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Isolation and identification of uridine(5′)-diphospho(1)-2,3-diacetamido-2,3-dideoxy-*α*-D-glucopyranuronic acid from *Pseudomonas aeruginosa* P1–III

Satoshi OKUDA,*‡ Shizuaki MURATA* and Nachiko SUZUKI†
*Chemical Laboratory, College of General Education, Nagoya University, Chikusa-ku, Nagoya 464, Japan, and †Institute of Medical Science, University of Tokyo, Shirokenedai, Minato-ku, Tokyo 108, Japan

A new uridine nucleotide was isolated from *Pseudomonas aeruginosa* P1–III (Habs serotype 5). On the basis of ^{13}C-n.m.r. and p.m.r. spectroscopy, mass spectrometry, i.r.-absorption spectroscopy and circular dichrometry, the structure of the new compound was unequivocally identified as uridine(5′)-diphospho(1)-2,3-diacetamido-2,3-dideoxy-α-D-glucopyranuronic acid.

INTRODUCTION

The mechanism of the biosynthesis of O-specific polysaccharide chains of the Enterobacteriaceae lipopolysaccharides, especially *Salmonella*, is now well established [1,2]. In the biosynthesis of *Salmonella* O-specific polysaccharide chains, sugar nucleotides containing component sugars of the O-specific chains act as glycosyl donors for the formation of the lipid-linked intermediates [1,2].

There has been little or no investigation on the mechanism of the biosynthesis of O-specific polysaccharide chains of *Pseudomonas aeruginosa* lipopolysaccharides, although the chemical structures of the O-specific polysaccharides have been elucidated progressively. The fact that the *Ps. aeruginosa* lipopolysaccharides, as well as Enterobacteriaceae lipopolysaccharides, consist of three regions, O-specific polysaccharide chains, a core and lipid A, suggests that the O-specific polysaccharide chains of *Ps. aeruginosa* lipopolysaccharides are synthesized in the manner similar to that proposed for *Salmonella* [1,2].

A new type of aminouronic acids, 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid [3,4], 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid [5] and 2,3-diacetamido-2,3-dideoxy-L-guluronic acid [6], has been found to be a constituent of the O-specific polysaccharides of some *Ps. aeruginosa* lipopolysaccharides. It seems reasonable to assume that the 2,3-diacetamido-2,3-dideoxyhexuronic acid residues in *Ps. aeruginosa* O-specific polysaccharides are derived from nucleotide-linked 2,3-diacetamido-2,3-dideoxyhexuronic acids. We attempted to find the sugar nucleotides containing the diaminouronic acids structurally related to the O-specific polysaccharide of the *Ps. aeruginosa* lipopolysaccharide from strain P1-III (Habs serotype 5 [7]) and were able to obtain a new uridine nucleotide sugar (designated as UDP-X). The present paper reports the isolation of UDP-X and its identification as uridine(5′)-diphospho(1)-2,3-diacetamido-2,3-dideoxy-α-D-glucopyranuronic acid (see Fig. 6).

EXPERIMENTAL

Organism and growth conditions

Pseudomonas aeruginosa P1–III (IID 1030) was used. The bacteria were inoculated in 390 bottles containing 150 ml of a medium [20 g of sodium glutamate, 5.6 g of Na_2HPO_4, 5 g of glucose, 0.25 g of KH_2PO_4, 0.1 g of $MgSO_4,7H_2O$ and 10 mg of $Ca(NO_3)_2$ per litre, adjusted to pH 7.6] and were grown under shaking for 18 h at 37 °C. The cells harvested by centrifugation at 7000 ***g*** for 20 min at 4 °C yielded 600 g of wet mass.

Materials

The following commercial materials were used: *Escherichia coli* orthophosphoric monoester phosphohydrolase (EC 3.1.3.1) from Worthington Corp.; snake-venom 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5), snake-venom dinucleotide nucleotidohydrolase (EC 3.6.1.9), UDP-*N*-acetylglucosamine and UDP-glucuronic acid from Sigma Chemical Co. Authentic compound, 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid was synthesized by the method described previously [3].

N.m.r. spectroscopy

P.m.r. spectra (at 400 MHz) and ^{13}C-n.m.r. spectra (at 100 MHz) were recorded on JEOL model FX-400FT spectrometer. P.m.r. spectra at 90 MHz were recorded on a JEOL model FX-90Q spectrometer. Chemical shifts were measured as δ in p.p.m. relative to the methyl signal of internal acetonitrile (2.03 for ^{1}H and 0.89 p.p.m. for ^{13}C).

M.s.

Fast-atom-bombardment m.s. was performed on a JEOL model DX-300 spectrometer equipped with a JMA-3500 computer system, utilizing a neutral beam of xenon atoms with an energy of 6 keV. A few drops of glycerol or triethanolamine as a matrix reagent were mixed with the aqueous solution of sample on a stainless-steel target. Peak masses were assigned by

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‡ To whom correspondence should be addressed.

comparison with the mass marker, which was calibrated by using the fast-atom-bombardment spectrum of perfluoroalkylphosphazine (Ultra Mark 1621, PCR).

Paper chromatography and electrophoresis

Paper chromatography (Toyo no. 50 paper, descending) was performed in solvent A [95% (v/v) ethanol/1 M-ammonium acetate, pH 7.2 (5:2, v/v)], solvent B [2-methylpropanoic acid/0.5 M-ammonia (5:3, v/v)] or solvent C [butan-1-ol/ethanol/water (53:32:16, by vol.)]. Paper electrophoresis (Toyo no. 51A paper, 30 V/cm, 75 min) was performed in apparatus constructed as described by Markham & Smith [8] in buffer D (0.05 M-ammonium acetate/0.03 M-acetic acid, pH 4.8).

Nucleotides were examined on paper chromatograms or electrophoretograms under an ultraviolet lamp, and phosphoric esters were detected by the $HClO_4$/molybdate reagent [9].

Miscellaneous methods

Optical rotations were determined with a JASCO model DIP-4 automatic polarimeter. C.d. spectra were recorded on a JASCO model J-500E spectropolarimeter. I.r.-absorption spectra were determined for KBr discs on a JASCO model A-102 spectrophotometer.

The procedures for measurements of phosphate [10], ribose in nucleotide [11] and reducing capacity [12] were modified to a micro scale so that the amounts of sample were in the range 0.02–0.1 μmol.

Isolation of UDP-X

The wet cells (600 g) suspended in 0.02 M-Tris/HCl buffer, pH 8.0, containing 0.02 M-$MgCl_2$ at a concentration of 0.5 g/ml were broken with a French press under 400 kg/cm^2 at 4 °C and then centrifuged at 9000 *g* for 30 min at 4 °C. The supernatant was freeze-dried, suspended in 1.3 litres of aq. 70% (v/v) ethanol and boiled for 20 min. The ethanolic extract was concentrated to 300 ml *in vacuo*. The nucleotide material was recovered from the extract with charcoal as described by Strominger [13]. The resulting eluate from the charcoal was concentrated to about 0.15 volume *in vacuo* and applied to a column (2.8 cm × 58 cm) of Dowex-1 (Cl^- form). Elution was carried out with a linear gradient of 0–0.3 M-NaCl in 7.4 litres of 0.01 M-HCl. Fractions (20 ml) were collected and analysed for absorbance at 260 nm. UDP-X was eluted in the second-peak fractions, which appeared at 2.7–5.2 litres (0.11–0.21 M-NaCl), and was recovered by the charcoal method [13]. The purification of UDP-X was achieved, in succession, by paper chromatography with solvent A, solvent B and solvent C, paper electrophoresis in buffer D, and paper chromatography with solvent C, essentially as described in a previous paper [14] (for R_{UMP} and m_{UMP} values of UDP-X see Table 1). The aqueous solution of purified UDP-X was passed through a column (0.3 cm × 3.5 cm) of Dowex-50 (H^+ form) at 5 °C. The effluent was adjusted to pH 8.5 with ammonia and evaporated to dryness *in vacuo*: yield 11.3 mg (15.9 μmol as uridine); $[\alpha]^{25}_{D}$ +33.2° (*c* 0.89 in water).

Hydrolysis of UDP-X with dinucleotide nucleotidohydrolase

UDP-X (12.4 μmol) was dissolved in 1.4 ml of a solution containing 0.07 M-Tris/HCl, pH 8.9, 0.8 mM-$MgCl_2$ and 10 units of dinucleotide nucleotidohydrolase. After 3 h incubation at 37 °C, the reaction was terminated by heating at 100 °C for 1 min. The precipitate was removed by centrifugation and discarded. The supernatant was subjected to paper electrophoresis in buffer D. Two phosphate-containing products, nucleoside monophosphate and sugar phosphate fragment (designated as X-1-*P*), were separated from each other on the paper electrophoretograms (for m_{UMP} values see Table 1).

The nucleoside monophosphate was eluted from the papers, converted into the sodium salt and crystallized from aqueous acetone: yield 4.0 mg (9.5 μmol as uridine); $[m]^{25}_{D}$ −43.0° (0.95 mmol in water).

Compound X-1-*P* was eluted from the papers with water and subjected to paper chromatography with solvent C (for desalting), in which the sugar phosphate has little mobility. Compound X-1-*P* was eluted with water and freeze-dried: yield 10.1 μmol, as estimated from the phosphate content; $[m]^{25}_{D}$ +208° (0.95 mmol in water).

Hydrolysis of compound X-1-*P* with orthophosphoric monoester phosphohydrolase

Compound X-1-*P* (9.5 μmol) was dissolved in 0.83 ml of a solution containing 0.05 M-Tris/HCl, pH 8.0, 1 mM-$MgCl_2$ and 2.6 units of orthophosphoric monoester phosphohydrolase. After 5 h incubation at 37 °C, the reaction was terminated by heating at 100 °C for 1 min. The precipitate was removed by centrifugation and discarded. Quantitative yield of inorganic phosphate was detected in the supernatant. After dilution with 0.4 ml of water, the supernatant was applied to a column (0.6 cm × 5 cm) of Dowex-1 (acetate form). Elution was carried out with 3 M-acetic acid. Fractions (3 ml) were collected and assayed for reducing sugar. The fractions containing the reducing sugar (tubes 1–3) were combined and evaporated to dryness *in vacuo*. Contaminating acetic acid was removed by co-evaporation with water and then toluene. The residue was dried over P_2O_5 and KOH: yield 2.7 mg.

RESULTS

UDP-X was extracted from *P. aeruginosa* P1–III protoplasm and isolated as an amorphous ammonium salt that gave a single spot on paper chromatography and electrophoresis with various solvent systems (Table 1). The u.v.-absorption spectrum of the nucleotide was identical with that of a uridine derivative. Chemical analysis of the material indicated that uridine, total phosphate, acid-labile phosphate and pentose (as D-ribose) were in the molar proportions 1.00:1.98:0.94:1.08.

The reducing value of UDP-X after hydrolysis with 0.01 M-HCl for 10 min at 100 °C was only 0.62 that of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid, indicating that UDP-X was much more stable than common sugar nucleotides. After hydrolysis for 45 min, it reached a maximum value, 0.92. Similar stability of UDP-*N*-acetylglucosaminuronic acid to hydrolysis with 0.01 M-HCl has been reported [15].

With UDP-X, a series of further identification procedures was undertaken. All of the evidence so far obtained indicated that UDP-X was uridine(5′)-diphospho(1)-2,3-diacetamido-2,3-dideoxy-α-D-glucopyranuronic acid. The evidence may be outlined as follows.

Table 1. Paper chromatography and paper electrophoresis of UDP-X and its degradation products

For experimental details see the text.

	Paper-chromatographic R_{UMP}		Paper-electrophoretic m_{UMP}
	Solvent A	Solvent B	Buffer D
Sample			
UDP-X	1.39	0.79	1.59
UMP from UDP-X	1.00	1.00	1.00
Compound X-1-*P*	1.33	1.07	1.62
Standard			
UDP-*N*-acetylglucosamine	1.82	0.59	1.27
UDP-glucuronic acid	0.64	0.30	1.70

(1) The positive fast-atom-bombardment mass spectrum of UDP-X (with glycerol as matrix) (Fig. 1) showed a peak at *m/z* 663 corresponding to the $(M+1)^+$ ion of the calculated values for free acid ($C_{19}H_{28}N_4O_{18}P_2$) of the proposed structure.

(2) The ^{13}C-n.m.r. spectrum of UDP-X (Fig. 2) indicated the presence of 19 C atoms, four of which were ascribable to the uracil, five to the ribose 5-phosphate and the remaining ten to the 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid 1-phosphate residues. The assignments were made by comparison with the spectra of authentic UMP and 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid.

(3) UDP-X was degraded into a nucleoside monophosphate and compound X-1-*P* by hydrolysis with dinucleotide nucleotidohydrolase. The nucleoside monophosphate was obtained as a crystalline sodium salt. The u.v.-absorption spectrum, i.r.-absorption spectrum and

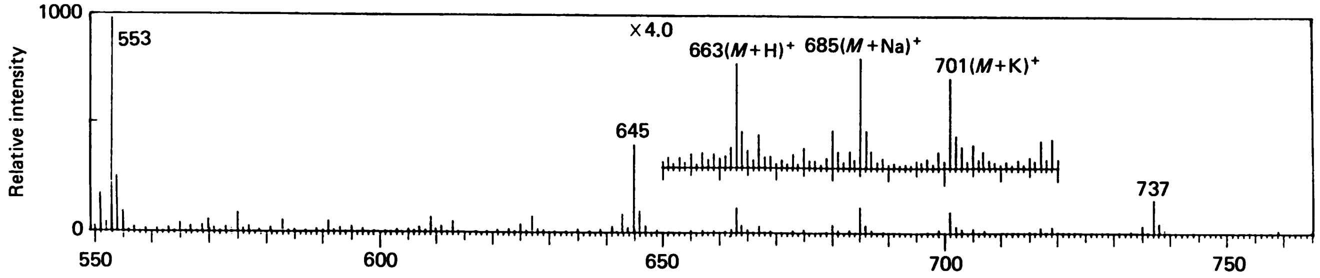

Fig. 1. Partial positive fast-atom-bombardment mass spectrum of UDP-X

Peaks at *m/z* 553, 645 and 747 are ascribed to glycerol used as matrix reagent: $(6\,M+1)^+$, $(7\,M+1)^+$ and $(8\,M+1)^+$ ion respectively.

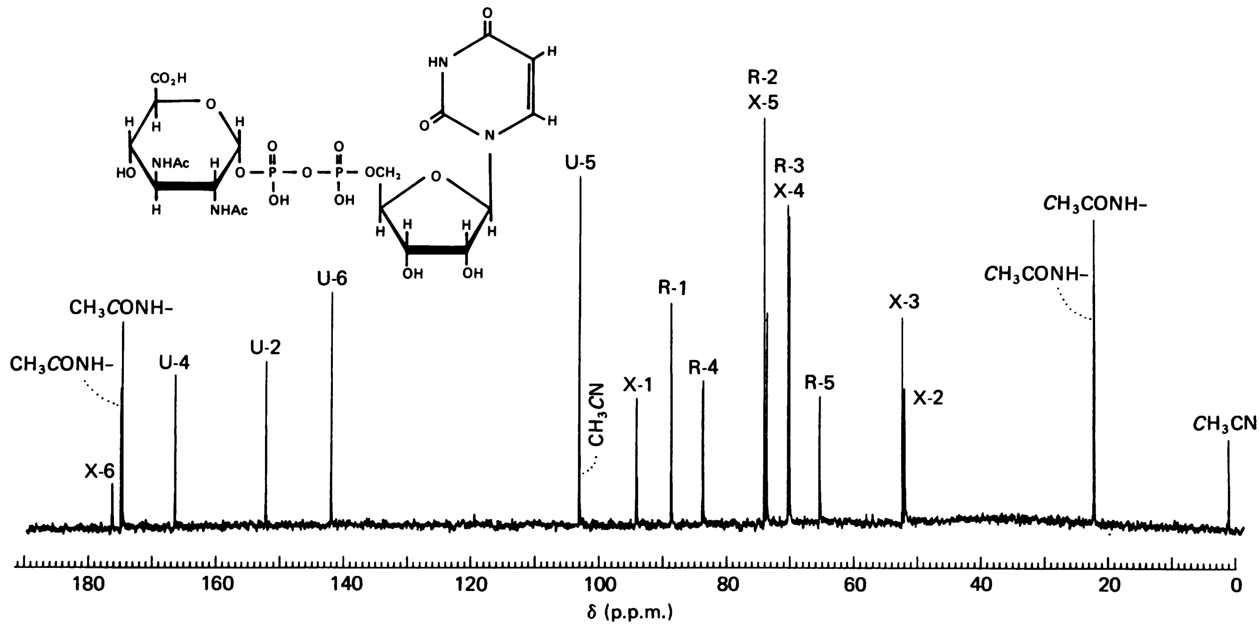

Fig. 2. Proton-decoupled ^{13}C-n.m.r. spectrum of UDP-X

The measurement was performed in 0.75 ml of ^2H_2O with 11 mg of sample (pD 4.8) at 25 °C: repetition time 1.0 s, pulse width 45°, data points 32k, sweep width 20000 Hz and 40037 scans. Major peaks occur at 176.14 (s, X-6), 174.78 (s, CH_3*C*ONH–), 174.56 (s, CH_3*C*ONH–), 166.22 (s, U-4), 151.89 (s, U-2), 141.69 (s, U-6), 102.74 (s, U-5), 93.77 (d, $J_{X\text{-}1,P(\beta)} = 6.1$ Hz, X-1), 88.36 (s, R-1), 83.39 (d, $J_{R\text{-}4,P(\alpha)} = 9.8$ Hz, R-4), 73.79* (s, R-2), 73.40* (s, X-5), 70.09§ (s, R-3), 69.83§ (s, X-4), 65.12 (d, $J_{R\text{-}5,P(\alpha)} = 6.1$ Hz, R-5), 52.18 (s, X-3), 51.80 (d, $J_{X\text{-}2,P(\beta)} = 8.5$ Hz, X-2), 22.10 (s, *C*H_3CONH–) and 21.96 p.p.m. (s, *C*H_3CONH–). U-, R- and X- signals are ascribed to uracil, ribose 5-phosphate and 2,3-diacetamido-2,3-dideoxy-α-D-glucopyranuronic acid 1-phosphate residue respectively. Signals marked * or § may be interchanged.

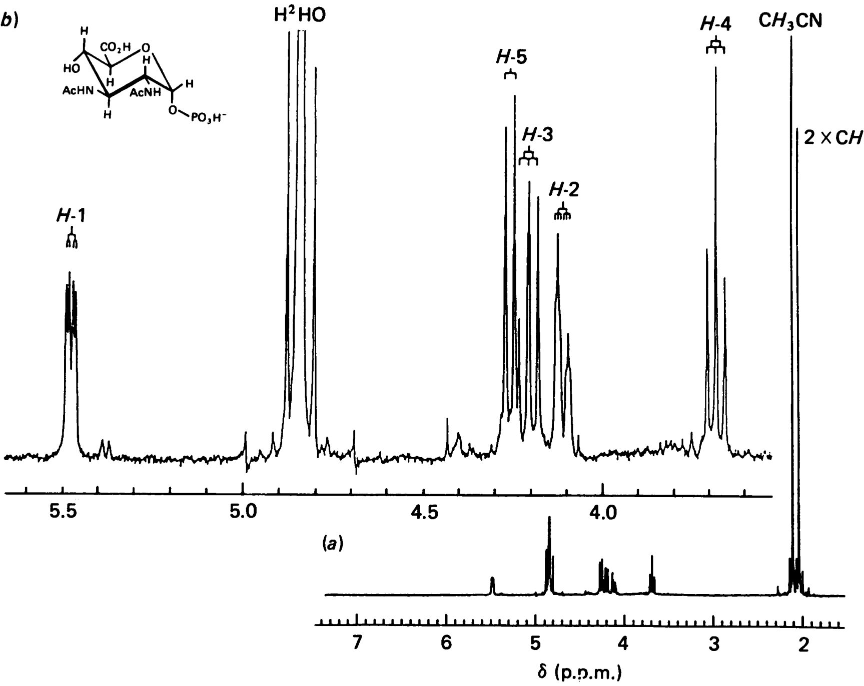

Fig. 3. 400 MHz p.m.r. spectrum of compound X-1-*P* (*a*) and its partial expansion (*b*)

The spectrum was recorded in 0.80 ml of ^2H_2O with 10.1 μmol of sample (pD 4.8) at 25 °C: δ_H (p.p.m.) 5.47 (1H, dd, $J_{1,2} = 3.1$ Hz, $J_{1,P} = 7.3$ Hz, *H*-1), 4.25 (1H, d, $J_{4,5} = 10.1$ Hz, *H*-5), 4.20 (1H, dd, $J_{2,3} = 11.3$ Hz, $J_{3,4} = 10.1$ Hz, *H*-3), 4.11 (1H, ddd, $J_{1,2} = 3.1$ Hz, $J_{2,3} = 11.2$ Hz, $J_{2,P} = 3$ Hz, *H*-2), 3.68 (1H, dd, $J_{3,4} = 10.1$ Hz, $J_{4,5} = 10.1$ Hz, *H*-4) and 2.03 (6H, s, $2CH_3CONH-$).

molar rotation of the sodium salt were all identical with those of UMP. Inorganic phosphate was liberated from the sample when it was exposed to the action of 5′-ribonucleotide phosphohydrolase. These results established that the nucleoside monophosphate was UMP.

Compound X-1-*P* was isolated as an amorphous solid. Its p.m.r. spectrum (Fig. 3) indicated the presence of 11 protons, six of which were compatible with *C*-methyl protons of two acetamido groups and the remaining five with ring protons, H-1, H-2, H-3, H-4 and H-5. The signals were assigned on the basis of double-resonance experiments. ^1H–^1H coupling constant data for the sugar moiety of compound X-1-*P* were consistent with a transdiaxial relationship for the H-2–H-3, H-3–H-4 and H-4–H-5 pairs and a *gauche* orientation for H-1–H-2, and thus indicated that the sugar moiety had the *α-glucopyrano* configuration. Further support for the assignment of α-anomeric configuration was provided by the couplings of the phosphorus nucleus with H-1 ($J_{1,P} = 7.3$ Hz) and H-2 ($J_{2,P} = 3$ Hz). It has been shown that in aqueous solution at pH 4 all of the α-anomers of common hexopyranose 1-phosphate have $^3J_{1,P}$ values between 7.0 and 8.8 Hz, and all of the α-hexopyranose 1-phosphate molecules carrying H-2 axial have $^4J_{2,P}$ values between 2.6 and 3.3 Hz [16].

(4) When compound X-1-*P* was further degraded by digestion with orthophosphoric monoester phosphohydrolase, a reducing sugar was formed with concomitant liberation of an equivalent amount of inorganic phosphate. The identity of the sugar with authentic sample of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid was established by the comparisons of their negative fast-atom-bombardment mass spectra (with triethanolamine as matrix), i.r.-absorption spectra, p.m.r. spectra (Fig. 4) and c.d. spectra (Fig. 5).

As a result, we concluded that the structure of UDP-X is uridine(5′)-diphospho(1)-2,3-diacetamido-2,3-dideoxy-α-D-glucopyranuronic acid (Fig. 6).

DISCUSSION

Uridine(5′)-diphospho(1)-2,3-diacetamido-2,3-dideoxy-α-D-glucopyranuronic acid is the first representative of the sugar nucleotides containing diaminouronic acids, a new class of sugar nucleotide, isolated for the first time from living cells.

Although 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid, -D-mannuronic acid and -L-guluronic acid have been found to be constituents of the O-specific polysaccharides of some *Ps. aeruginosa* lipopolysaccharides [3–6], the corresponding diaminouronic acids have never been isolated in the free state from the acid hydrolysates of the polysaccharides [3–6]. The glycosidic bonds of the diaminouronic acids on the polysaccharides are reported to be very stable under acidic conditions [4–6]. However, those of the diaminouronic acids having a *threo* configuration at C-3–C-4 are assumed to be cleaved, resulting in the formation of corresponding γ-lactam. Indeed, such a γ-lactam compound was isolated from the acid hydrolysate of *Ps. aeruginosa* P14 lipopolysaccharide [3]. In the present study we succeeded in isolating 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid in the free state, which formed the corresponding γ-lactam under acidic conditions [3].

The presence of UDP-2,3-diacetamido-2,3-dideoxy-

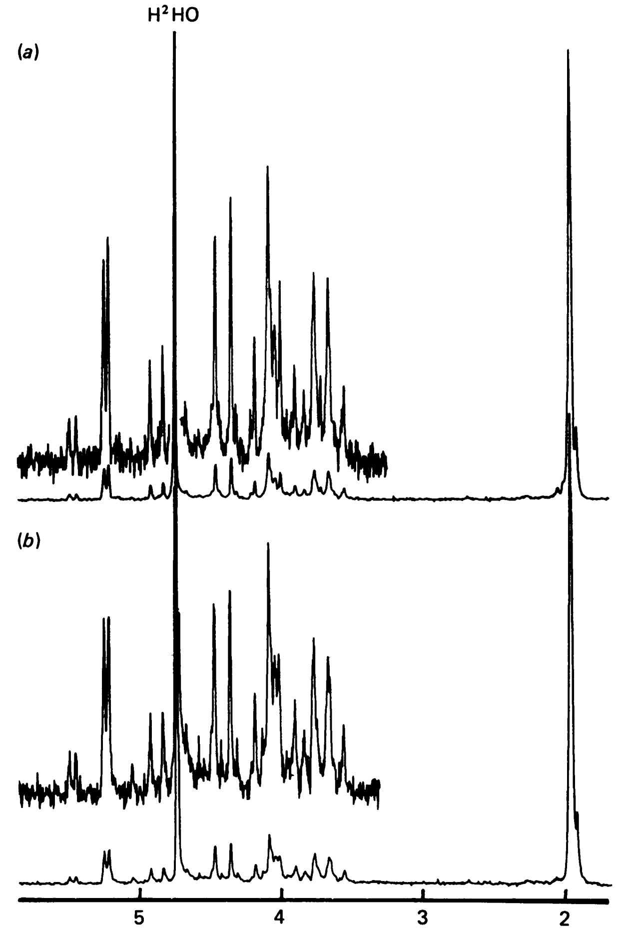

Fig. 4. 90 MHz p.m.r. spectra of the reducing sugar liberated from compound X-1-*P* (*a*) and 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid (*b*)

Each spectrum was recorded in 0.45 ml of ^2H_2O with 2.7 mg of sample. Assignments were not made.

D-glucuronic acid in *Ps. aeruginosa* P1–III cells indicates the existence of reactions leading to the formation of this activated diaminouronic acid *in vivo*. One may speculate that the formation of UDP-2,3-diacetamido-2,3-dideoxy-D-glucuronic acid takes place through the enzymic oxidation of UDP-*N*-acetylglucosamine to UDP-*N*-acetylglucosaminuronic acid followed by the conversion of the 3-hydroxy group into an acetamido group. Thus UDP-*N*-acetylglucosamine is converted into UDP-*N*-acetylglucosaminuronic acid by an enzyme similar to the UDP-acetylglucosamine dehydrogenase found in other bacteria [17,18]. Then the formation of UDP-2,3-diacetamido-2,3-dideoxy-D-glucuronic acid from UDP-*N*-acetylglucosaminuronic acid takes place by the pathway analogous to those proposed for the enzymic syntheses of dTDP-4-acetamido-4,6-dideoxyhexoses [19–23] and dTDP-3-acetamido-2,6-dideoxyhexose [24] from dTDP-glucose and of UDP-2-acetamido-4-amino-2,4,6-trideoxyhexose from UDP-*N*-acetylglucosamine [25].

Knirel *et al.* have proposed the structures of the O-specific polysaccharides of *Ps. aeruginosa* serogroup O:3 according to Lanyi's classification [5,6]. Recently we have found that the ^{13}C-n.m.r. spectrum and specific rotation of the O-specific polysaccharide of P1–III lipopolysaccharide are identical with those of *Ps. aeruginosa* 170006 (Lanyi serotype O:3,a,d,e) reported by Knirel *et al.* [6] (N. Suzuki & S. Okuda, unpublished work). Therefore the structure of P1–III O-specific polysaccharide can be regarded as

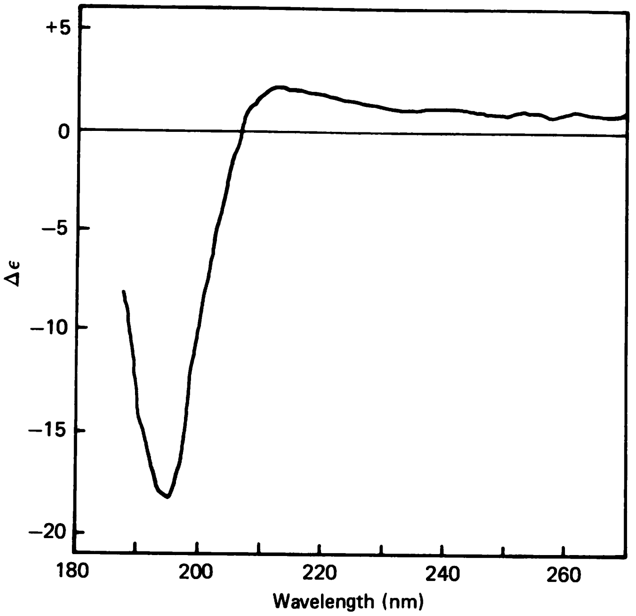

Fig. 5. C.d. spectrum of the reducing sugar liberated from compound X-1-*P*

The measurement was performed in 2 ml of water with 11 μg of sample: $\lambda_{\text{max.}_1}$ 195 nm, $\Delta\epsilon_1$ −18.3; $\lambda_{\text{max.}_2}$ 212 nm, $\Delta\epsilon_2$ +2.34. The spectrum of 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid was identical with this spectrum: $\lambda_{\text{max.}_1}$ 195 nm, $\Delta\epsilon_1$ −18.9; $\lambda_{\text{max.}_2}$ 212 nm, $\Delta\epsilon_2$ +2.33.

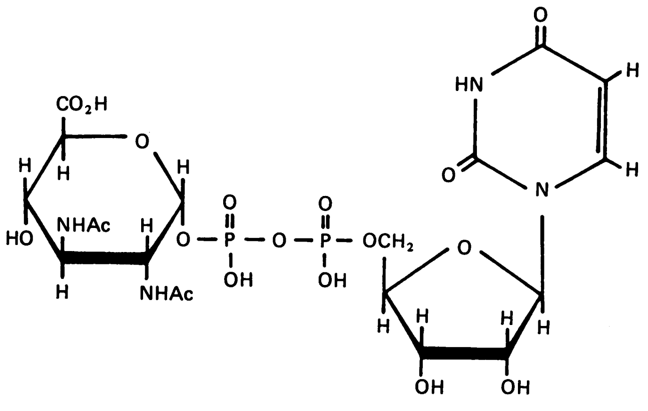

Fig. 6. Proposed structure of UDP-X

$\{4)$DManImA(β1–4)LGul(NAc)$_2$A(α1–3)DFucNAc(α1$\}_n$

where DManImA represents 2,3-(1-acetyl-2-imidazolino-5,4)-2,3-dideoxy-D-mannuronic acid, LGul(NAc)$_2$A represents 2,3-diacetamido-2,3-dideoxy-L-guluronic acid and DFucNAc represents 2-acetamido-2,6-dideoxy-D-galactose. This structure does not contain a 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid residue.

Summarizing these considerations, it seems likely that the possible physiological role of UDP-2,3-diacetamido-2,3-dideoxy-D-glucuronic acid in *Ps. aeruginosa* P1–III cells is not a glycosyl donor in the biosynthesis of O-specific polysaccharide chains of the lipopolysaccharide, but an intermediate in the enzymic conversion of UDP-*N*-acetylglucosamine into the donor nucleotide(s).

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