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Identification of 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine), isolated from the cell walls of the alkaliphilic *Bacillus* sp. Y-25, by 500-MHz ^{1}H NMR spectroscopy

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ABSTRACT

Cell walls of alkaliphilic *Bacillus* strain Y-25 are composed of γ-peptidoglycan and two acidic polymers. An amino sugar, which was a main component of one acidic polymer, did not correspond to any of the commercially available hexosamines. The amino sugar was isolated from the hydrolysate of the acidic polymer, purified, and identified as D-quinovosamine (2-amino-2,6-dideoxy-D-glucose) by 500-MHz NMR spectroscopic analysis and polarimetry.

INTRODUCTION

Cell walls of alkaliphilic strains of *Bacillus* spp. have been analysed[1]. The amounts of acidic compounds (uronic acids and acidic amino acids) found in the cell walls of several strains are enhanced when the strains are grown at an alkaline pH. The acidic polymers were extracted from *Bacillus* sp. strain Y-25 and separated chromatographically into two fractions. One was composed mainly of glutamic acid and glucuronic acid. The other was composed of glutamic acid, galacturonic acid, and an unidentified compound. After separation by cellulose TLC, the pure compound was a ninhydrin-positive, reducing sugar and gave a positive Elson–Morgan reaction, indicating that it was a 2-amino-2-deoxy sugar.

The amino sugar was isolated from a hydrolysate of the acidic polymer with HCl. Analysis of the 500-MHz NMR spectrum and the mass spectrum and measurement of the optical rotation revealed that the amino sugar was D-quinovosamine. This paper presents data on the purification and characterisation of the amino sugar.

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EXPERIMENTAL

General methods.—The ^1H NMR spectra were recorded at room temperature with a Varian VXR-500S spectrometer; ^1H chemical shifts (δ) are reported for solutions in D_2O with reference to Me_4Si. The optical rotation was determined at 25°C with a Jasco-DIP 140 digital polarimeter. Fast-atom-bombardment (FAB)-mass spectrometry was recorded with a JEOL JMS-HX110. Dowex 1-X4 (200–400 mesh; Cl^- form) and Dowex 50W-X4 (200–400 mesh; H^+ form) resins were used for ion-exchange chromatography. Cellulose CC-31 (Whatman) was used for partition chromatography, and Avicel cellulose thin-layer (Funakoshi) was used for TLC.

Organism and cell-wall preparation.—The alkaliphilic *Bacillus* sp. Y-25 was grown at an alkaline pH as described previously[1]. The culture medium contained (g per liter of deionised water): K_2HPO_4, 13.7; KH_2PO_4, 5.9; citric acid, 0.34; $MgSO_4 \cdot 7H_2O$, 0.05; Na_2CO_3, 10.6; D-glucose, 5; peptone, 5; yeast extract, 0.5. The pH of this medium was ~10. The organism was grown at 30°C aerobically with shaking. Cells in the early stationary phase of growth (A_{660} 2.0) were harvested by centrifugation at 8000*g* for 10 min at 4°C, and their walls were prepared, as described previously[1].

Purification of the amino sugar.—The acidic non-peptidoglycan components were extracted from cell walls of the alkaliphilic *Bacillus* sp. Y-25 with 5% (w/v) trichloroacetic acid (TCA) and the extraction with TCA was repeated three times. The TCA extracts were combined and exhaustively dialysed against running water. The solution containing the non-diffusible material from the extract was adjusted to pH 5.0 with acetic acid and loaded on a DEAE-cellulose column (2.5 × 50 cm) which had been equilibrated with 50 mM acetic acid–NaOH buffer (pH 5.0). The column was eluted at the same rate with 250 mL of the buffer containing 0.2 M NaCl, followed by a linear gradient elution from 0.2 to 0.6 M NaCl in the buffer (700 mL) at a flow rate of 60 mL/h. Fractions (15 mL) were collected and assayed for uronic acids, amino compounds, and NaCl. Fraction 1 (see legend of Fig. 1) was dialysed against deionised water and concentrated in a rotary evaporator at 43°C. Samples (6 mL) were applied to a column of Fractogel TSK-HW65S (2.5 × 100 cm) which had been equilibrated with 0.2 M NaCl. The column was eluted with 0.2 M NaCl at a flow rate of 30 mL/h. Fractions (8.5 mL) were assayed for uronic acids and amino sugars. The fractions containing these substances from a number of gel chromatography runs were combined, dialysed against deionised water, and freeze-dried. A 200-mg portion of the fraction-1 compound was hydrolysed in 12 mL of 4 M HCl at 100°C for 5 h. This hydrolysate was concentrated to dryness in a rotary evaporator at 43°C. The residue was dissolved in 9 mL of deionised water and filtered. The filtrate was passed through a column (0.5 cm × 15 cm) of Dowex 1 (Cl^-) resin. The column was washed with 50 mL of deionised water. The effluent and wash were combined and applied to a column (1 × 11 cm) of Dowex 50 W (H^+) resin. The column was washed with 50 mL of

deionised water and then 100 mL of 0.05 M HCl at a flow rate of 45 mL/h. A linear gradient of 0.05–0.80 M HCl (200 mL) was applied to the column at a flow rate of 15 mL/h. Fractions (2 mL) containing the amino sugar were pooled, concentrated to dryness in a rotary evaporator, and dried in vacuo over NaOH. The residue was dissolved in 0.2 mL of water and loaded on to a column (2.5 × 55 cm) of CC-31 cellulose that had been equilibrated with EtOAc–pyridine–H_2O–acetic acid (5:5:3:1). The column was eluted with 400 mL of the same solvent. Fractions (2.5 mL) containing the amino sugar were pooled, washed with diethyl ether, and concentrated to dryness. The residue was redissolved in 1.1 mL of deionised water and was completely converted into the hydrochloride salt by the addition of a small amount of M HCl followed by lyophilization.

Structural analysis of the amino sugar.—A 2-mg portion of the purified amino sugar hydrochloride was dissolved in 0.5 mL of D_2O. ^1H NMR spectra were recorded with a 500-MHz NMR spectrometer. The dry weight of the amino sugar was measured by heating at 43°C in vacuo until a constant weight was obtained.

Chemical analysis.—Amino compounds, uronic acids, and neutral sugars were determined as described previously[1]. Samples purified by chromatography were hydrolysed in 4 M HCl for 16 h at 100°C in sealed tubes. The hydrolysates were also analysed with an amino acid analyser. Sodium was determined with a Shimazu atomic absorption spectrometer model AA-670 (Japan).

Avicel cellulose TLC of the sample was carried out as described previously[2], with the solvents EtOAc–pyridine–H_2O–acetic acid (5:5:3:1) or 2-propanol–H_2O–acetic acid (9:2:1). Compounds were located with ninhydrin or alkaline silver nitrate.

RESULTS AND DISCUSSION

Purification of the amino sugar.—The extraction with TCA (four times) solubilised 99% of the uronic acids in the walls. The non-dialysable fraction of the TCA extracts from the walls was separated into two fractions by DEAE-cellulose column chromatography (Fig. 1). One fraction (fraction-1) was eluted at 0.35–0.41 M NaCl and the other (fraction-2) at 0.42–0.50 M NaCl. The acidic compound found in fraction-1 was composed mainly of glutamic acid, galacturonic acid, and a 2-amino-2-deoxy sugar (Table I). The other was composed mainly of glutamic acid and glucuronic acid (data not shown).

The fraction-1 compound was concentrated and subjected to gel chromatography on Fractogel TSK HW-65S. Fraction 1 gave a single peak of glutamic acid, galacturonic acid, and the amino sugar.

Under the conditions of hydrolysis, recovery of the amino sugar was ~ 83% of the total. The uronic acid was destroyed to form a dark-brown insoluble material and was removed by filtration. Most of the glutamic acid was removed by passage through a column of Dowex 1 resin. Quinovosamine was eluted from the column of Dowex 50W resin by 0.06–0.20 M HCl (Fig. 2). Other ninhydrin-positive com-

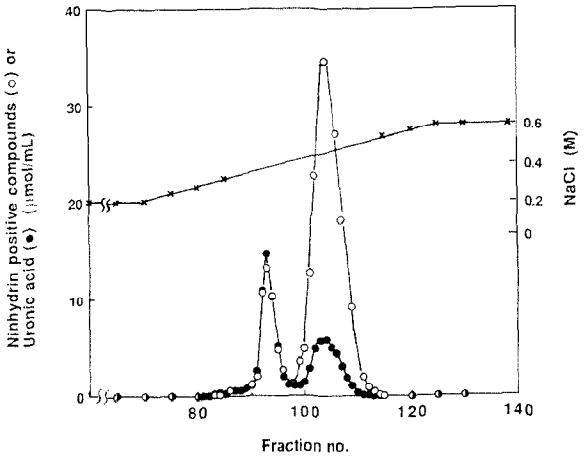

Fig. 1. DEAE-Cellulose chromatography of the non-dialysable fraction of TCA extracts. The solution containing the non-dialysable material from the extracts was adjusted with acetic acid to pH 5.0 and loaded on a column (2.5×50 cm) of DEAE-cellulose equilibrated with the same buffer. The column was eluted as described in the Experimental section. Fractions (15 mL) were collected and assayed for uronic acids (●) by the carbazole method with D-glucuronic acid as the reference standard, and for amino groups (○) by the ninhydrin method with D-glucosamine as the reference standard. Concentration of NaCl (×) was determined by refractive index. Fractions 89–98 (fraction-1) and 99–114 (fraction-2) were pooled.

pounds (mainly glutamic acid) were eluted by 0.22–0.33 M HCl. Avicel cellulose TLC revealed that this fraction contained a small amount of glutamic acid but no uronic acids. Quinovosamine (169 μmol) was further purified by cellulose column chromatography and was eluted in fractions 115–130 mL. Concomitant glutamic acid was eluted in fractions 200–225 mL.

Identification of the amino sugar as D-quinovosamine.—Fig. 3 shows the ^1H NMR spectrum of the amino sugar hydrochloride measured at 500 MHz. Doublet signals for Me groups were found at 1.28 and 1.32 ppm relative to Me_4Si. Three doublet signals and six quartet signals for methine groups were found between 2.9

TABLE I

Composition of the fraction-1 compound purified by DEAE-cellulose and gel chromatography

Component	Composition of fraction-1 compound [μmol/(mg of acidic polymer)]
Glutamic acid	3.63
L-Glutamic acid	0.75
Galacturonic acid	2.17
2-Amino-2-deoxy sugar	1.08
Galactosamine	0.02
Neutral sugar	ND [a]
Sodium	2.7

[a] Not detected.

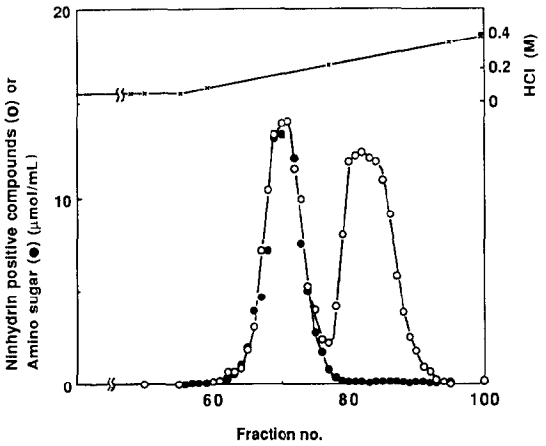

Fig. 2. Ion-exchange chromatography of the hydrolysate of the acidic polymer. The acid hydrolysate of the acidic polymer, after removal of HCl, was passed through a small column of Dowex 1 (Cl^- form) and loaded on a column (1×11 cm) of Dowex 50W-X4 (200–400 mesh; H^+ form). The column was eluted with deionised water, 0.05 M HCl, and then a linear gradient of HCl as described in the Experimental section. Fractions (2 mL) were collected and assayed for amino sugars (●) by the Elson–Morgan reaction and for amino groups (○) by the ninhydrin method with D-glucosamine as the reference standard. Concentration of HCl (×) was determined by titration. Fractions 64–76 containing amino sugar were pooled.

and 5.4 ppm. These signals were assigned, as shown in Table II, with multiplicities and spin-coupling constants as shown in Table III. This assignment was confirmed by decoupling experiments (results not shown).

These data suggested that the sample was a type of hexosamine existing as two anomers. The coupling constants were consistent with the configurations and conformations shown in Fig. 4. This structure is analogous to that of quinovose. The ^1H NMR spectrum of authentic D-quinovose was recorded under the same conditions. Quinovose and the sample had different values for the chemical shifts of H-1, H-2, and H-3, and for $J_{2,3}$, as shown in Tables II and III. These chemical shift values suggested that the C-2 position of the sample was aminated. Therefore, the sample could be identified as 2-amino-2,6-dideoxyglucose, so-called quinovosamine.

This suggestion was supported by the fact that the amino sugar gave the Elson–Morgan reaction, generally thought to require a free 2-amino-2-deoxy sugar[4]. A positive FAB-mass spectrum was recorded for the sample (0.1 mg). An aqueous solution of the compound was deposited in a small quantity of glycerol that had been placed on a gold-plated copper, sample-probe tip. The sample was then bombarded with a beam of neutral fast atoms. A mixture of sodium chloride was added to induce attachment-ion formation, in order to aid in the interpretation of the resulting FAB-mass spectrum[5]. The positive FABMS of the sample showed a molecular ion at m/z 164.0919. The molecular formula of quinovosamine is $C_6H_{13}NO_4$ $[(M + H)^+$, calcd for $C_6H_{13}NO_4$, 164.0923].

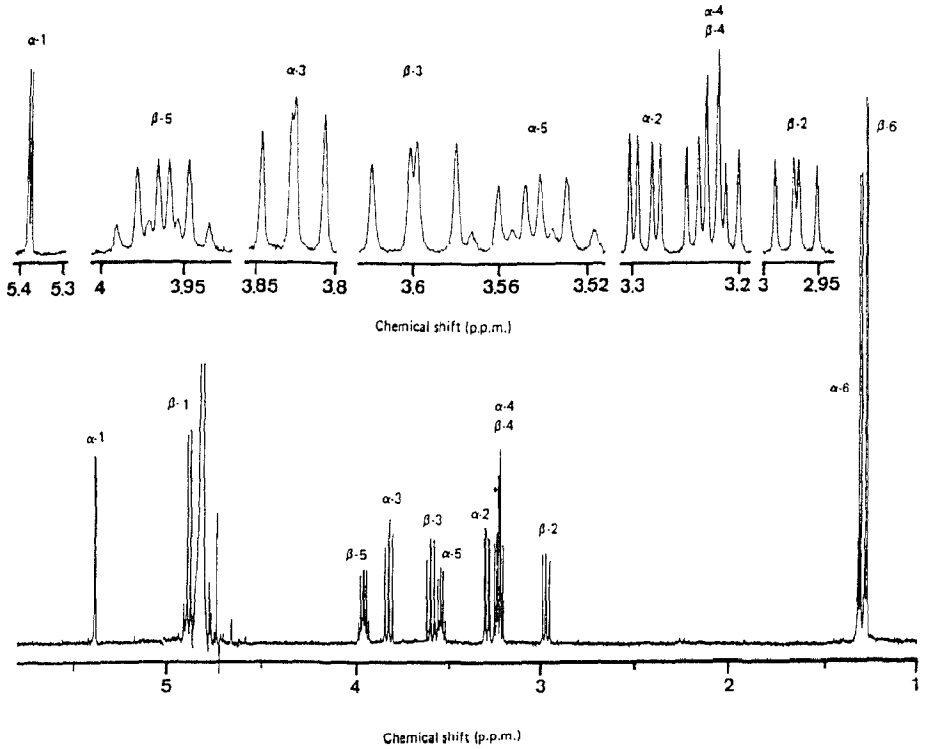

Fig. 3. 500-MHz ^{1}H NMR spectrum of the amino sugar hydrochloride isolated from the acidic polymer of cell walls.

TABLE II

Assignment of chemical shift values of the amino sugar isolated from the acidic polymer

Sugar	Chemical shift [a] (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
α Anomer of the sample	5.38	3.30	3.83	3.23	3.55	1.32
β Anomer of the sample	4.89	2.97	3.60	3.23	3.96	1.28
α-D-Quinovose	5.18	3.54	3.66	3.17	3.50	1.29
β-D-Quinovose	4.63	3.25	3.44	3.17	3.90	1.26

[a] Each signal found in Fig. 3 was assigned with its multiplicity and spin-coupling constant. Chemical shift values of authentic D-quinovose were obtained from the NMR spectrum measured under the same conditions as those for the sample.

TABLE III

Spin-coupling constants of quinovosamine, isolated from the acidic polymer, and authentic D-quinovose

Sugar	Spin-coupling constant (Hz)				
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
α Anomer of the sample	3.50	10.50	9.00	9.50	6.00
β Anomer of the sample	8.00	11.50	9.00	10.50	6.50
α-D-Quinovose	4.00	9.50	9.00	9.50	6.00
β-D-Quinovose	8.00	9.00	9.50	10.00	6.00

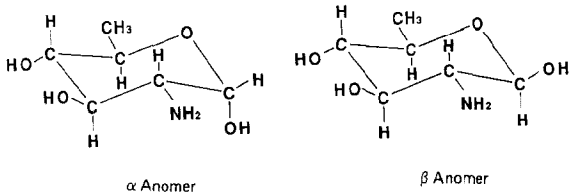

Fig. 4. Proposed structure for the amino sugar isolated from the acidic polymer of the cell walls.

The absolute configuration of quinovosamine isolated from the acidic polymer was determined by measurement of the optical rotation of the sample: $[\alpha]_D$ +48.0° (*c* 0.92, H_2O) was found. Kuhn et al.[6] reported $[\alpha]_D$ +53° for synthetic D-quinovosamine hydrochloride, Mortel[7] reported $[\alpha]_D$ +55° for synthetic D-quinovosamine hydrochloride, and Jann et al.[8] reported $[\alpha]_D$ +45° for D-quinovosamine hydrochloride isolated from *Vibrio cholerae*.

These results confirmed that the amino sugar is D-quinovosamine.

As far as we know, quinovosamine is not available commercially and its ^1H NMR spectrum has not been measured. We had to purify large amounts of the amino sugar in order to analyse it structurally. Although quinovosamine rarely appears as a structural component of cell walls, D and L forms of quinovosamine are known to occur in the capsular polysaccharide of *Bacteroides fragilis*[9]. The D form of the sugar is known to be present in the lipopolysaccharides of *Vibrio cholerae*[8], *Salmonella* group S and 58, *Arizona* 1.33, and *Proteus vulgaris*[10]. The L form of the sugar is present in the O-specific polysaccharide of *Proteus vulgaris* 5/43 (ref. 11). The D form has now been isolated from the cell walls of the alkaliphilic *Bacillus* sp. Y-25, but not from cell walls of other *Bacillus* spp.

Cell walls of the strain Y-25 are composed of γ-peptidoglycan[12] and two acidic polymers. The acidic polymer containing quinovosamine is composed mainly of glutamic acid, galacturonic acid, and quinovosamine in the molar ratios (7:4:2) (Table I). Structural analysis of the acidic polymer is under study.

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