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Note

Identification of 2-acetamido-3-*O*-(3-acetamido-3,6-dideoxy-β-D-glucopyranosyl)-2-deoxy-D-galactopyranose isolated after degradation of the lipopolysaccharide from *Vibrio parahaemolyticus* Serotype O12

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3-Amino-3,6-dideoxy-D-glucose (3,6dGlcN) has been reported as a constituent of some enterobacterial lipopolysaccharides (LPS)[1–3] and was also identified in the LPS of *Vibrio parahaemolyticus*[4]. We now describe the isolation and identification of 2-acetamido-3-*O*-[3-acetamido-3,6-dideoxy-β-D-glucopyranosyl]-2-deoxy-D-galactopyranose (**1**), which contains this sugar and occurs as

TABLE I

^{1}H-N.M.R. DATA[a] FOR **2α** (360 MHz, D_2O)

Assignment	*δ (p.p.m.)*	J *(Hz)*
3,6dGlc*p*NAc		
H-1′	4.45(d)	$J_{1',2'}$ 7.9
H-3′	3.68(ddd)	$J_{3',4'}$ 10.0, $J_{3',5'}$ 1.8
H-5′	3.42(dd)	$J_{5',6'}$ 6.0
H-2′	3.18(dd)	$J_{2',3'}$ 10.8
H-4′	3.03(dd)	$J_{4',5'}$ 9.6
H-6′,6′,6′	1.17(d)	
Gal*p*NAc		
H-1	4.66(d)	$J_{1,2}$ 3.8
H-2	4.22(ddd)	$J_{2,3}$ 10.9
H-4	4.06(dd)	$J_{4,5}$ ~1
H-3	3.90(dd)	$J_{3,4}$ 3.0
H-5	3.83(dd)[b]	$J_{5,6a}$ 4.7, $J_{5,6b}$ 7.4
H-6a	3.68–3.62[c]	
H-6b		

[a]Other signals: δ 3.27 (s, OMe). [b]Broad signal. [c]Unresolved multiplet.

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TABLE II

^{13}C-N.M.R. DATA[a] FOR **2α** AND METHYL 2-ACETAMIDO-2-DEOXY-α-D-GALACTOPYRANOSIDE (90.6 MHz, D_2O)

Assignment	*δ (p.p.m.)*	
2 (3,6dGlc*p*NAc)		
C-1′	105.50	
C-4′	74.13	
C-5′	73.90	
C-2′	72.20	
C-3′	57.70	
C-6′	17.70	
2 (Gal*p*NAc)		αGal*p*NAc-OMe
C-1	99.20	99.10
C-3	78.30	68.70
C-5	71.40	71.60
C-4	69.50	69.40
C-6	62.10	62.10
C-2	49.40	50.80

[a]Assignments by ^{1}H,^{13}C-COSY n.m.r. spectroscopy.

a unit of the core-oligosaccharide of *V. parahaemolyticus* LPS (serotype O12).

Periodate oxidation of the LPS followed by methanolysis gave the αβ-mixture of the methyl glycosides (**2**), which were isolated by h.p.l.c. on 3-aminopropyl silica gel (ZORBAX^R-NH_2). The ^{1}H- and ^{13}C-n.m.r. data are shown in Tables I and II together with those for methyl 2-acetamido-2-deoxy-α-D-galactopyranoside.

The $J_{1',2'}$ value of 7.9 Hz for the major isomer **2α** indicates a β-interglycosidic linkage. The resonance signal of C-3 (78.3 p.p.m.) in **2** (Table II) is shifted downfield by 9.6 p.p.m. compared with that of C-3 (68.7 p.p.m.) in methyl 2-acetamido-2-deoxy-α-D-galactopyranoside, indicating a 3-substituted Gal*p*NAc moiety. These inferences are confirmed (*a*) by the ^{1}H-n.m.r. data in Table III for the tetra-acetate (**4**) of **2α** and for 3-acetamido-1,2,4-tri-*O*-acetyl-3,6-dideoxy-β-D-mannopyranose (**9**) isolated from amphotericin B, and (*b*) the ^{13}C-n.m.r. data in Table IV which show that the resonance for C-3 (73.5 p.p.m.) in **4** is shifted upfield by 4.7 p.p.m. relative to the signal (68.8 p.p.m.) for C-3 of methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranoside, and that the $J_{C\text{-}1',H\text{-}1'}$ value of 163 Hz indicates[5] the 3,6dGlc*p*NAc moiety to be β.

G.l.c.–m.s. analysis of the methylated[6] derivative (**3**) of **2** and also the tetra-*O*-acetyl derivative **4** gave the results shown in Table V. E.i. fragments of **3** at *m/z* 230 and 260 were assigned to the methylated 3,6dGlc*p*NAc and Gal*p*NAc monosaccharide moieties, respectively. C.i.-(ammonia)-m.s. indicated the mol. wt. of **3** to be 506 ($[M + H]^+$ *m/z* 507; $[M + NH_4]^+$ *m/z* 524) and **4** to be 590 ($[M + H]^+$ *m/z* 591; $[M + NH_4]^+$ *m/z* 608).

Acid hydrolysis of **2** followed by *N*-acetylation[8], reduction ($NaBH_4$ or NaB^2H_4), and methylation gave **5a** and **5b**. On g.l.c.–m.s. of **5a** (Table V), the e.i.

TABLE III

^{1}H-N.M.R. DATA (360 MHz, BENZENE-d_6) FOR **4**[a] AND **9**[b]

Assignment	δ (*p.p.m.*)	J (*Hz*)		*Assignment*	δ (*p.p.m.*)	J (*Hz*)	
4 (3,6dGlc*p*NAc)				**9**			
NH-3′	5.61(d)	$J_{NH,3'}$	9.6	H-1	5.75(d)	$J_{1,2}$	~1
H-2′	4.88(dd)	$J_{2',3'}$	9.6	NH-3	5.83(d)	$J_{NH,3}$	9.4
H-1′	4.84(d)	$J_{1',2'}$	7.3	H-2	5.64(dd)	$J_{2,3}$	3.2
H-4′	4.66(dd)	$J_{4',5'}$	9.7	H-4	4.99(dd)	$J_{4,5}$	10.6
H-3′	4.52(ddd)	$J_{3',4'}$	9.6	H-3	4.58(ddd)	$J_{3,4}$	10.6
H-5′	3.64(dd)	$J_{5',6'}$	6.0	H-5	3.38(ddd)	$J_{5,6}$	6.2
H-6′,6′,6′	1.19(d)			H-6	1.09		
4 (Gal*p*NAc)							
NH-2	6.37(d)	$J_{NH,2}$	9.2				
H-4	5.66(dd)	$J_{4,5}$	~1				
H-2	4.96(ddd)	$J_{2,3}$	10.8				
H-1	4.76(d)	$J_{1,2}$	3.8				
H-6a	4.33(dd)	$J_{6a,6b}$	11.3				
H-6b	4.20(dd)	$J_{5,6b}$	7.2				
H-3	4.04(dd)	$J_{3,4}$	3.3				
H-5	3.97(dd)	$J_{5,6a}$	5.6				

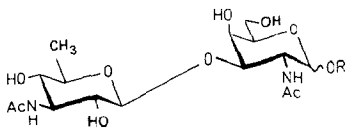
[a]Other signals: δ 2.00–1.70 (6 s, 4 OAc, 2 NAc), 3.00 (s, OMe). [b]Other signals: δ 1.80 (NAc), 1.60–1.44 (OAc).

TABLE IV

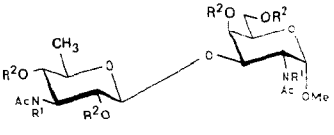
^{13}C-N.M.R. DATA[a] FOR **4**, **9**, AND METHYL 2-ACETAMIDO-3,4,6-TRI-*O*-ACETYL-2-DEOXY-α-D-GALACTOPYRANOSIDE (*A*) (90.6 MHz, BENZENE-d_6)

Assignment	δ (*p.p.m.*)	
4 (3,6dGlc*p*NAc		**9**
C-1′	101.2	91.2 ($J_{C\text{-}1,H\text{-}1}$ 163 Hz)
C-4′	74.1	71.8
C-2′	71.4	70.1
C-5′	71.0	71.8
C-3′	53.2	51.6
C-6′	17.4	17.3
4 (Gal*p*NAc)		*A*
C-1	99.1	99.2
C-3	73.5	68.8
C-4	69.4	67.7
C-5	67.3	66.9
C-6	62.8	62.0
C-2	48.9	48.1

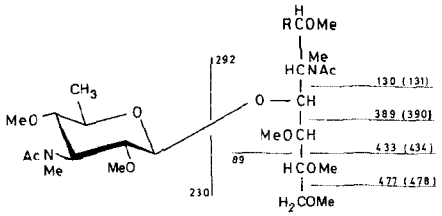
[a]Assignments by ^{1}H,^{13}C-COSY n.m.r. spectroscopy.



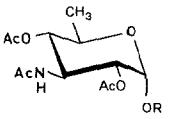
1 R = H
2 R = Me



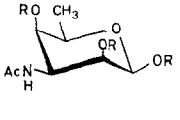
3 $R^1 = R^2 = Me$
4 $R^1 = H, R^2 = Ac$



5a R = H (mol. wt. 522)
5b R = D (mol. wt. 523)



6 R = (−)-^{s}Bu
7 R = (+)-^{s}Bu



8 R = H
9 R = Ac

fragments *m/z* 156, 198, and 230 were assigned to the 3,6 dGlc*p*NAc moiety (*m/z* 230) with subsequent eliminations of methanol (−32). The relatively high intensity of the fragments at *m/z* 433 and 477 indicated a 3-substituted GalNAc-ol moiety, since the presence of MeO groups at C-4,5,6 resulted in preferential cleavage[9] of

TABLE V

G.L.C. AND G.L.C.–M.S. DATA FOR **3**, **4**, **5a**, AND **5b**

Derivative	*Mol.wt.*[a]	*Retention time*[b]	*Characteristic fragments*[c]
3	506	2.53	125(62.4), 129(36.9), 142(19.8), 156(21.9), 157(20.3), 186(7.6), 198(11.8), 230(100.0), 260(16.7), 332(6.1), 442(1.9)
4	590	2.85	111(65.2), 140(37.2), 153(67.0), 170(30.5), 184(8.2), 200 (40.1), 230(6.8), 272(100.0), 302(7.8), 316(10.3), 470(6.2)
5a	522	2.31	88(53.0), 125(51.9), 130(54.9), 156(15.5), 157(15.6), 198(12.0), 230(100.0), 246(5.7), 276(9.0), 389(0.8), 433(7.8)
5b	523	2.31	89(27.0), 125(37.8), 131(32.7), 156(12.4), 157(16.0), 198(9.7), 230(100.0), 246(7.1), 277(8.7), 330(0.5), 390(0.7), 477(1.4), 478(0.5)

[a]Determined by c.i. (ammonia)-m.s. on the basis of peaks at *m/z* for $(M + 1)^+$ and $(M + 18)^+$. [b]Relative to that of 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol, using a fused-silica capillary column (25 m x 0.32 mm i.d.) with chemically bonded SE-54, a temperature programme of 200° for 3 min and then 5°/min to 300°, and H_2 as carrier gas (0.08 MPa). [c]Determined by e.i.-m.s. at 70 eV.

the C-4–C-5 and C-5–C-6 bonds. This interpretation was further supported by the mass spectrum of **5b** (mol. wt. 507), where the signals for the fragments of the GalNAc-ol moiety (*m/z* 390, 434, and 478) reflected the presence of the deuterium at C-1. The fragmentation pattern of the 2-acetamido-2-deoxygalactitol residue in **5a** was similar to that of the corresponding 3-substituted 2-acetamido-2-deoxyglucitol[7].

Methylation analysis of **5a** gave 1,5-di-*O*-acetyl-3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)glucitol (from terminal 3,6dGlc*p*NAc) and 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol (from 3-substituted Gal*p*NAc). 1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-(1-^{2}H)galactitol was obtained from **5b**.

The absolute configuration of the 3-acetamido-3,6-dideoxyglucose was determined[10] by g.l.c.–m.s. of the acetylated (−)- (**6**) and (+)-*sec*-butyl (**7**) glycosides. The retention times on OV-210 of 5.42 (±0.03) and 5.62 (±0.03) min, respectively, were identical to those of the corresponding derivatives of 3-amino-3,6-dideoxy-D-glucose isolated from *Campylobacter coli*[3].

3-Amino-3,6-dideoxy-D-glucose has been identified as a component of *V. parahaemolyticus* O7 LPS, the sugar composition of which is similar to O12 LPS[4,11]. However, **1** was not detected in the LPS of serotype O7. In SDS-polyacrylamide gel electrophoresis (15% polyacrylamide), O7 and O12 LPS yielded banding patterns that were identical to that of the LPS of a rough mutant of *Salmonella minnesota* (chemotype Ra). Therefore, no repeating oligosaccharide units (O-specific chain) are present and the core oligosaccharide is made up of 10–12 sugar residues[12] which determine the serological specificity and the substantial cross-reactivity[13]. It is likely that **1** might be part of the epitope(s) responsible for the serological specificity of *V. parahaemolyticus* serotype O12 LPS.

EXPERIMENTAL

General. — *V. parahaemolyticus* serotypes O12 (strain OP204, K19) and O7 (strain Ta 11, K19) were provided by Dr. T. Miwatani (Institute of Microbial Diseases, Osaka University, Osaka, Japan) and Drs. M. Ohashi and K. Ohta (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan), respectively. Cultivation of the microbes and extraction of LPS by hot phenol–water have been described[11]. Methylation was done according to Ciucanu and Kerek[6]. G.l.c. was carried out on a Varian gas chromatograph (model 3700) equipped with a fused-silica capillary column (25 m × 0.22 mm i.d.) and chemically bonded SE-54 or OV-210 (film thickness, 0.35 μm), with temperature programmes *A*, 3 min at 130° then to 300° at 3°/min; *B*, 3 min at 200° then to 300° at 5°/min; and *C*, 3 min at 150° then to 250° at 3°/min. G.l.c.–m.s. analysis was performed as described[14]. The ^{1}H- and ^{13}C-n.m.r. spectra were recorded for solutions (0.5 mL) in D_2O (**2**) and benzene-d_6 (**6**) with Me_4Si (^{1}H) as internal standard and acetonitrile (^{13}C, 1.70 p.p.m.) as reference signal, respectively. Sodium dodecylsulfate–poly-

acrylamide gel electrophoresis (SDS–PAGE) was performed by the Laemmli system (15% acrylamide) as described[15].

Degradation of the LPS. — The LPS (250 mg) was treated with 0.02M sodium periodate (300 mL; 4°, 120 h). Oxidation was stopped by adding ethyleneglycol (3.9 g) and stirring for 1 h at room temperature. After reduction (10 g of $NaBH_4$ in 50 mL of water), the solution was brought to pH 7 with M HCl and then lyophilized prior to methanolysis (methanolic 0.5M HCl, 20 h, 37°). The methanolysate was concentrated to dryness, and a solution of the residue in water (1.5 mL) was dialyzed three times against water (100 mL each). The diffusates were collected, concentrated, and eluted from a column (2.5 × 100 cm) of Sephadex G-50 with water. Fractions (5 mL) that gave a positive Morgan–Elson reaction (after hydrolysis in 4M HCl; 100°, 16 h) were freeze-dried to give crude **2** (25 mg).

Crude **2** was eluted from a column (2.6 × 92 cm) of TSK 40HW(S) (particle size, 25–40 μm; Merck) at 3.3 mL/min with water at 0.1 MPa. Fractions (3.3 mL) which were positive in the Morgan–Elson reaction (after hydrolysis as above) were freeze-dried to give **2** (8.0 mg). H.p.l.c. of **2** was performed on a column (9.4 × 250 mm) of silica gel with chemically bonded 3-aminopropyl groups ($ZORBAX^R$-NH_2, DuPont) by elution with aq. 70% acetonitrile at 40° and 2.5 mL/min. The eluate was monitored by its u.v.-absorbance at 205 nm. The appropriate fractions were combined and concentrated to give methyl 2-acetamido-3-*O*-(3-acetamido-3,6-dideoxy-β-D-glucopyranosyl)-2-deoxy-β-D-galactopyranoside) (**2β**, 1.0 mg), *T* 7.1 min; and the α anomer (**2α**, 2.3 mg), *T* 7.8 min, $[\alpha]_D$ +37° (*c* 0.2, water).

Derivatives of **2**. — Compound **2** (100 μg) was methylated by the method of Ciucanu and Kerek[6].

Crude **2** (25 mg, prepared from 250 mg of LPS) was treated with 2:1 pyridine–acetic anhydride (2 mL), in the presence of 4-dimethylaminopyridine as catalyst, at room temperature. The product, isolated in the usual manner, was purified by h.p.l.c. on a column (20 × 500 mm) of Partilsil-Silica (10 μ, Bischoff, Leonberg) by elution with 20:1 chloroform–methanol at 10 mL/min. The eluate was analyzed by t.l.c. [Kieselgel 60 F_{245} (Merck), 9:1 chloroform–methanol] for the tetra-*O*-acetyl derivative **4**, R_F 0.35. Concentration of the appropriate fractions gave methyl 2-acetamido-3-*O*-(3-acetamido-2,4-di-*O*-acetyl-3,6-dideoxy-β-D-glucopyranosyl)-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranoside (**4**, 7.3 mg), $[\alpha]_D$ +55° (*c* 0.6, chloroform); and the β anomer (2.6 mg).

Compound **2** (100 μg) was hydrolyzed (0.5M HCl, 100°, 30 min), and the product was reduced ($NaBH_4$), *N*-acetylated[8], and methylated[6] to give 2-deoxy-3-*O*-[3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)-β-D-glucopyranosyl]-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol (**5a**). Reduction with NaB^2H_4 gave **5b**.

For methylation analysis, **5a** (or **5b**) (100 μg) was acetolyzed[16], reduced ($NaBH_4$), acetylated, and analyzed by g.l.c.–m.s.

3-Amino-3,6-dideoxy-D-mannopyranose (**8**). — Amphotericin B (0.1 mmol, Sigma) was hydrolyzed with M trifluoroacetic acid at 100° for 1 h. The hydrolyzate

was concentrated and the residue was eluted from a column (0.5 × 7 cm) of Amberlite IR-120 (H^+) resin with 6 mL of M HCl. The appropriate fractions were concentrated, and the residue was treated with 2:1 pyridine–acetic anhydride (5 mL) in the presence of 4-dimethylaminopyridine. H.p.l.c. of the product, as for **4**, gave 3-acetamido-1,2,4-tri-*O*-acetyl-3,6-dideoxy-β-D-mannopyranose (4.8 mg) and the α anomer (5 mg).

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