2,3-Diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam from the hydrolysate of *Pseudomonas aeruginosa* P14 lipopolysaccharide

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An unknown amino sugar, U-7, which had been detected in the hydrolysate of the polysaccharide fraction (F-A) of *Pseudomonas aeruginosa* P14 lipopolysaccharide, was isolated from the hydrolysate of whole cells of this micro-organism and converted into the *N*-acetyl derivative (U-7NAc). On the basis of i.r.-absorption spectrometry, ¹³C-n.m.r. and ¹H-n.m.r. spectroscopy and mass spectrometry, the structure of compound U-7NAc was identified as 2-acetamido-3-amino-2,3-dideoxyhexofuranurono-6,3-lactam. The configuration of compound U-7NAc was then unequivocally identified as 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam by comparing the synthetic and natural compounds. Compound U-7 and synthetic 2,3-diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam showed the same behaviour on chromatography. G.l.c.–mass-spectral analyses of fraction F-A and synthetic 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid after methanolyses and trimethylsilylations showed the presence of the same derivative. It was concluded that the amino sugar U-7 was produced from the 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid residue present in fraction F-A.

The endotoxic lipopolysaccharides of *Pseudomonas aeruginosa* cell walls contain unusual amino sugars together with D-glucosamine and D-galactosamine. Our group has previously reported the isolation of DL-fucosamine from the lipopolysaccharide of strain N10 (Suzuki, 1969*a,b*) and of D-quinovosamine from that of strain P14 (Suzuki *et al.*, 1970). Wilkinson & Welbourn (1975) reported the presence of 2-amino-2-deoxygalacturonic acid (later determined to be the L-isomer) in the lipopolysaccharide of strain N.C.T.C. 8505, and Wilkinson (1977) isolated D-bacillosamine from the same lipopolysaccharide. The identification of these compounds made progress in several serological, biological and structural investigations of *Ps. aeruginosa* lipopolysaccharides (e.g. Chester *et al.*, 1973; Wilkinson & Galbraith, 1975; Horton *et al.*, 1977). However, other substances in the lipopolysaccharides have still remained unidentified. We have previously found an unknown 2-amino sugar U-7 in the hydrolysate of the polysaccharide fraction (F-A) of strain P14 lipopolysaccharide (Suzuki, 1974; Suzuki *et al.*, 1977). The present paper reports that compound U-7 has the structure 2,3-diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam.

Our preliminary report stated that 2,3-diamino-

2,3-dideoxy-D-glucuronic acid was synthesized and then, by comparison of it with compound U-7, the amino sugar U-7 was identified as the same compound (Suzuki & Okuda, 1982). But further studies by n.m.r. spectrometry showed that the synthetic compound possessed a furanoid structure. The structure of compound U-7 has consequently been corrected to a lactam in the present paper.

Results and discussion

When acid hydrolysate of polysaccharide fraction (F-A) or dry cells of *Ps. aeruginosa* P14 was submitted to ion-exchange chromatography on Dowex 50 (H⁺ form), compound U-7 could be detected by the Boas modification of Elson–Morgan reaction, as shown in Fig. 1.

Since a substantial loss of the material occurred during the isolation and purification of fraction F-A, the isolation of compound U-7 was performed directly from whole-cell hydrolysate. After the purification procedures compound U-7 was isolated as an amorphous solid that gave a single spot on paper chromatography and electrophoresis with various solvent systems (Table 1). Compound U-7

Fig. 1. Chromatography on Dowex 50 (H^+ form) of hydrolysates of polysaccharide fraction (F-A) (a) and dry cells (b) of *Ps. aeruginosa* P14

Fractions were analysed for 2-amino sugars by the Boas modification of Elson–Morgan reaction. Details are presented in the text. Peaks are numbered as described previously (Suzuki *et al.*, 1970): peak 1, unidentified compound U-1; peak 2, unidentified compound U-2; peak 3, D-glucosamine; peak 4, D-galactosamine (plus muramic acid in b); peak 5, D-quinovosamine; peak 6, D-fucosamine; peak 7, compound U-7.

was a hygroscopic and labile compound, but on *N*-acetylation it gave a stable crystalline product (U-7NAc) having m.p. 223–228°C (decomp.) and $[\alpha]_D^{24} +13 \pm 1^\circ$ (*c* 0.18' in water). Compound U-7NAc also migrated as a single spot on paper chromatography and electrophoresis with various solvent systems (Table 1). It was detected as a white spot when a black spot obtained with alkaline $AgNO_3$ was treated with 5% $Na_2S_2O_3$, but did not react with ninhydrin. No colour was obtained in the Morgan–Elson reaction performed as described by Reissig *et al.* (1955).

With compound U-7NAc, a series of identification procedures was undertaken. All of the evidence so far obtained indicated that compound U-7NAc was 2-acetamido-3-amino-2,3-dideoxyhexofuranurono-6,3-lactam. The evidence may be outlined as follows.

(1) In the i.r.-absorption spectrum of compound U-7NAc, two strong bands at 1655 and 1545 cm^{-1} were readily attributed to C=O stretching and N–H bending of a secondary amide group respectively. A strong band at 1695 cm^{-1} was assigned to C=O stretching of a γ -lactam group on the basis of literature data (Bellamy, 1958; Hanessian, 1969).

(2) In the ^{13}C -n.m.r. spectrum of compound U-7NAc (Fig. 2), two sets of signals due to the α - and β -anomers were observed. The assignments were made on the basis of the off-resonance proton-decoupling experiment together with comparison with literature data on methyl D-hexofuranosides (Ritchie *et al.*, 1975), 2-acetamido-2-deoxyhexoses (Bundle *et al.*, 1973; Perkins *et al.*, 1977) and 2,3-diaminopentoses involved in aminoglycoside antibiotics (McAlpine *et al.*, 1977; Ellestad *et al.*, 1978).

(3) The ^1H -n.m.r. spectrum of compound U-7NAc in $^2\text{H}_2\text{O}$ (Fig. 3) indicated the presence of eight protons, three of which were compatible with the C-methyl protons of one acetamido group and

Table 1. Paper-chromatographic and paper-electrophoretic data for compounds U-7 and U-7NAc
For experimental details see the text. — indicates the migration from origin to anode. Abbreviation: n.d., not determined.

Sample	Paper-chromatographic R_F			Paper-electrophoretic m_{GlcN}	
	Solvent A	Solvent B	Solvent C	Buffer D	Buffer E
Compound U-7	0.04	0.34	0.31	1.05	1.10
Compound U-7NAc	0.17	0.56	0.54	0	0
Standard					
D-Glucosamine	0.08	0.42	0.45	1.00	1.00
N-Acetyl-D-glucosamine	0.23	0.59	0.65	0	0
D-Glucuronic acid	n.d.	n.d.	n.d.	−0.91	−0.56
2,3-Diacetamido-2,3-dideoxy-D-glucuronic acid (III)	0.10	0.45	0.50	−0.72	−0.49
2,3-Diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (IV)	0.03	0.34	0.31	1.05	1.10
2-Acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (V)	0.17	0.56	0.54	0	0

Fig. 2. Proton-decoupled ^{13}C -n.m.r. spectrum of compound U-7Nac

The measurement was performed in 0.4 ml of $^2\text{H}_2\text{O}$ with 13 mg of sample at 23°C : repetition time 1.0 s, pulse width $5\mu\text{s}$ (90°), data points 8000, sweep width 6024 Hz, and 38 300 scans. Major peaks occur at 176.84 and 176.66 (C-6 α and -6 β), 174.49 and 174.07 (α - and β - NHCOCH_3), 102.15 and 96.75 (C-1 α and -1 β), 77.97 and 75.56 (C-4 α and -4 β), 70.16 and 69.96 (C-5 α and -5 β), 60.88, 60.65, 60.00 and 58.77 (C-2 α and -2 β , C-3 α and -3 β) and 21.90 p.p.m. (α - and β - NHCOCH_3). The spectrum of 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (V) was identical with this spectrum.

the remaining five with the ring protons, H-1, H-2, H-3, H-4 and H-5.

(4) The field-desorption mass spectrum of compound U-7Nac gave a peak at m/z 217 ($M^+ + 1$), indicating the same molecular mass as that of the structure proposed.

(5) As shown in Fig. 4, the g.l.c.-mass spectrum of the *O*-trimethylsilyl derivative of compound U-7Nac exhibited a molecular-ion peak at m/z 360. The increase in the molecular mass caused by trimethylsilylation confirmed the presence of two hydroxy groups in compound U-7Nac.

(6) The peracetate of compound U-7Nac revealed a molecular-ion peak at m/z 384 in the mass spectrum and showed the presence of one *N*-acetyl, one *N*-diacetyl and two *O*-acetyl groups in the ^1H -n.m.r. spectrum. The signals for ring protons of the α -anomer of the peracetate showed downfield shifts of +0.84 (H-1), +0.55 (H-2), +1.24 (H-3), +0.62 (H-4) and +1.38 (H-5) p.p.m. relative to the chemical shifts of the corresponding protons of compound U-7Nac. These results indicated that compound U-7Nac possessed, not a pyranoid structure, but a furanoid structure, showing that the molecule contains two hydroxy groups attached to C-1 and C-5.

The bicyclic structure was indicative of a *threo*-

configuration at C-3-C-4, a *gluco*-, *manno*-, *gulo*- or *ido*-configuration. Among these four, the *gluco*-configuration seemed to be most probable, because each coupling constant in the ^1H -n.m.r. spectrum of compound U-7Nac (Fig. 3) was in fair agreement with the corresponding value in the spectral data of methyl β -D-glucofuranosidurono-6,3-lactone reported by Weidmann & Dax (1971). Therefore the chemical synthesis of 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (V) was performed from 2,3-diacetamido-2,3-dideoxy- α -D-glucuronic acid (III) via 2,3-diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam hydrochloride (IV) (Scheme 1), all of which were new sugars.

The identity of compound U-7Nac and compound (V) was established by comparisons of their i.r.-absorption spectra, ^{13}C - and ^1H -n.m.r. spectra, paper-chromatographic and electrophoretic behaviours, and specific rotations. Compound U-7 and compound (IV) were found to have the same behaviour on comparison of both compounds on chromatography [paper chromatography, t.l.c. and column chromatography on Dowex 50 (H^+ form)] and paper electrophoresis and the use of colour reactions with ninhydrin and with Elson-Morgan reagent.

The fact that acid hydrolysis of compound (III)

Scheme 1. Synthetic pathway of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid, 2,3-diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam and 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam
Reagents: (i) O_2 (Pt/C); (ii) H_2 (Pd/C); (iii) H^+ ; (iv) acetic anhydride/triethylamine. $R = CH_2C_6H_5$.

Fig. 3. 270 MHz ^1H -n.m.r. spectrum of compound U-7NAc (a) and its partial expansion (b)
The spectrum was recorded in 0.4 ml of ^2H_2O with 9 mg of sample at 25°C: δ_H (p.p.m.) (A as standard) 5.59 (1H, d, $J_{1,2}$ 4.8 Hz, H-1 α), 5.39 (1H, d, $J_{1,2}$ <0.5 Hz, H-1 β), 4.89 (1H, dd, $J_{4,5}$ 6.2 Hz, H-4 β), 4.82 (1H, dd, $J_{4,5}$ 6.2 Hz, H-4 α), 4.39 (2H, d, H-5 α and -5 β), 4.18 (1H, dd, $J_{2,3}$ 2.1 Hz, H-2 α), 4.13 (1H, dd, $J_{2,3}$ <0.5 Hz, H-2 β), 4.10 (1H, dd, $J_{3,4}$ 4.8 Hz, H-3 β), 4.02 (1H, dd, $J_{3,4}$ 4.8 Hz, H-3 α), 1.98 (3H, s, α -NHCOCH₃) and 1.94 (3H, s, β -NHCOCH₃). The signals were assigned on the basis of double-resonance experiments. Assignments of anomeric protons were tentatively made in accordance with the Lemieux & Lineback (1963) and Hruska *et al.* (1970) procedures.

Fig. 4. G.I.c.-mass spectrum of a trimethylsilyl derivative of compound U-7Nac by electron-impact technique. Compound U-7Nac (1 mg) was trimethylsilylated with 0.1 ml of pyridine/hexamethyldisilazane/trimethylchlorosilane (6:3:1, by vol) at 60°C for 20 min. The spectrum was scanned from 2 μ l of the reaction mixture introduced via a glass column (3 m $\times$ 2 mm, A) at 200°C: m/z 360 (M^+), 345 ($M^+ - \text{CH}_3$), 342 ($M^+ - \text{H}_2\text{O}$), 301 ($M^+ - \text{CH}_3\text{CONH}_2$), 286 ($M^+ - \text{CH}_3 - \text{CH}_3\text{CONH}_2$), 273, 270 ($M^+ - \text{Me}_3\text{SiOH}$), 255 ($M^+ - \text{CH}_3 - \text{Me}_3\text{SiOH}$), 196 ($M^+ - \text{CH}_3 - \text{CH}_3\text{CONH}_2 - \text{Me}_3\text{SiOH}$), 73 (base peak, Me_3Si).

yielded compound (IV) led us to believe that compound U-7 might originate from a 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid residue in fraction F-A. G.I.c.-mass-spectrometric studies of fraction F-A and compound (III) after methanolysis and trimethylsilylation proved the presence of a diacetamido uronic acid residue in fraction F-A.

During the preparation of the present manuscript, the tentative identification of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid from strain 170014 (Lanyi serotype 0:6) (Dmitriev *et al.*, 1982) was reported. Their proof was made by different methods from ours.

Hydrolysates of strains of the Habs-serotype classification from type 1 to type 13 were analysed for the presence of compound U-7. Compound U-7 was found in those from type 1 (16%) and type 12 (3%).

Experimental

Organisms

Pseudomonas aeruginosa P14 and a series of strains of the Habs-serotype classification, 5933 (type 1), 4821 (type 2), 13264 (type 3), 5936 (type 4), A486 (type 5), 5939 (type 6), A443 (type 7), 5940 (type 8), T6370 (type 9, Verder & Evans),

U3433 (type 10), 121 (type 11), 5945 (type 12) and 6092 (type 13, Veron) in our previous report (Suzuki *et al.*, 1977), were used.

General methods

Melting points were determined with a Yanaco apparatus and uncorrected. Optical specific rotations were determined with a JASCO model DIP-4 automatic polarimeter. Elementary analyses were performed by the Analytical Section, Meijo University, Nagoya, Japan. I.r.-absorption spectra were determined for KBr discs on a JASCO model A-102 spectrophotometer.

N.m.r. spectroscopy

^1H -n.m.r. spectra were recorded on a JEOL model FX100 (at 100 MHz) or Bruker WH-270 (at 270 MHz) spectrometer. Chemical shifts determined for $^2\text{H}_2\text{O}$ solution were quoted relative to an internal standard of methanol (A) or 2-methylpropan-2-ol (B), which produced its resonance signal at 3.33 or 1.23 p.p.m. downfield from the 3-(trimethylsilyl)propane-1-sulphonate reference respectively, and for ^{12}H pyridine solution, to internal tetramethylsilane. ^{13}C -n.m.r. spectra at 22.5 MHz were recorded on a JEOL FX100 instrument in $^2\text{H}_2\text{O}$ solutions and reported as chemical shifts relative to an internal

standard of methanol (49.023 p.p.m. relative to external tetramethylsilane).

Mass spectrometry

G.I.c.—mass spectra were obtained on Shimadzu Auto 6020 mass spectrometer, equipped with a glass column [Chromosorb W (HP-AW-PMCS, 80–100 mesh) containing 3% OV-101 (A), 3% OV-17 (B) or 0.3% OV-1 (C)]. The injector temperature was 260°C and the carrier gas was He at flow rate of 30 ml/min. The temperatures of the ion source and molecular separator were 270°C. For the electron-impact technique (e.i.), the ionizing voltage was 70 eV and the trap current was 60 μ A. For the chemical-ionization technique (c.i.), the box current was 200 μ A and the ionizing voltage was 150 eV.

Field-desorption mass spectra were obtained on a JEOL model JMS-DX300 mass spectrometer coupled to a JEOL model JMA 3500 computer system by using a carbon emitter. The acceleration voltage was 3 kV and the cathode voltage was –5.5 kV. The emitter current was raised from 5 to 30 mA at a rate of 1 mA/min. The spectra were calibrated with the electron-impact spectrum of Fomblin L oil.

Paper chromatography and electrophoresis

Paper chromatography (Toyo no. 51A paper, descending) was performed in solvent *A* (butan-1-ol/ethanol/water, 4:1:1, by vol.), solvent *B* (ethyl acetate/pyridine/acetic acid/water 5:5:1:3, by vol.) and solvent *C* (propan-2-ol/pyridine/acetic acid/water, 8:8:4:1, by vol.). Paper electrophoresis (Toyo no. 51A paper, 25 V/cm, 60 min) was performed in apparatus constructed as described by Markham & Smith (1952) in buffer *D* (pyridine/acetic acid/water, 5:2:43, by vol., pH 5.2) and buffer *E* (pyridine/formic acid/acetic acid/water, 2:3:20:180, by vol., pH 2.8). Compounds were detected with alkaline AgNO₃ (Trevelyan *et al.*, 1950) or ninhydrin.

Detection of compound U-7 from fraction F-A and dry cells

Fraction F-A was isolated by mild acid hydrolysis (0.1 M-HCl, 100°C, 30 min) from *Ps. aeruginosa* P14 lipopolysaccharide and purified by fractionations on Sephadex G-50 and G-200 as described previously (Suzuki, 1974). Fraction F-A (1.7 mg) was hydrolysed with 0.45 ml of 4 M-HCl at 100°C for 4 h. The hydrolysate was applied to a column (43 cm $\times$ 1.3 cm) of Dowex 50-HW-X8 (H⁺ form) equilibrated with 0.33 M-HCl. Elution was carried out with 0.33 M-HCl. Fractions of volume 11.4 ml were collected. A portion (3 ml) of each fraction was analysed for 2-amino sugar by the Boas (1953) modification of the Elson–Morgan reaction. The elution value of compound U-7 was 2.5 relative to glucosamine, and the reaction product of compound

U-7 gave an orange colour with an absorption curve having a maximum at 522 nm. On t.l.c. of the hydrolysate of fraction F-A on silica gel 60 in butan-1-ol/pyridine/water/acetic acid (60:40:30:3, by vol.), galactosamine, quinovosamine, fucosamine and compound U-7 were detected as spots having R_{GAIN} values of 1.0, 2.0, 1.6 and 0.6 respectively. Compound U-7 gave a blue colour when treated with ninhydrin, and it reduced alkaline AgNO₃/NH₃ (Partridge, 1948), yielding a dark spot.

Dry cells (98 mg) of strain P14 were hydrolysed with 1 ml of 4 M-HCl at 100°C for 4 h. The evaporated residue was dissolved in 0.3 ml of water and chromatographed as described above.

Detection of compound U-7 in hydrolysates of a series of strains of the Habs-serotype classification

The method for the detection of compound U-7 was the same as in the analysis of 2-amino sugar composition described previously (Suzuki *et al.*, 1977), except that 70 chromatographic fractions were collected and that total 2-amino sugar content detected in these 70 fractions was regarded as 100%.

Isolation of compound U-7

Bacteria (strain P14) were inoculated on plates containing 20 ml of nutrient agar in the individual plate and grown for 18 h at 37°C. The culture from 700 plates yielded 340 g of wet cells. The cells, suspended in a half-volume of 6 M-HCl, were heated at 100°C for 4 h. The hydrolysate was filtered through a sintered-glass filter (G2), neutralized with Amberlite IR-45 (OH[–] form) and evaporated *in vacuo* to give a syrup at 55°C. The syrup was chromatographed on a column (6.5 cm $\times$ 53 cm) of Dowex 50 (H⁺ form) with 0.33 M-HCl as described above. Fractions that contained compound U-7 (fractions 20–27) were pooled, neutralized with Amberlite IR-45 (OH[–] form) and evaporated *in vacuo*. The residue was dissolved in water (30 ml) and subjected to paper chromatography on 12 sheets of paper (40 cm $\times$ 40 cm) (solvent *A* for 16 h). Compound U-7 was eluted from the papers with four 100 ml portions of water. The evaporated residue was purified by using a column (0.9 cm $\times$ 40 cm) of Dowex 50 (K⁺ form) equilibrated with 0.1 M-potassium acetate buffer, pH 5.6, as described by Wheat *et al.* (1964). After neutralization with Amberlite IR-45 (OH[–] form), the concentrated eluate (6 ml) was desalted by using a column (1 cm $\times$ 100 cm) of Sephadex G-10. The filtrate was freeze-dried. The residue was subjected to rechromatography on a column (1.2 cm $\times$ 46 cm) of Dowex 50 (H⁺ form) as described above. The resulting residue was triturated with ethanol, evaporated *in vacuo* and dried. Compound U-7 was obtained as a nearly colourless solid (60 mg).

N-Acetylation of compound U-7

Compound U-7 (33 mg) was *N*-acetylated with acetic anhydride in the presence of triethylamine as described by Kuhn *et al.* (1959). A crystalline product (U-7Nac) was obtained (9 mg, m.p. 283–288°C (decomp.), $[\alpha]_D^{25} +13 \pm 1^\circ$ (c 0.18 in water), field-desorption mass spectrum m/z 217 ($M^+ + 1$), δ_H (p.p.m.) (^2H)pyridine, 100 MHz) 6.01 (1H, d, $J_{1,2}$ 5 Hz, *H*-1 α), 5.15 (1H, dd, $J_{4,5}$ 6 Hz, *H*-4 α), 4.70 (1H, d, *H*-5 α), 4.67 (1H, dd, $J_{2,3}$ 2 Hz, *H*-2 α), 4.19 (1H, dd, $J_{3,4}$ 5 Hz, *H*-3 α), 2.12 (3H, s, α -NHCOCH₃), 6.02 (1H, d, $J_{1,2}$ <0.5 Hz, *H*-1 β), 5.23 (1H, dd, $J_{4,5}$ 6 Hz, *H*-4 β), 4.93 (1H, dd, $J_{2,3}$ <0.5 Hz, *H*-2 β), 4.70 (1H, d, *H*-5 β), 4.38 (1H, dd, $J_{3,4}$ 5 Hz, *H*-3 β), 2.08 (3H, s, β -NHCOCH₃).

Peracetylation of compound U-7Nac

Acetic anhydride (0.6 ml) was added to 3 mg of compound U-7Nac. The mixture was heated at 100°C for 2 h by the method of Stellner *et al.* (1973). The reagent was removed by evaporation under a stream of N₂. The purification was carried out by using the same procedure for the permethylated product as described by Iwamori & Nagai (1981): yield 2 mg, g.l.c.–mass spectrum (column, 50 cm $\times$ 2 mm, C) (100–230°C, 8°C/min): c.i. (NH₃ gas) m/z 402 ($M^+ + NH_4$); e.i. m/z (rel. intensity) 384 (1) (M^+), 342 (16) ($M^+ - CH_2CO$), 324 (6) ($M^+ - CH_3CO_2H$), 300 (73) ($M^+ - 2 \times CH_2CO$), 299 (62) ($M^+ - CH_2CO - CH_3CO$), 282 (100) ($M^+ - CH_2CO - CH_3CO_2H$), 257 (44) ($M^+ - CH_3CO - 2 \times CH_2CO$), 240 (64), 223 (33), 198 (24), 181 (44), 169 (89). ^1H -n.m.r. spectrum: δ_H (p.p.m.) (^2H)pyridine, 100 MHz) 6.85 (1H, d, $J_{1,2}$ 6.5 Hz, *H*-1 α), 6.08 (1H, d, *H*-5 α), 5.77 (1H, dd, $J_{4,5}$ 5.0 Hz, *H*-4 α), 5.43 (1H, dd, $J_{3,4}$ 4.5 Hz, *H*-3 α), 5.22 (1H, dd, $J_{2,3}$ 1.0 Hz, *H*-2 α), 2.54 (6H, s, $2 \times \alpha$ -COCH₃), 2.41 (3H, s, α -COCH₃), 2.11 (3H, s, α -COCH₃), 1.95 (3H, s, α -COCH₃), 6.69 (0.3H, d, $J_{1,2}$ 4.0 Hz, *H*-1 β). The assignments were confirmed by double-resonance experiments, and those of signals in the anomeric region were tentatively made in accordance with the Bundle & Lemieux (1976) and Stevens & Fletcher (1968) procedures.

(Benzyl 2,3-diacetamido-2,3-dideoxy- α -D-glucopyranoside)uronic acid (II)

A suspension of benzyl 2,3-diacetamido-2,3-dideoxy- α -D-glucopyranoside (I) (0.7 g) prepared by the method of Meyer zu Reckendorf (1972), NaHCO₃ (0.3 g) and 5% platinum on carbon (2.2 g) in water (80 ml) were stirred with bubbling with O₂ at 90°C as described by Yoshimura *et al.* (1969). The product was recrystallized from water; yield 0.43 g, m.p. 234–235°C (decomp.), $[\alpha]_D^{25} +96 \pm 1^\circ$ (c 0.9 in water), (Found: C, 54.4; H, 6.2; N, 7.6;

loss at 110°C, 2.4. Calc. for C₁₇H₂₂N₂O₇, $\frac{1}{2}H_2O$: C, 54.4; H, 6.3; N, 7.5; H₂O, 2.4%), ν_{max} 3300 (NH and OH), 2940 (CH), 1725 (CO₂H), 1645 and 1550 (NHAc), and 740 cm⁻¹ (phenyl), field-desorption mass spectrum m/z 367 ($M^+ + 1$).

2,3-Diacetamido-2,3-dideoxy- α -D-glucopyranuronic acid (III)

Compound (II) (500 mg) in water (60 ml) was hydrogenated with 5% palladium on carbon (500 mg) for 15 h. The product was recrystallized from aq. 90% (v/v) ethanol/diethyl ether; yield 268 mg, m.p. 199–201°C (decomp.), $[\alpha]_D^{25} -6^\circ \rightarrow -12 \pm 1^\circ$ (c 1.1 in water), (Found: C, 43.3; H, 6.1; N, 10.0. Calc. for C₁₀H₁₆N₂O₇: C, 43.5; H, 6.1; N, 10.1%), ν_{max} 3410 and 3320 (NH and OH), 2950 (CH), 1740 (COOH), and 1650 and 1555 cm⁻¹ (NHAc), field-desorption mass spectrum m/z 277 ($M^+ + 1$), δ_H (p.p.m.) (^2H_2O , 100 MHz, B as standard) 5.22 (1H, d, $J_{1,2}$ 3.0 Hz, *H*-1 α), 4.38 (1H, d, $J_{4,5}$ 9.8 Hz, *H*-5 α), 4.23–3.63 (3H, superimposed, *H*-2 α , *H*-3 α , *H*-4 α), 2.02 (6H, s, $2 \times \alpha$ -NHCOCH₃).

2,3-Diamino-2,3-dideoxy-D-glucofuranurono-6,3-lactam hydrochloride (IV)

Compound (III) (25 mg) was hydrolysed (4 ml of 3M-HCl, 100°C, 2 h), and the hydrolysate was subjected to chromatography on a column (1 cm $\times$ 10 cm) of Dowex 50 (H⁺ form) as described under 'Isolation of compound U-7'. The product was obtained as a colourless brittle film: yield 15 mg, $[\alpha]_D^{25} +15 \pm 1^\circ$ (c 0.69 in water), ν_{max} 3370 (OH and NH), 1695 (γ -lactam) and 1620 cm⁻¹ (NH₃⁺), δ_C (p.p.m.) 176.92 and 176.29 (C-6 α and -6 β), 100.21 and 95.77 (C-1 α and -1 β), 77.49 and 75.86 (C-4 α and -4 β), 69.38 (C-5 α and -5 β), 60.21, 58.34 and 57.99 (C-2 α and -2 β , C-3 α and -3 β), δ_H (p.p.m.) (^2H_2O , 100 Hz, B as standard) 5.80 (1H, d, $J_{1,2}$ 4.9 Hz, *H*-1 α), 5.69 (0.3H, d, $J_{1,2}$ <0.5 Hz, *H*-1 β), 4.99 (1H, dd, $J_{4,5}$ 5.6 Hz, *H*-4 α), 4.52 (1.3H, d, *H*-5 α and -5 β), 4.36 (1H, dd, $J_{2,3}$ 2.0 Hz, *H*-2 α), 3.88 (1H, dd, $J_{3,4}$ 4.6 Hz, *H*-3 α). The ring structure was confirmed by the small coupling constant between H-4 and H-5 (not diaxial).

2-Acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,6-lactam (V)

Compound (III) (200 mg) was hydrolysed as described above. The evaporated residue was *N*-acetylated by the method of Kuhn *et al.* (1959). The product was recrystallized from methanol: yield 65 mg, m.p. 228–229°C, $[\alpha]_D^{25} +13 \pm 1^\circ$ (c 0.73 in water), (Found: C, 44.5; H, 5.9; N, 12.9. Calc. for C₈H₁₂N₂O₅: C, 44.4; H, 5.6; N, 13.0%).

Identification of compound (III) as a component of fraction F-A

Compound (III) (1 mg) and fraction F-A (1 mg) were separately treated with 0.05 M-HCl in methanol

(1 ml, 80°C, 10h), trimethylsilylated with 0.1 ml of a mixture of hexamethyldisilazane, trimethylchlorosilane and pyridine (2:1:10, by vol.) at 60°C for 20 min and analysed by g.l.c.-mass spectrometry (column 1.7 m $\times$ 2 mm, B) (140–250°C at a rate of 8°C/min after holding for 4 min).

The product from compound (III) gave a following mass spectrum: m/z (rel. intensity) 376 (1) (M^+), 361 (9) ($M^+ - \text{CH}_3$), 344 (7) ($M^+ - \text{CH}_3\text{OH}$), 329 (4) ($M^+ - \text{CH}_3 - \text{CH}_3\text{OH}$), 317 (11) ($M^+ - \text{CO}_2\text{CH}_3$, $M^+ - \text{CH}_3\text{CONH}_2$), 313 (7) ($M^+ - \text{CH}_3\text{OH} - \text{CH}_3\text{O}$), 302 (3) ($M^+ - \text{CH}_3 - \text{CO}_2\text{CH}_3$, $M^+ - \text{CH}_3 - \text{CH}_3\text{CONH}_2$), 287 (13) ($M^+ - \text{Me}_3\text{SiO}$), 270 (8) ($302 - \text{CH}_3\text{OH}$), 257 (55), 226 (15), 215 (53), 196 (15), 183 (27), 168 (10), 155 (92), 146 (17), 144 (18), 141 (17), 131 (11), 125 (12), 113 (66), 102 (11), 89 (23), 83 (11), 73 (100) (base peak, Me_3Si), 71 (11), 59 (17) (CO_2CH_3 , CH_3CONH_2), 45 (13), 43 (66) (CH_3CO), 28 (13). The analysis proved that the product obtained with compound (III) corresponded to the 4-*O*-trimethylsilyl ether of methyl (methyl 2,3-diacetamido-2,3-dideoxy-D-glucopyranosid)uronate. In the spectrum of the products from fraction F-A, a peak at the same retention time possessed ions that identify the compound with the product obtained with (III).

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References

- Bellamy, L. J. (1958) *The Infra-red Spectra of Complex Molecules*, 2nd edn., pp. 213–215. Methuen, London
- Boas, N. F. (1953) *J. Biol. Chem.* **204**, 553–563
- Bundle, D. R. & Lemieux, R. U. (1976) *Methods Carbohydr. Chem.* **7**, 79–86
- Bundle, D. R., Jennings, H. J. & Smith, I. C. P. (1973) *Can. J. Chem.* **51**, 3812–3819
- Chester, I. R., Meadow, P. M. & Pitt, T. L. (1973) *J. Gen. Microbiol.* **78**, 305–318
- Dmitriev, B. A., Kocharova, N. A., Knirel, Y. A., Shashkov, A. S., Kochetkov, N. K., Stanislavsky, E. S. & Mashilova, G. M. (1982) *Eur. J. Biochem.* **125**, 229–237
- Ellestad, G. A., Cosulich, D. B., Broschard, R. W., Martin, J. H., Kunstmann, M. P., Morton, G. O., Lanchester, J. E., Fulmor, W. & Lovell, F. M. (1978) *J. Am. Chem. Soc.* **100**, 2515–2524
- Hanessian, S. (1969) *J. Org. Chem.* **34**, 675–681
- Horton, D., Rodemeyer, G. & Haskell, T. H. (1977) *Carbohydr. Res.* **55**, 35–47
- Hruska, F. E., Grey, A. A. & Smith, I. C. P. (1970) *J. Am. Chem. Soc.* **92**, 4088–4094
- Iwamori, M. & Nagai, Y. (1981) *J. Biochem. (Tokyo)* **89**, 1253–1264
- Kuhn, R., Bister, W. & Dafeldecker, W. (1959) *Justus Liebigs Ann. Chem.* **628**, 186–192
- Lemieux, R. U. & Lineback, D. R. (1963) *Annu. Rev. Biochem.* **32**, 155–184
- Markham, R. & Smith, J. D. (1952) *Biochem. J.* **52**, 552–557
- McAlpine, J. B., Sinclair, A. C., Egan, R. S., DeVault, R. L., Stanaszek, R. S., Cirovic, M., Mueller, S. L., Goodley, P. C., Mauritz, R. J., Wideburg, N. E., Mitscher, L. A., Shirahata, K., Matsushima, H., Sato, S. & Iida, T. (1977) *J. Antibiot.* **30**, 39–49
- Meyer zu Reckendorf, W. (1972) *Methods Carbohydr. Chem.* **6**, 266–269
- Partridge, S. M. (1948) *Biochem. J.* **42**, 238–249
- Perkins, S. J., Johnson, L. N., Phillips, D. C. & Dwek, R. A. (1977) *Carbohydr. Res.* **59**, 19–34
- Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959–966
- Ritchie, R. G. S., Cyr, N., Korsch, B., Koch, H. J. & Perlin, A. S. (1975) *Can. J. Chem.* **53**, 1424–1433
- Stellner, K., Saito, H. & Hakomori, S. (1973) *Arch. Biochem. Biophys.* **155**, 464–472
- Stevens, J. D. & Fletcher, H. G., Jr. (1968) *J. Org. Chem.* **33**, 1799–1805
- Suzuki, N. (1969a) *Biochim. Biophys. Acta* **177**, 371–373
- Suzuki, N. (1969b) *J. Jpn. Biochem. Soc.* **41**, 769–776
- Suzuki, N. (1974) *FEBS Lett.* **48**, 301–305
- Suzuki, N. & Okuda, S. (1982) *Jpn. J. Exp. Med.* **52**, 107–110
- Suzuki, N., Suzuki, A. & Fukasawa, K. (1970) *J. Jpn. Biochem. Soc.* **42**, 130–134
- Suzuki, N., Tsunematsu, Y., Matsumoto, H. & Tazaki, T. (1977) *Infect. Immun.* **15**, 692–697
- Trevelyan, W. E., Proctor, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Weidmann, H. & Dax, K. (1971) *Montash. Chem.* **102**, 877–884
- Wheat, R., Rollins, E. L. & Leatherwood, J. (1964) *Nature (London)* **202**, 492–493
- Wilkinson, S. G. (1977) *Biochem. J.* **161**, 103–109
- Wilkinson, S. G. & Galbraith, L. (1975) *Eur. J. Biochem.* **52**, 331–343
- Wilkinson, S. G. & Welbourn, A. P. (1975) *Biochem. J.* **149**, 783–784
- Yoshimura, J., Sato, T. & Ando, H. (1969) *Bull. Chem. Soc. Jpn.* **42**, 2352–2356