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IDENTIFICATION OF 2-AMINO-6-*O*-(2-AMINO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-DEOXY-D-GLUCOSE AS A MAJOR CONSTITUENT OF THE HYDROPHOBIC REGION OF THE *Bordetella pertussis* ENDOTOXIN

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ABSTRACT

2-Amino-6-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose substituted on the amino group of the reducing 2-amino-2-deoxy-D-glucose unit by a 3-hydroxytetradecanoyl group was shown to be a major constituent of the "Lipid A" fragment obtained by acid hydrolysis of the *Bordetella pertussis* endotoxin.

INTRODUCTION

The disaccharide 2-deoxy-6-*O*-[2-deoxy-2-(3*R*)-3-(hydroxytetradecanamido)-β-D-glucopyranosyl]-2-(3*R*)-3-(hydroxytetradecanamido)-D-glucose (**1**), esterified by substituted phosphate groups at O-1 and -4′ and fatty acids at the remaining hydroxyl groups, is believed to represent the hydrophobic region ("Lipid A") of the endotoxin (lipopolysaccharide, LPS) of several Enterobacteria[1–3]. More elaborate structures derived from disaccharide **1** have been found to be present in the endotoxins of *Chromobacterium violaceum*[4], *Rhodospirillum tenue*[5], and *Vibrio cholerae*[6], whereas a different structure containing 2,3-diamino-2,3-dideoxy-D-glucose was identified in the endotoxins of *Rhodopseudomonas viridis*[1,7] and *Rhodopseudomonas palustris*[8]. The present paper describes the identification of disaccharide **1** in the "Lipid A" fragment[9] of the *Bordetella pertussis* endotoxin.

RESULTS AND DISCUSSION

The Lipid A of *B. pertussis* used contained about 14% of 2-amino-2-deoxy-D-glucose as estimated after acidic hydrolysis by the method of Rondle and Morgan[10]. When determination of the amount of reducing 2-amino-2-deoxy-D-glucose present in the pertussis Lipid A was attempted by the method of Reissig *et al.*[11], the time of heating required to obtain maximal color yield was 25 min, rather than the prescribed 3 min (Fig. 1); the values thus obtained correspond to ~55% of the total 2-amino-2-deoxy-D-glucose content of the Lipid A. As a corollary, when Lipid A, dispersed in aqueous potassium tetraborate of pH 9.4, was treated with sodium borohydride

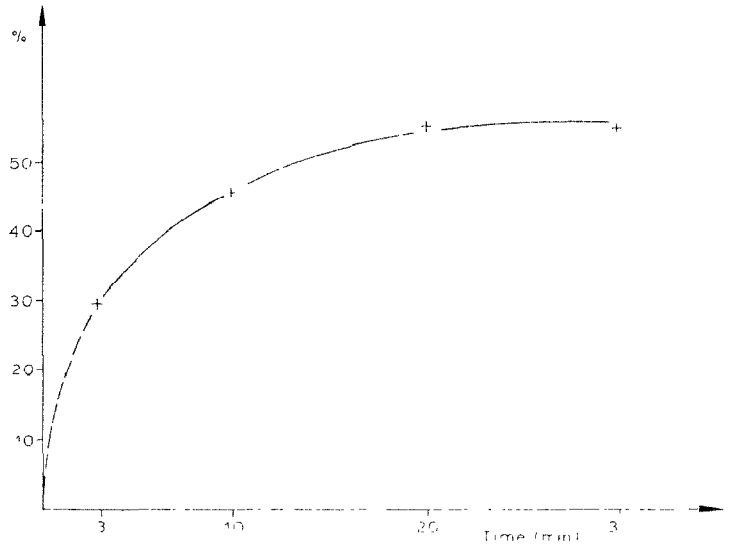


Fig. 1. Kinetics of color development as a function of time in the modified[11] Morgan–Elson test applied to Lipid A of *B. pertussis*. The amount of reducing 2-amino-2-deoxy-D-glucose is expressed as a percent of the total amount of 2-amino-2-deoxy-D-glucose.

(30 min at 100°) and then hydrolyzed, only ~45% of the original 2-amino-2-deoxy-D-glucose content survived. In the borohydride-treated Lipid A, the ratio of 2-amino-2-deoxy-D-glucose to -D-glucitol was ~2:3; the relatively low content of 2-amino-2-deoxy-D-glucose is very probably due to incomplete hydrolysis and partial destruction of the sugar; it could be shown, by paper electrophoresis, that at the point where the highest value for 2-amino-2-deoxy-D-glucose was measured by Rondle and Morgan's method[10], some 2-amino-2-deoxy-D-glucose phosphate was still present in the hydrolyzate. Unequivocal identification of 2-amino-2-deoxy-D-glucose and -D-glucitol in this hydrolyzate was accomplished by g.l.c.–m.s. of the peracetylated mixture.

It has been observed previously that the reduction of the reducing 2-amino-2-deoxy-D-glucose units present in enterobacterial Lipid A preparations to 2-amino-2-deoxy-D-glucitol units was difficult. Hase and Rietschel[3] treated such Lipid A preparations with sodium borohydride for 16 h at pH 10 and 50° to reduce the aldose into the alditol; similar observations were made by Tharanathan *et al.*[5], and Blache *et al.*[12]; it was suggested that the slow rate of reduction was due to steric factors. We established that reduction of *B. pertussis* Lipid A with sodium borohydride in the presence of sodium dodecyl sulfate was complete within 60 min at room temperature, *i.e.*, it proceeded at a rate similar to that found for 2-acetamido-2-deoxy-D-glucose. This result suggests that the slow rate of reduction observed in the absence of detergent is due to the micellar nature of Lipid A preparations in aqueous medium, and rules out such alternative explanation as the presence of an alkali-labile substituent at C-1 of the reducible 2-amino-2-deoxy-D-glucose units.

TABLE I

AMINO SUGAR COMPONENTS OF LIPID A AND DERIVATIVES[a]

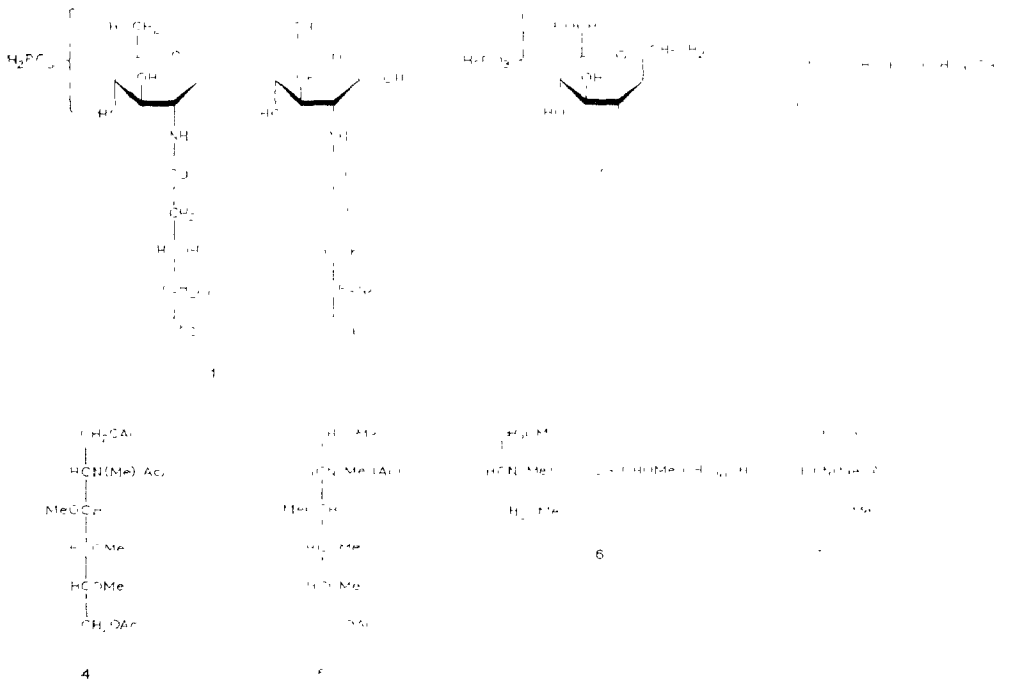
Compound	*Total 2-amino-2-deoxy-D-glucose*		*2-Amino-2-deoxy-D-glucitol*[c]	*Reducing 2-acylamino-2-deoxy-D-glucose*[d]
	[b]	[c]		
Lipid A	797	805	0	476
Reduced Lipid A	446	321	488	0
Periodate-oxidized, reduced Lipid A	381	274	0	0

[a]Nanomol per mg of Lipid A. [b]Method of Rondle and Morgan[10]. [c]Amino acid analyzer. [d]Method of Reissig *et al.*[11].

The 2-amino-2-deoxy-D-glucitol residues formed upon treatment of Lipid A with sodium borohydride were degraded when the reduced material was treated with periodate, whereas the 2-amino-2-deoxy-D-glucose units were not (Table I). Treatment of the periodate-oxidized material with sodium borohydride gave 2-deoxy-2-(3-hydroxytetradecanamido)glycerol (**3**), which was isolated by t.l.c. and identified by g.l.c.–m.s. after methylation. This result proved that 3-hydroxytetradecanoic acid was bound to the amino group of the reducing 2-amino-2-deoxy-D-glucose residues of the Lipid A of *B. pertussis*, as it has been established, by deuteration, that the 2-amino-2-deoxyglycerol fragment was derived from the alditol residues present in the reduced Lipid A.

This was, indeed, the case, as shown by treating reduced Lipid A with hydrazine[3] to remove all ester- and amide-bound fatty acids and phosphate groups. In a separate experiment, it was established that, under identical conditions, methyl α-D-glucopyranoside 6-phosphate releases 75% of its phosphate content. The water-soluble part of the hydrazinolyzed material was acetylated and analyzed by g.l.c.–m.s. 2-Acetoxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranoside was the sole product identified, and it represented 40% of the 2-amino-2-deoxy-D-glucose content of the material as estimated by g.l.c. Co-chromatography with an authentic sample established that it was the β-D anomer, and it was well separated from the α-D anomer[13]. When the reduction following the periodate-oxidation step was performed with sodium borodeuteride, the 2-hydroxyethyl part of **2** was labeled, thus proving that **2** was formed as a result of the oxidative cleavage. Finally, 2-amino-6-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucitol was isolated and identified as its *N*-acetylated, permethylated derivative by g.l.c.–m.s.; it gave a mass spectrum identical to that found by Jensen *et al.*[14]; when chemical-ionisation (ammonia) was used the molecular peak (M + 1, *m/z* 553), which was not detectable by electron-impact ionisation[14], was the base peak (42% of the total ion-current).

We have reported previously[5] that acid hydrolysis or methanolysis demethylated 2-deoxy-1,3,4,5,6-penta-*O*-methyl-2-(*N*-methylacetamido)hexoses at O-1 and



yielded, after acetylation, 1-*O*-acetyl-2-deoxy-3,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)hexitols. Accordingly, when reduced Lipid A was methylated, hydrolyzed, and acetylated, the sole product derived from 2-amino-2-deoxy-glucitol that could be identified by g.l.c.–m.s. was 1,6-di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol (**4**), and not 6-*O*-acetyl-2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol (**5**). Similarly, treatment of 2-deoxy-1,3-di-*O*-methyl-2-[3-methoxy-(*N*-methyl)-tetradecanamido]glycerol (**6**) with 4M hydrochloric acid for 2.5 h at 100°, in order to cleave the amide bond, gave, following acetylation, 1-*O*-acetyl-2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)glycerol (**7**) as the sole aminoglycerol derivative. Thus, *O*-demethylation of a primary methyl ether group adjacent to an *N*-methylamino (or amino) group in aminopolyols is confirmed, and this reaction appears to be characteristic for this class of compounds. It is noteworthy, however, that only one of the primary methyl ether groups of the 2-aminoglycerol derivative was *O*-demethylated.

EXPERIMENTAL

Materials. — Bacteria, grown in Cohen and Wheeler's liquid medium[16], were harvested by centrifugation and extracted with phenol–water[17]. The endotoxin containing both constituent lipopolysaccharides[18] was isolated as described previously[9].

The "Lipid A" fragment used was that described by Le Dur *et al.*[9], *i.e.*, a

mixture of the hydrophobic regions of LPS-1 and LPS-2 that was obtained by acid hydrolysis (0.25M hydrochloric acid, 30 min, 100°) of the "glycolipid"[9]. "Lipid A-1" (the hydrophobic region of LPS-1) was isolated by extraction of the "glycolipid" with acidified (by hydrochloric acid) 10:1 (v/v) oxolane–methanol. "Lipid A-2" was the sediment obtained after acid hydrolysis (0.25M hydrochloric acid, 30 min, 100°) of the "glycolipid" from which most of the "Lipid-A-1" had been extracted[19]. Results obtained with "Lipid A" were repeated and confirmed with both "Lipid A-1" and "Lipid A-2".

Methods. — Reducing, *N*-acylated 2-amino-2-deoxy-D-glucose present in "Lipid A" preparations was estimated by the method of Reissig *et al.*[11], but with an incubation period of 25 min at 100° (*cf.* Fig. 1); synthetic 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-D-glucose was used as reference. Total 2-amino-2-deoxy-D-glucose content was estimated on hydrolyzed (4M hydrochloric acid, 6 h, 100°) Lipid A samples by Rondle and Morgan's method[10], but substituting 0.5M sodium carbonate for the 0.25M solution prescribed. The conditions of hydrolysis mentioned gave the highest values for hexosamine-content. Phosphorus was estimated by the method of Chen *et al.*[20].

G.l.c.–m.s. was performed as previously described[15], except that a stainless-steel column (3.2 × 2000 mm) packed with 3% OV-1 on Gaschrom Q (100–120 mesh) was also used; thermal conditions are specified for individual analyses. G.l.c.–m.s.c.i. (chemical ionisation with ammonia) was performed with a NERMAG R 10-10 instrument equipped with a CP-Sil 5 column (25 m, 260°, isothermal). The ratio of 2-amino-2-deoxy-D-glucose to -D-glucitol was determined with a Beckman Multichrom amino acid analyzer. High-voltage electrophoresis was carried out on Whatman 3MM paper (Savant flat-bed instrument, 75 cm) in 16:119:973 (v/v) pyridine–acetic acid–water buffer, pH 5. Silica gel H (Merck) was used for preparative layer-chromatography.

Preparation and analysis of reduced Lipid A. — For the transformation of the reducing 2-amino-2-deoxy-D-glucose units into the corresponding alditol units, a solution of sodium borohydride (7.5 mg) in water (1 mL) was added to Lipid A (3 mg), dispersed in a potassium borate solution (0.2 mL, 244 g of $K_2B_4O_7 \cdot 2\,H_2O$/L; apparent pH 9.8). The mixture was boiled under reflux with magnetic stirring for 30 min at 100°; foam formation was controlled by addition of a few drops of ether when necessary. Nitrogen gas was bubbled through the cooled mixture to remove any residual ether, and excess borohydride was destroyed by addition of acetic acid. Following dialysis, the retentate was lyophilized and the 2-amino-2-deoxy-D-glucose content estimated: 45% of the initial amount was recovered. A sample of the material was hydrolyzed with 4M hydrochloric acid for 6 h at 100°, the acid was removed, and the dry residue was acetylated. Analysis by g.l.c.–m.s. (OV-1, 200°, isothermal) showed peaks for the per-*O*-acetyl derivatives of 2-amino-2-deoxy-D-glucose (~8.5 min) and -D-glucitol (~9.5 min). Analysis of an aliquot of the dry residue before acetylation by the amino acid analyzer indicated a molar ratio of alditol to aldose of 1.5:1.

Transformation of the reducing 2-amino-2-deoxy-D-glucose units of Lipid A into 2-amino-2-deoxy-D-glucitol units in the presence of sodium dodecyl sulfate – To a sample (15 mg) of Lipid A was added an aqueous solution of sodium dodecyl sulfate (1%, w/v, 1.5 mL), and the mixture kept for 5 min at 100°. The opalescent solution was cooled to room temperature and treated with a 0.5% w/v solution of sodium borohydride (6 mL). From the well-stirred mixture samples (1 mL) were withdrawn at 40, 60, and 120 min, and 12M hydrochloric acid (0.5 mL) was immediately added. The samples were kept for 6 h at 100°. Free fatty acids were removed by extraction with hexane, the aqueous solution was evaporated to dryness, and the residue was acetylated with acetic anhydride–sodium acetate for 1 h at 100°. After removal of the solvents, the residue was extracted with ethyl acetate and analyzed by g.l.c. (OV-1, as above). It was found that reduction (~50% within 30 min) was complete after 60 min; the ratio of alditol to aldose was 1.25:1 as estimated from the peak areas.

Identification of the amino group substituent of the reducing 2-amino-2-deoxy-D-glucose units of Lipid A. – A dispersion of Lipid A (10 mg) in potassium borate (0.7 mL) was treated in the absence of sodium dodecyl sulfate with sodium borohydride (25 mg) in water (3.5 mL) as just described. The material was dialyzed, and the retentate lyophilized ("reduced Lipid A"). Treatment of a sample according to Reissig *et al.*[11] gave no color, which indicated the absence of reducing amino sugar residues. A second sample was hydrolyzed with 4M hydrochloric acid, and the ratio of aldose to alditol was determined with the amino acid analyzer. A third sample was hydrolyzed and the 2-amino-2-deoxy-D-glucose content estimated by the method of Rondle and Morgan[10] (Table I).

Reduced Lipid A (10 mg) was dispersed in 10mM aqueous sodium periodate (7 mL) at room temperature; the kinetics of the oxidation were monitored by the method of Avigad[21] using 20-μL aliquots; the periodate uptake ceased after 2 h. Excess periodate was destroyed by addition of 0.1M 1,2-ethanediol (0.7 mL). The ratio of aldose to alditol was determined with the amino acid analyzer, and the 2-amino-2-deoxy-D-glucose content by the method of Rondle and Morgan[10]; no 2-amino-2-deoxy-D-glucitol, but almost all of the 2-amino-2-deoxy-D-glucose was present (Table I). The bulk of the oxidation mixture was treated with a large excess of sodium borohydride (14 mg) in water (7 mL), the solution dialyzed, and the retentate lyophilized. The solid residue was extracted with ethyl acetate (3 × 5 mL), and the extract submitted to p.l.c. (200-mm plate; silica gel, 3:2, v/v, ethyl acetate–ethanol). The plate was horizontally divided into 20-mm bands that were scraped off and eluted with 3:2 (v/v) ethyl acetate–ethanol; the eluates were evaporated to dryness and methylated[22,23], and the resulting products analyzed by g.l.c.–m.s. (OV-1, 200–290°, 2°/min). The eluate of the band corresponding to R_I 0.5–0.6 gave a peak at 12.5 min (225°): its mass spectrum established the structure as 2-deoxy-1,3-di-*O*-methyl-2-[(*N*-methyl)-3-methoxytetradecanamido]glycerol (**6**). Acetylation could not be substituted for methylation, as the acetylated product gave no interpretable gas–liquid chromatogram.

A sample of the methylated product was treated with 4M hydrochloric acid for 2.5 h at 100°, the acid was removed, and the dry residue acetylated and treated with ethereal diazomethane. Analysis by g.l.c.–m.s. gave two peaks (OV-1, 100–250°, 2°/min) corresponding to 1-*O*-acetyl-2-deoxy-3-*O*-methyl-2-*N*-(methylacetamido)-D-glycerol (**7**) (12.5 min) and methyl 3-methoxytetradecanoate (30 min).

*Determination of the point of substitution of the 2-amino-2-deoxy-*D*-glucitol units present in reduced Lipid A.* — Reduced Lipid A (9 mg), dried in the presence of phosphorus pentaoxide, was dissolved (ultrasonic bath) in dimethyl sulfoxide (3 mL) and methylated. The solution was diluted with water (15 mL) and dialyzed. The retentate was lyophilyzed, the residue was hydrolyzed with 4M hydrochloric acid for 6 h at 100°, and the acid was removed. The aqueous solution of the residue was passed through a column (5 × 1 cm) of Dowex 50 (H^+) cation-exchange resin, the column was washed with 1:5 (v/v) methanol–water (20 mL) and eluted with 1:5 (v/v) methanol–M hydrochloric acid (20 mL). The eluate was evaporated, the residue acetylated, and the product analyzed by g.l.c.–m.s. (SE-30, 140–270°, 2°/min). The main product (15 min, 170°) was 1,6-di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol (**4**); it was identified by comparison of its retention time and mass spectrum with those of an authentic sample obtained as described in the next paragraph.

*1,6-Di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-2-(N-methylacetamido)-D-glucitol* (**4**). — Chlorotriphenylmethane (150 mg) was added to 2-acetamido-2-deoxy-D-glucitol (50 mg) dried in the presence of phosphorus pentaoxide *in vacuo* and dissolved in anhydrous pyridine (1 mL). The mixture was kept for 48 h at 20°; the solvent was removed, and the 1,6-bis(triphenylmethyl) ether recovered by p.l.c. (silica gel; 3:2, v/v, ethyl acetate–ethanol). The product was methylated[22,23], and the crude product, recovered from the chloroform solution, detritylated with 50% aqueous acetic acid (1.5 mL, 1 h, 100°). The mixture was evaporated to dryness, and toluene was added to and evaporated from the residue several times. The residue was acetylated with acetic anhydride–sodium acetate for 1 h at 100°. After removal of the solvent, the residue was extracted with ethyl acetate, and the concentrated extract analyzed by g.l.c.–m.s. as just described; mass spectrum: *m/z* (%) 364 (M + 1) (0.6), 303 (12.6), 290 (0.9), 288 (2.2), 272 (2.8), 271 (1.7), 258 (1.7), 246 (4.8), 214 (7.3), 202 (13.5), 183 (1.3), 173 (4.7), 161 (7.3), 158 (21.7), 142 (18), 117 (20), 116 (25.3), 101 (13.5), 99 (24.6), 98 (100), 88 (5.1), 87 (3), 74 (6.8), and 43 (33).

Identification of the substituent at O-*6 of the 2-amino-2-deoxy-*D*-glucitol units of reduced Lipid A.* — A sample (2 mg) of reduced Lipid A was treated with sodium periodate and then with sodium borodeuteride as described earlier. The dry residue was treated with hydrazine hydrate (0.3 mL) for 60 h at 100°. Acetone (0.3 mL) was added to the cooled mixture, which was then evaporated *in vacuo*; the residue was extracted with water (2 × 5 mL). Water was evaporated from the extract, the residue (dried in the presence of phosphorus pentaoxide) acetylated, and the product analyzed by g.l.c.–m.s. (SE-30, 220–270°, 4°/min). A single peak (2 min) was detected; it had the retention time and the mass spectrum of 2-acetoxy[^{2}H]ethyl 2-acetamido-

3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside[13] and was well separated from the α-D anomer. The substituents originally present in the 2-amino-2-deoxy-D-glucopyranosyl residue were not identified.

Identification of 2-amino-6-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucitol as a constituent of reduced Lipid A. Lipid A (25 mg) was converted into reduced lipid A. The material was dissolved in 0.5M barium hydroxide solution (3 mL) and kept for 48 h at 95°. The pH of the cooled mixture was brought to 2.5 by addition of M sulfuric acid, and free fatty acids were extracted with hexane. The pH was adjusted to 7 with dilute sodium hydroxide and the precipitate centrifuged off (15 min, 1085*g*). The supernatant solution was concentrated and submitted to paper electrophoresis (pH 5, 3500 V·75 cm). The ninhydrin positive material (side bands) migrating like synthetic 2-amino-6-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucitol was eluted with water and methylated. Upon analysis by g.l.c.–m.s. (CP-Sil 5, 25 m, 260°, isothermal), a single peak was observed that had a mass spectrum in agreement with that given by an authentic sample[14]; in m.s.c.i. (ammonia), the M + 1 peak (*m/z* 553) appeared and was the base peak.

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REFERENCES

1 O. Lüderitz, C. Galanos, V. Lehmann, H. Mayer, E. T. Rietschel, and J. Weckesser, *Naturwissenschaften*, 65 (1978) 578–585.
2 M. R. Rosner, J.-Y. Tang, I. Barzilay, and H. G. Khorana, *J. Biol. Chem.*, 254 (1979) 5906–5917.
3 S. Hase and E. T. Rietschel, *Eur. J. Biochem.*, 63 (1976) 101–107.
4 S. Hasl and E. T. Rietschel, *Eur. J. Biochem.*, 75 (1977) 23–34.
5 R. N. Tharanathan, J. Weckesser, and H. Mayer, *Eur. J. Biochem.*, 84 (1978) 385–394.
6 K. W. Broady, E. T. Rietschel, and O. Lüderitz, *Eur. J. Biochem.*, 115 (1981) 463–468.
7 G. Keilich, J. Roppel, and H. Mayer, *Carbohydr. Res.*, 51 (1976) 129–134.
8 J. Roppel, H. Mayer, and J. Weckesser, *Carbohydr. Res.*, 40 (1975) 31–40.
9 A. Le Dur, M. Caroff, R. Chaby, and L. Szabó, *Eur. J. Biochem.*, 84 (1978) 579–589.
10 C. J. M. Rondle and W. T. J. Morgan, *Biochem. J.*, 61 (1955) 586–589.
11 J. L. Reissig, J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.*, 217 (1955) 259–266.
12 D. Blache, M. Brunetaud, and G. Michel, *Biochimie* 62 (1980) 191–194.
13 S. R. Sarfati and P. Szabó, *Carbohydr. Res.*, 72 (1979) 297–300.
14 M. Jensen, D. Borowiak, H. Paulsen, and E. T. Rietschel, *Biomed. Mass Spectrom.*, 6 (1979) 559–565.
15 M. Caroff and L. Szabó, *Carbohydr. Res.*, 84 (1980) 43–52.
16 S. M. Cohen and M. W. Wheeler, *Am. J. Publ. Health*, 36 (1946) 371–376.
17 O. Westphal, O. Lüderitz, and F. Bister, *Z. Naturforsch.*, 76 (1952) 148–155.
18 A. Le Dur, R. Chaby, and L. Szabó, *J. Bacteriol.*, 143 (1980) 78–88.
19 M. Caroff and L. Szabó, unpublished results.
20 P. S. Chen, T. Y. Toribara, and M. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
21 G. Avigad, *Carbohydr. Res.*, 11 (1969) 119–123.
22 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
23 P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lonngren, *Chem. Commun., Univ. Stockholm*, 8 (1976) 1–75.