = Me$, $R^2 = R^3 = Ac$

those reported[2,3,11,13–16]. The base peak and other characteristic fragment ions obtained in EIMS of GlcNAc3NAc-ol derivatives are listed in Table II. The following generalisations can be drawn. (*a*) In EIMS of acetylated partially methylated derivatives of GlcNAc3NAc-ol, cleavage of C(OMe)–C(OMe) bonds is more prevalent than of C(OMe)–C(OAc) or C(OAc)–C(OAc) bonds, as in derivatives of neutral hexitols[17]. (*b*) An *N*-methylacetamido group promotes cleavage between adjacent carbons regardless of the substituents attached thereto. (*c*) Cleavage between the *N*-methylacetamido groups (C-2–C-3 bond), and primary or secondary fragments therefrom, predominates and may yield the base peaks. (*d*) CI(ammonia)-MS yields more intense $[M + H]^+$ than $[M + NH_4]^+$ ions, as do acetylated partially methylated derivatives of GlcNAc-ol.

Based solely on compositional analyses, lipid A's from several sources have been reported to contain either GlcN3N monosaccharides[11] or disaccharides[6,7], the latter expressing a hybrid backbone (also termed[6,7] mixed lipid A backbone). However, the linkage of the disaccharide has been postulated only in one instance[9] as β, solely on the basis of serological cross-reactivity with *Salmonella* lipid A. Moreover, the position of the 4′-phosphate was also postulated only on the basis of serological data. However, application of the procedures described herein confirmed for the first time three hybrid backbones (**1**, **3**, and **5**) in the lipid A of *C. jejuni*. Surprisingly, the hybrid backbone β-D-Glc*p*N-(1 → 6)-D-Glc*p*N3N was not detected, although it is known that the enzyme (lipid A synthase) that catalyses the formation of the disaccharide[8] accepts a variety of GlcN analogues as substrates. The position of the 4′-phosphate could be determined by combined chemical degradation and methylation analysis. Thus, the methodologies reported here provide a basis for elucidation of the structure of GlcN3N-containing hybrid lipid A backbones.

TABLE I

GLC and CI-MS data (see Experimental)

Compound	Systematic name	Mol wt [a]	Retention time	
			Absolute (min)	Relative [b]
16	6-*O*-Acetyl-2,3-dideoxy-1,4,5-tri-*O*-methyl-2,3-di-(*N*-methylacetamido)glucitol	376	21.47	1.26
17	1,5-Di-*O*-acetyl-2,3-dideoxy-4,6-di-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol	405	22.07	1.30
18	1,6-Di-*O*-acetyl-2,3-dideoxy-4,5-di-*O*-methyl-2,3-di-(*N*-methylacetamido)glucitol	404	23.02	1.35
19	1,4,5-Tri-*O*-acetyl-2,3-dideoxy-6-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol	432	23.70	1.39
20	1,5,6-Tri-*O*-acetyl-2,3-dideoxy-4-*O*-methyl-2,3-di-(*N*-methylacetamido)-[1-^{2}H]glucitol	432	25.26	1.49

[a] Determined by CI(ammonia)-MS on the basis of peaks at m/z for $[M+H]^+$ and $[M+NH_4]^+$. [b] Relative to that of 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-[1-^{2}H]glucitol (17.00 min/1.00).

TABLE II

Characteristic fragment ions in EIMS

Compound	Base peak	Primary fragment ions (m/z)								Characteristic daughter ions (m/z)
		C-1/2	C-1/3	C-1/4	C-1/5	C-2/6	C-3/6	C-4/6	C-5/6	
16	117	130	215	259	– [a]	331	246	–	117	88, 112, 172, 183, 186, 204, 214, 227, 271
17	246	159	244	288	–	–	246	161	117	99, 112, 117, 144 154, 184, 186, 204, 214, 228, 246, 256, 300
18	117	158	243	287	331	–	246	161	117	98, 112, 116, 129, 172, 183, 186, 204, 214, 227, 255, 289
19	112	159	244	–	–	–	274	189	117	99, 112, 117, 172, 184, 202, 214, 228, 232, 242, 246, 256, 328
20	112	159	244	–	–	–	274	189	145	99, 112, 117, 142, 172, 184, 202, 214, 232, 246, 256, 300, 318

[a] Primary fragment ion was not observed.

EXPERIMENTAL

Lipopolysaccharide (LPS).—LPS was extracted from *C. jejuni* CCUG 10936 (serotype O:2) by a modified phenol–$CHCl_3$–light petroleum method[18].

Synthetic compounds.—The crystalline tris(hydroxymethyl)aminomethane ("Tris") salts of 2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-α-D-glucopyranosyl phosphate (**8**), 2,3-dideoxy-2,3-di[(*R*)-3-hydroxytetradecanamido]-D-glucopyranose 4-phosphate (**9**), and 2-deoxy-6-*O*-{2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-β-D-glucopyranosyl}-2-[(*R*)-3-hydroxytetradecanamido]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-α-D-glucopyranosyl phosphate (**11**) were synthesised as described[8].

Degradation and modification procedures.—Free lipid A (lacking the 1-substituent) was obtained by treatment of LPS with HCl (0.1 M, 100°, 30 min). Compounds **1**, **3**, and **5** were obtained by subjecting lipid A to a chemical degradation pathway developed[10,12] for the analysis of the lipid A backbone. Preparative dephosphorylation of LPS, **8** (→ **7**), and **11** (→ **10**) was performed with aq 48% HF at 4° for 48 h with vigorous stirring. Dephosphorylated lipid A was obtained by treatment of dephosphorylated LPS with mild acid (0.1 M HCl, 100°, 1 h).

Methylation analysis.—Methylation was performed using NaOH–Me_2SO–MeI[19]. Reduction with $NaBH_4$ or NaB^2H_4 was accomplished in water at room temperature for 2 h (monosaccharides), and in aq 0.02% triethylamine (lipid A, dephosphorylated LPS, and dephosphorylated lipid A) or in 1:1 EtOH–dioxane (**7** and **10**) at 56° for 16 h. Acetolysis involved[2] 0.25 M H_2SO_4 in 95% acetic acid. Acetylation was performed with acetic anhydride–pyridine (1:1, by vol; 100°, 30 min).

GLC–MS.—GLC was performed on 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 (Weeke, Mülheim, FRG). The carrier gas was H_2 at 2.5 mL/min and 0.08 MPa. The temperature of the injector port and flame-ionisation detector was 290°. Temperature programmes: monosaccharides, 150° for 3 min, then 5°/min to 300°; disaccharides, 250° for 3 min, then 5°/min to 320°.

GLC–MS was performed on a Hewlett–Packard 5985 instrument equipped with the above column and an HP-1000 data system. Helium was the carrier gas (0.1 MPa). EIMS spectra were recorded at 70 eV and CIMS spectra were obtained with ammonia as the reactant gas. The ion-source temperature was 200°.

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