Carbohydrate Research, 83 (1980) 129–134
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

ISOLATION AND IDENTIFICATION OF 3,6-DIDEOXY-3-(L-GLYCEROYL-AMINO)-D-GLUCOSE, A CONSTITUENT OF THE ANTIGENIC POLY-SACCHARIDE OF *Eubacterium saburreum*, STRAIN V5

WATARU KONDO, FUTOSHI NAKAZAWA,
Department of Oral Microbiology, School of Dentistry, Niigata University, Gakko-cho-dori, Niigata (Japan)
AND TEIICHIRO ITO
Research Laboratories of Meiji Seika Kaisha Ltd., Morooka, Kohoku-ku, Yokohama (Japan)

(Received August 23rd, 1979; accepted for publication in revised form, January 25th, 1980)

ABSTRACT

3,6-Dideoxy-3-(L-glyceroylamino)-D-glucose has been identified, for the first time, as a sugar component of the antigenic polysaccharide of *Eubacterium saburreum* strain V5, principally by n.m.r. and mass spectrometry.

INTRODUCTION

The cell-wall antigenic polysaccharides produced by *Eubacterium saburreum*, strains L44 (ref. 1), L49 (ref. 2), L452 (ref. 3), and L32 (ref. 4) contain several unusual sugar residues, namely, D-*glycero*-D-*galacto*-heptopyranosyl, 6-deoxy-D-*altro*-heptofuranosyl, D-ribofuranosyl, D-fucofuranosyl, and tyvelopyranosyl. In this report, we describe the occurrence of 3,6-dideoxy-3-(L-glyceroylamino)-D-glucose as a sugar component of the antigenic polysaccharide produced by another strain, V5, of the same organism.

RESULTS AND DISCUSSION

Acid hydrolysis of the antigenic polysaccharide yielded four components, three of which were neutral sugars, and the fourth was an amino sugar. The neutral sugars were identified as L-rhamnose, D-galactose, and a heptose. L-Rhamnose and D-galactose were identified by optical rotation and by g.l.c.–m.s. of the per-*O*-trimethylsilylalditol derivatives, the retention times and mass spectra of which were indistinguishable from those given by authentic samples. The mass spectrum of the per-*O*-trimethylsilylalditol derivative of the third component showed it to be a heptose derivative. The amino sugar gave a brownish-yellow color upon staining with ninhydrin. It did not react with either the Rondle–Morgan[5] or the Levvy–McAllan[6] reagent, but, after *N*-acetylation, the 3-acetamido-3-deoxyhexose reaction[7] was positive.

0008-6215/80/0000–0000/$ 02.25,

The mass spectrum of its *N*-acetyl per-*O*-trimethylsilylalditol derivative (**1**) was identical with that of a sample prepared from 3-amino-3,6-dideoxy-D-galactose, which was isolated from the cell-wall of *Xanthomonas campestris*[8]. These data indicate that the compound is a 3-amino-3,6-dideoxyhexose. In both mass spectra we detected two prominent peaks, at *m/e* 217 and 246. They are secondary fragments, but the origins and the fragmentation sequences that result in their formation are not clear, except that they do not show a deuterium effect. However, it seems that these two strong ions are characteristic for the per-*O*-trimethylsilyl derivatives of 3-acetamido-3,6-dideoxyhexitols.

The ^{1}H-n.m.r. spectrum showed that the ratio of α- to β-pyranose form, at equilibrium in deuterium oxide solution, was 2:3. The chemical shifts and coupling constants of the anomeric protons were almost identical with those of D-glucose (α anomer: δ 5.24, $J_{1,2}$ 3.6 Hz; β anomer: δ 4.67, $J_{1,2}$ 7.6 Hz). In order to confirm the *gluco* configuration of the sugar, the *N*-acetyl-tri-*O*-acetyl derivative was prepared. The ^{1}H-n.m.r. spectrum of the solution in chloroform-*d* indicated that the compound was present mainly as β anomer, contaminated with $\sim$10% of α anomer. The spectrum showed a signal for H-3 at δ 4.38 ($J_{2,3} = J_{3,4} = 10$ Hz), thus confirming the *gluco* configuration. The optical rotation of the sugar was identical to that of 3-amino-3,6-dideoxy-L-glucose hydrochloride[9], but opposite in sign. These results suggest the structure of 3-amino-3,6-dideoxy-D-glucose. 3-Amino-3,6-dideoxy-D-hexoses are not uncommon as components of lipopolysaccharides from Gram-negative bacteria[8,10–12], but it is the first time that a 3-amino-3,6-dideoxyhexose is observed in a polysaccharide from a Gram-positive bacterium.

When the antigenic polysaccharide was subjected to mild, acid hydrolysis and the products were separated by p.c., a new, neutral reducing-sugar (**2**) was isolated. Prolonged hydrolysis with M hydrochloric acid gave two components. One was a ninhydrin-positive, reducing sugar, which was identified as 3-amino-3,6-dideoxy-D-glucose by its retention time in g.l.c. and the mass spectrum of its *N*-acetyl-per-*O*-trimethylsilylalditol derivative. The other component was an acid and it was indistinguishable from an authentic sample of glyceric acid in p.c. and g.l.c. The ^{1}H-n.m.r. spectrum of the calcium salt was identical with that of authentic calcium DL-glycerate. Comparison of the mass spectra of the *O*-trimethylsilyl derivatives of the authentic and of the isolated compounds provided further evidence for glyceric acid as the acid component. The optical rotation of the calcium salt indicated that it was L-glyceric acid.

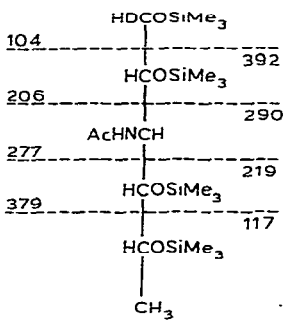
The ^{13}C-n.m.r. spectrum of **2** (Table I) showed twelve strong signals, three of which were assigned to the carbon atoms of the glyceric acid residue and the remaining nine to those of the 3-amino-3,6-dideoxy-D-glucose residue. The i.r. spectrum showed absorptions at 1640 and 1535 cm^{-1}, thus indicating the presence of an amide linkage. These data strongly suggest that the sugar is 3-amino-3,6-dideoxy-D-glucose *N*-acylated with L-glyceric acid (**2**). In order to confirm this structure, the sugar was reduced with sodium borodeuteride, trimethylsilylated, and analyzed by g.l.c.–m.s. The mass spectrum of the resulting compound **3** was closely related to the mass

TABLE I

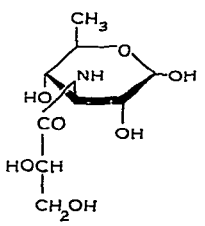
^{13}C-N.M.R. DATA FOR CALCIUM GLYCERATE, 3-ACETAMIDO-3,6-DIDEOXY-D-GLUCOSE, AND 3,6-DIDEOXY-3-(L-GLYCEROYLAMINO)-D-GLUCOSE

Carbon atom	*Chemical shift (δ)*		
	Calcium L-glycerate	*3-Acetamido-3,6-dideoxy-D-glucose*	*3,6-Dideoxy-3-(L-glyceroylamino)-D-glucose*
C-1		92.6(α), 97.5(β)	92.6(α), 97.5(β)
C-2		70.8	71.0
C-3		51.8(α), 55.7(β)	51.5(α), 55.3(β)
C-4		70.2	70.5
C-5		71.2(α), 72.7(β)	71.3(α), 72.8(β)
C-6		16.4	16.4
C′-1	179.2		174.5
C′-2	74.4		73.1
C′-3	64.9		64.2
Acetyl		22.8, 174.9	

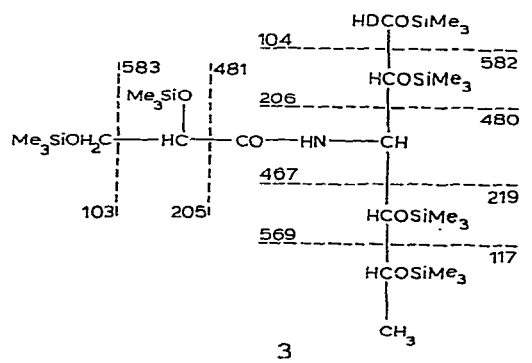
spectrum of **1**. The primary fragments containing C-3, at *m/e* 467, 480, 569, 582, and 671 ($M^+ - 15$), showed values higher by 190 mass units than the corresponding fragments of **1** at *m/e* 277, 290, 379, 392, and 481 ($M^+ - 15$), respectively. The same is true for the secondary fragments containing C-3, at *m/e* 377 (187 + 190), 390 (200 + 190), 436 (246 + 190), and 479 (289 + 190). Both compounds **1** and **3** gave identical primary fragments that do not contain C-3, at *m/e* 104, 117, 206, and 219. The difference of 190 mass units between the corresponding fragments containing C-3 of **1** and **3** can only be explained by *N*-acylation of 3-amino-3,6-dideoxy-D-glucose with a $CO\text{-}CHOSiMe_3\text{-}CH_2OSiMe_3$ group (233) for **3** and an acetyl group (43) for **1**, indicating a 3,6-dideoxy-3-(L-glyceroylamino)-D-glucose structure (**2**) for the new component isolated from *E. saburreum* strain V5. This structure has not been previously shown in Nature.



1



2



3

EXPERIMENTAL

Culture conditions. — *E. saburreum* strain V5 was grown anaerobically in the following medium: proteose peptone No. 3 (Difco) (10 g), yeast extract (Difco) (5 g), sodium chloride (5 g), dipotassium hydrogenphosphate (2.5 g), D-glucose (10 g), and distilled water (1 L), pH 7.0. Following incubation for 24 h at 37°, the cells were centrifuged off, washed twice with saline solution, and kept as lyophilized cells.

Antigen extraction and purification methods. — The antigenic polysaccharide was extracted essentially as described by Krause and McCarthy[13]. Formamide (20 mL) was added to lyophilized cells (1 g), and the extraction was carried out with continuous stirring for 15 min at 150° (oil bath). After extraction, removal of the insoluble material by centrifugation was facilitated by the addition of 2 vol. of acid alcohol (19 : 1, v/v, ethanol–M hydrochloric acid). The polysaccharide was precipitated from the alcoholic supernatant by the addition of acetone (5 vol.). The acetone-precipitable polysaccharide was dissolved in 0.02M Tris–hydrochloric acid buffer containing 15mM calcium chloride, pH 7.8 (10 mL). It was digested with Pronase (1 mg/mL) for 24 h at 37°, and the digest was adsorbed on a column (2.4 × 140 cm) of Sephadex G-100 and eluted with 0.02M phosphate buffer, pH 7.4, containing sodium azide (0.02%). The elution volume of the serologically active polysaccharide was found to be 326 mL (V_e/V_o 1.48). The lyophilized cells (1 g) yielded 43 mg of antigenic polysaccharide, $[\alpha]_D^{25}$ +92° (*c* 1.3, water).

General analytical methods. — Optical rotations were determined with a Perkin–Elmer 241 photoelectric polarimeter. G.l.c. was performed with a Hitachi 063 instrument with flame-ionization detector, and g.l.c.–m.s. with a Hitachi RMU7M instrument. The per-*O*-trimethylsilyl derivatives were analyzed by use of a glass column (0.3 × 300 cm) packed with 3% OV-17 on Shimalite W (80–100 mesh), the temperature being raised from 150 to 250° at a rate of 5°/min. N.m.r. spectra were recorded with a Varian XL-100 spectrometer equipped with an F.t. system, tetramethylsilane being the external standard for ^{1}H-spectra in D_2O or in chloroform-*d*, and 1,4-dioxane the internal standard for ^{13}C-spectra in D_2O. I.r. spectra were determined with a Hitachi Model 215 apparatus for Nujol mulls. Paper chromatography was performed on Whatman No. 1 paper with 6 : 4 : 3 (v/v) 1-butanol–pyridine–water as irrigant.

Isolation and identification of components. — (*a*) *Strong acid hydrolysis.* The antigenic polysaccharide was hydrolyzed with M hydrochloric acid for 5 h. After cooling of the mixture, the excess of hydrochloric acid was removed by treatment with Dowex-1 (AcO^-) anion-exchange resin, and the resulting solution was passed through a column of Dowex-50 (H^+) cation-exchange resin. The effluent and water washings were concentrated to a small volume (neutral sugar fraction). The amino sugar was then eluted with 2M hydrochloric acid (3 bed vols.).

The neutral-sugar fraction was reduced with sodium borohydride, per-*O*-trimethylsilylated, and analyzed by g.l.c.–m.s. G.l.c. of the product showed three

components with mobilities (*t* value) relative to per-*O*-trimethylsilylinositol of 0.61, 0.82, and 1.09. The first-eluted component was identified as L-rhamnose. It showed $[\alpha]_D^{25}$ +6.3° (*c* 1.5, water); lit.[14] $[\alpha]_D$ +8.9°. The second component was identified as D-galactose. It showed $[\alpha]_D^{25}$ +75° (*c* 0.16, water); lit.[15] $[\alpha]_D$ +80.2°. The third component was identified as a heptose; $[\alpha]_D^{25}$ +18° (*c* 0.5, water); m.s.: *m/e* 701 (0.1, $M^+ - 15$), 626 (0.1), 536 (0.2), 523 (2), 446 (1), 433 (2), 421 (10), 331 (14), 319 (100), 307 (27), 217 (45), 205 (64), 147 (41), 129 (11), 117 (13), 103 (28), and 73 (71).

The amino sugar was identified as 3-amino-3,6-dideoxy-D-glucose[9] hydrochloride; $[\alpha]_D^{24}$ +56.3° (*c* 6.0, water) {$[\alpha]_D$ of L isomer[9]; −57° (*c* 2.8, water)}; ^{1}H-n.m.r.: δ 5.26 ($J_{1,2}$ 3.6 Hz, H-1α), 4.67 ($J_{1,2}$ 7.6 Hz, H-1β), 3.43 ($J_{2,3}$ 10.4 Hz, H-2), 1.29, and 1.22 ($J_{5,6}$ 5 Hz, H-6). It was treated with Dowex-1 (AcO^-) anion-exchange resin, *N*-acetylated, reduced with sodium borodeuteride, and per-*O*-trimethylsilylated to give **1**; g.l.c.: *t* 0.94; m.s.: *m/e* 481 (12, $M^+ - 15$), 392 (2), 379 (7), 290 (3), 289 (3), 277 (9), 260 (36), 246 (79), 219 (29), 217 (96), 206 (6), 200 (14), 187 (44), 147 (35), 129 (22), 117 (39), 104 (6), and 73 (100).

The *N*-acetylated amino sugar (10 mg) was dissolved in pyridine–acetic anhydride (1:1, 0.2 mL), and the solution kept for 24 h at room temperature. The mixture was then poured into ice–water and extracted with chloroform. The chloroform solution was washed with water, dried, and evaporated to give a crystalline product (11.6 mg); n.m.r. (after deuteration of NH group, chloroform-*d*): δ 5.74 (d, 1 H, $J_{1,2}$ 8 Hz, H-1β), 5.24 (m, 1 H, H-4), 5.01 (q, 1 H, $J_{2,3}$ 10 Hz, H-2), 4.38 (t, 1 H, $J_{3,4}$ 10 Hz, H-3), 3.70 (m, 1 H, H-5), 2.19 (s, 3 H, OAc), 2.12 (s, 3 H, OAc), 2.06 (s, 3 H, OAc), 1.90 (s, 3 H, NDAc), and 1.16 (d, 3 H, *J* 7 Hz, Me). The analysis corresponded to 3-acetamido-1,2,4-tri-*O*-acetyl-3,6-dideoxy-D-glucopyranose.

Anal. Calc. for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39; N, 4.23. Found: C, 51.18; H, 6.70; N, 4.11.

(*b*) *Mild acid hydrolysis.* The antigenic polysaccharide was hydrolyzed under mild conditions (0.25M sulfuric acid, 25 min, 100°), and the solution was neutralized with barium carbonate, and evaporated. The hydrolyzate was then applied to a column (1.4 × 100 cm) of Sephadex G-10 and eluted with water. The fractions eluted in the disaccharide region were pooled. Paper-chromatographic examination of the concentrated fractions revealed the presence of 3,6-dideoxy-3-(L-glyceroylamino)-D-glucose (**2**) which crystallized as needles, m.p. 190–196°, $[\alpha]_D^{28}$ +60.8° (*c* 2.6, water; equil.); R_{Gal} 1.56; ν_{max}^{Nujol} 1640 (Amide I) and 1535 cm^{-1} (Amide II); g.l.c. of reduced and per-*O*-trimethylsilylated derivative: *t* 1.35.

Anal. Calc. for $C_9H_{17}NO_7$: C, 43.02; H, 6.82; N, 5.58. Found: C, 42.71; H, 6.76; N, 5.07.

Compound **2** was isolated by p.c., and hydrolyzed under strong acid conditions (M hydrochloric acid, 100°, 5 h). After cooling of the mixture, the excess of hydrochloric acid was removed by repeated addition and evaporation of water. The residue was *N*-acetylated, reduced with sodium borodeuteride, per-*O*-trimethylsilylated, and analyzed by g.l.c.–m.s. G.l.c. revealed the disappearance of the original sugar (*t* 1.35)

and the formation of two components (t 0.25 and 0.94). M.s. of first component: m/e 322 (1, M^+), 307 (15, $M^+ - 15$), 292 (30), 219 (2), 217 (3), 205 (18), 189 (30), 175 (4), 147 (42), 133 (22), 117 (14), 103 (25), and 73 (100). The m.s. of the second component was identical with that of 3-amino-3,6-dideoxy-D-glucose. The first component was identified as L-glyceric acid. The optical rotation of its calcium salt, $[\alpha]_D^{25}$ −14.2° (c 0.6, water), was identical with that of authentic calcium L-glycerate[16], $[\alpha]_D^{20}$ −14.6° (c 5.0, water). Compound **2** was reduced with sodium borodeuteride and per-*O*-trimethylsilylated; m.s.: m/e 671 (7, $M^+ - 15$), 583 (0.4), 582 (2), 569 (2), 540 (1), 481 (2), 480 (0.5), 479 (3), 467 (15), 436 (14), 390 (4), 377 (13), 364 (13), 219 (12), 217 (30), 206 (8), 205 (12), 147 (26), 117 (46), 104 (7), 103 (10), and 73 (100).

ACKNOWLEDGMENT

We are indebted to Dr. Gilbert Ashwell, National Institutes of Health, Bethesda, Maryland, for a generous gift of 3-acetamido-3,6-dideoxy-D-galactose.

REFERENCES

1 J. HOFFMAN, B. LINDBERG, S. SVENSSON, AND T. HOFSTAD, *Carbohydr. Res.*, 35 (1974) 49–53.
2 J. HOFFMAN, B. LINDBERG, J. LÖNNGREN, AND T. HOFSTAD, *Carbohydr. Res.*, 47 (1976) 261–267.
3 J. HOFFMAN, B. LINDBERG, T. HOFSTAD, AND H. LYGRE, *Carbohydr. Res.*, 58 (1977) 439–442.
4 J. HOFFMAN, B. LINDBERG, T. HOFSTAD, AND N. SKAUG, *Carbohydr. Res.*, 66 (1978) 67–70.
5 C. J. M. RONDLE AND W. T. J. MORGAN, *Biochem. J.*, 61 (1955) 586–589.
6 G. A. LEVVY AND A. MCALLAN, *Biochem. J.*, 73 (1959) 127–132.
7 G. ASHWELL, N. C. BROWN, AND W. A. VOLK, *Arch. Biochem. Biophys.*, 112 (1965) 648–652.
8 G. ASHWELL AND W. A. VOLK, *J. Biol. Chem.*, 240 (1965) 4549–4555.
9 A. C. RICHARDSON AND K. A. MCLAUCHLAN, *J. Chem. Soc.*, (1962) 2499–2506.
10 B. JANN, K. JANN, AND E. MÜLLER-SEITZ, *Nature (London)*, 215 (1967) 170–171.
11 O. LÜDERITZ, E. RUSCHMANN, O. WESTPHAL, R. RAFF, AND R. WHEAT, *J. Bacteriol.*, 93 (1967) 1681–1687.
12 R. RAFF AND R. WHEAT, *J. Biol. Chem.*, 242 (1967) 4610–4613.
13 R. M. KRAUSE AND M. MCCARTHY, *J. Exp. Med.*, 114 (1961) 127–140.
14 M. WINDHOLZ (Ed.), S. BUDAVARI, L. Y. STROUMTSOS, AND M. N. FERTIG (Assist. Eds.), *The Merck Index*, 9th edn., Merck and Co., Rahway, NJ, U.S.A., 1976, p. 1060.
15 Ref. 14, p. 559.
16 Ref. 14, p. 581.