4-AMINO-4,6-DIDEOXY-*D*-MANNOSE (*D*-PEROSAMINE) : A COMPONENT OF THE LIPOPOLYSACCHARIDE OF *VIBRIO CHOLERAE* 569B (INABA)

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1. Introduction

Recent accounts [1,2] of analytical studies of the lipopolysaccharides of *Vibrio cholerae* strains have drawn attention to the fact that some 30% of the total weight of the complexes was unaccounted for. We now report the presence in the lipopolysaccharide of *Vibrio cholerae* 569B (Inaba) of a highly-labile sugar, 4-amino-4,6-dideoxy-*D*-mannose (*D*-perosamine).

2. Experimental

2.1. *General*

Lipopolysaccharide from *Vibrio cholerae* 569B (Inaba) was prepared as previously reported from acetone-dried cells kindly provided by Dr R. A. Finkelstein (University of Texas). NMR spectra were measured on a Varian Associated XL-100 spectrometer and optical rotations on a Perkin-Elmer 141 polarimeter.

Gel chromatography was carried out using a column (15 X 850 mm) eluted with 0.1 M pyridinium acetate buffer (pH 5.75). Chromatography on sulphonic acid resin was performed as by Gardell [3]. The eluates were monitored by a Pye Unicam LCM-2 detector and fractions were examined for neutral sugar content using the phenol–sulphuric acid reagent [4] and for amino groups using trinitrobenzene sulphonic acid [5].

Thin-layer chromatography was carried out using Merck cellulose plates eluted with: (a) 1-butanol–pyridine–water (4:3:3, by vol.); (b) 1-butanol–ethanol–water (13:8:4, by vol.); (c) formic acid–butanone–2-methyl-2-propanol–water (15:25:35:25, by vol.) and preparative thin-layer chromatography using Merck Kieselgel G plates eluted with ether–acetone (9:1, v/v) and visualized with iodine vapour.

2.2. *Hydrolysis of Lipopolysaccharide*

Lipopolysaccharide (0.50 g) was dispersed in 10 M hydrochloric acid (5 ml) and heated at 80°C for 25 min. The dark mixture was then cooled, diluted with water (50 ml), filtered through a pad of Celite and evaporated at reduced pressure at below 50°C.

2.3. *Acetylation of hydrolysate*

The crude hydrolysate was treated with a solution of anhydrous zinc chloride (0.01 g) in acetic acid (5 ml) followed by acetyl chloride (3 ml) and the mixture boiled under gentle reflux for 2 hr before evaporating to dryness at below 50°C. Acetic anhydride (3 ml) was then added, followed by pyridine (2 ml) and the mixture allowed to stand at 20°C for 16 hr. After evaporation to dryness, the residue was partitioned between chloroform and water and the organic layer dried and concentrated. Preparative thin-layer chromatography of the resultant syrup gave D-perosamine α-peracetate (R_F = 0.24) as an amorphous powder (0.060 g).

2.4. *Transesterification*

A solution of D-perosamine α-peracetate (0.060 g) in dry methanol (2 ml) at 0°C was treated with a small lump of sodium and maintained at this temperature for 30 min before neutralization with dry ice and evaporation. Extraction of the residue with ethanol gave, in quantitative yield, *N*-acetyl-D-perosamine as a gum, $[\alpha]_{578}^{21}$ = +33°C (previously reported [6] : $[\alpha]_D^{23}$ = +34°C).

3. Results and discussion

Although the phenol–sulphuric acid test indicated the presence of approx. 30% neutral sugars (expressed as glucose) in the lipopolysaccharide, much lower values of these components have been obtained by gas chromatography [1,2]. Moreover, monitoring of the release of reducing sugars on treatment of the complex with 1 M sulphuric acid showed that a limit (equivalent to 17% glucose) was reached after 4.5 hr at 100°C. Longer treatment caused extensive darkening, indicating decomposition. When a 4.5-hr hydrolysate was subjected to chromatography on Sephadex G-10, an oligomeric fraction, just included by the gel, was obtained in addition to the monosaccharide fraction. It was not possible to hydrolyse this material further by 'normal' acid treatment, *e.g.*, 1 M sulphuric acid, 100°C, 16 hr; 2 M trifluoroacetic acid, 100°C, 16 hr [7] or 90% acetic acid, 100°C, 16 hr [8]. When, however, the lipopolysaccharide was treated briefly with 10 M hydrochloric acid, complete fragmentation to monosaccharides resulted.

Chromatography of this hydrolysate on an Amberlite CG-120 column [3] gave a fraction (V_{GlcNH_2} = 1.46) which was unreactive to trinitrobenzene sulphonic acid but reactive to the phenol–sulphuric acid reagent. This lack of reactivity of the amino group suggests its involvement in a ring under the alkaline conditions of the test [5]. The fraction was homogeneous by thin-layer chromatography in all systems, gave a yellow colour when visualized with ninhydrin and a brown colour, with a pale blue fluorescence, when the untreated chromatogram was heated briefly at 120°C. Similar behaviour has been noted for 4-amino-4-deoxy-L-arabinose [9].

To carry out detailed structural studies, it was desired to prepare an acetate of the sugar. Preliminary studies revealed that brief warming of the sugar with pyridine led to rapid darkening. Such behaviour is typical of that of 4-amino sugars which undergo ready cyclodehydration to pyrrolines [10]. That the compound gives a positive Ehrlich test [11] is consistent with this view. In order, therefore, to prevent such cyclization by the highly nucleophilic amino group of the sugar, the esterification of the hydroxyl groups was carried out in a highly acidic medium. The amino group was acetylated in a second, basic, step.

The pure fraction obtained by preparative thin-layer chromatography of the resultant acetate mixture was examined by NMR spectrometry. The spectral details summarized in table I permit unequivocal assignment of the structure of the peracetate as 4-acetamido-4,6-dideoxy-α-mannopyranose, 1,2,3-triacetate (in the Cl conformation). This peracetate has already been prepared by acetolysis of the antibiotic perosamine [12], but the 60 MHz NMR spectrum was not exhaustively analysed. The spectrum of the acetylated methyl α-glycoside has, however, been described in more detail and has many features in common with that of the peracetate [12,13].

In spin-decoupling experiments, irradiation of the methyl doublet at 1.28 δ caused collapse of the multiplet

Table 1
NMR data of D-perosamine peracetate in $CDCl_3$

Signal	Integration	Chemical shift (δ)	Coupling constant (Hz)	Assignment
Doublet	3	1.28	$J_{5,6}$ = 6.1	5-methyl
Singlet	3	1.97	–	NH-Ac (eq)
Singlet	3	2.04	–	O-Ac (eq)
Singlet	3	2.13	–	O-Ac (ax)
Singlet	3	2.18	–	O-Ac (ax)
Multiplet	1	3.82	$J_{4,5}$ = 10.2	H-5 (ax)
Multiplet	1	4.26	$J_{3,4}$ = 10 (approx.)	H-4 (ax)
Multiplet	2	5.10-5.40	$J_{2,3}$ = 2 (approx.)	H-3 (ax) and H-2 (eq)
Doublet (broad)	1	5.70	$J_{4,N}$ = 9.6	N-H
Doublet	1	6.03	$J_{1,2}$ = 1.6	H-1 (eq)

at 3.82 δ to a doublet with $J_{4,5}$ = 10.2 Hz, indicating a trans diaxial disposition of protons 4 and 5. Furthermore, when the N-H proton at 5.70 δ was irradiated, the multiplet at 4.26 δ collapsed to a symmetrical triplet (J = 10 Hz), indicating an essentially equal coupling of H-4 to both H-3 and H-5. Proton 3 must therefore also be axially disposed. Irradiation of the narrow doublet at 6.03 δ simplified part of the multiplet at 5.10–5.40 δ to a broad singlet, indicating a small $J_{2,3}$ of approx. 2 Hz. Since H-3 is axial, H-2 must therefore be equatorially directed. The magnitude of $J_{1,2}$ does not in itself allow definite assignment of the configuration of H-1. Other workers have observed, however, that the chemical shift of the anomeric proton of a pyranose peracetate depends on the configuration at carbons 1 and 2 [14]. In particular, the resonance of H-1 at 6.03 δ indicates that both H-1 and H-2 are equatorially directed. The resultant structure requires one equatorial acetamido, one equatorial acetoxy and two axial acetoxy groups. The chemical shifts of the acetyl singlets are in accord with this conclusion [15].

The 4-amino-4,6-dideoxymannose was placed in the D-series on the basis of the optical rotation of its known *N*-acetyl derivative [6].

The amount of peracetate isolated from the lipopolysaccharide indicates a D-perosamine content of at least 6%. In view of the evident decomposition during hydrolysis, the real value is undoubtedly much higher. The D-gluco and D-galacto isomers have been found in the lipopolysaccharides of *E. coli* strains [16]. It was, however, possible to release these other sugars from the complexes under much milder hydrolytic conditions than we have found necessary, probably reflecting a difference in linkage types. This matter is at present under detailed study.

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References

[1] Jackson, G. D. F. and Redmond, J. W. (1971) FEBS Lett. 13, 117–120.
[2] Jann, B., Jann, K. and Beyaert, G. O. (1973) Eur. J. Biochem. 37, 531–534.
[3] Gardell, S. (1958) in: Methods of Biochemical Analysis (Glick, D., ed.), Vol. 6, pp. 310–312, Interscience, N.Y.
[4] Hodge, J. E. and Hofreiter, B. T. (1962) in: Methods in Carbohydrate Chemistry (Whistler, R. L. and Wolfrom, M. L., eds.), Vol. 1, pp. 388–389, Acad. Press, N. Y.
[5] Kellaher, P. C. and Smith, C. J. (1968) J. Chromatog. 34, 7–13.
[6] Lee, C. H. and Schaffner, C. P. (1966) Tetrahedron Lett. 5837–5840.
[7] Albertsheim, P. A., Nevins, N. J., English, P. D. and Karr, A. (1967) Carbohyd. Res. 5, 340–343.
[8] Hellerquist, C. G. and Lindberg, A. A. (1971) Carbohyd. Res. 16, 39–48.
[9] Volk, W. A., Galanos, C. and Lüderitz, O. (1970) Eur. J. Biochem. 17, 223–229.
[10] Paulsen, H., Steinert, K. and Heyns, K. (1970) Chem. Ber. 103, 1599–1620.
[11] Distler, J., Kaufman, B. and Roseman, S. (1966) Arch. Biochem. Biophys. 116, 466–478.
[12] Lee, C. H. (1966), Ph. D. Thesis, Rutgers, The State University, New Brunswick, N.J.
[13] Lee, C. H. and Schaffner, C. P. (1969) Tetrahedron 25, 2229–2232.
[14] Hall, L. D. (1964) Tetrahedron Lett. 1457–1460.
[15] Lichtenthaler, F. W. (1963) Chem. Ber. 96, 2047–2051.
[16] Jann, B. and Jann, K. (1967) Eur. J. Biochem. 2, 26–31.