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Note

5,7-Diamino-5,7,9-trideoxynon-2-ulosonic acid: a novel sugar from a phytopathogenic *Pseudomonas* lipopolysaccharide

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Dedicated to the memory of Professor Guido Sodano.

Abstract

Preliminary results on the structure of a novel sugar from a lipopolysaccharide from *Pseudomonas corrugata*, a plant pathogenic bacterium whose several aspects of phytopathogenic mechanism are under investigation, are described. This is a 5,7-diamino-5,7,9-trideoxynon-2-ulosonic acid, isolated as an O-glycoside from the Smith degradation of the O-chain. The structure was obtained both with NMR and MS methodologies. To the best of our knowledge, this is the first example of 3-hydroxylated non-2-ulosonic acid. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: 5,7-Diamino-5,7,9-trideoxynon-2-ulosonic acid; Lipopolysaccharide; *Pseudomonas corrugata*; Bacteria–plant interaction

The interest in lipopolysaccharides (LPSs) from bacterial plant pathogens arises from involvement of this component of the outer membrane in host–parasite interactions. An updated report on this topic was recently published by Dow et al.[1] Studies on the LPS structure of some phytopathogenic Pseudomonads resulted in the identification of the O-chains[2]. In this regard, we were prompted to investigate the LPS fraction from *Pseudomonas corrugata*, the causal agent of tomato pith necrosis[3]. The pathogenetic mechanism of this bacterium has been already investigated by Chun and Leary,[4] who related the elicitation of the disease to the production of a heat-labile phytotoxin. Afterwards, Emanuele et al.[5] isolated and identified in cultures of the bacterium two lipodepsipeptides containing 22 amino acid residues, the corpeptins, which showed some phytotoxic and antibacterial activities. Investigations on the LPS of this pathogen may lead to some advances in understanding of bacteria–plant interactions.

During the investigation of the O-chain from LPS of *P. corrugata*, we found for the first time a 3-hydroxylated non-2-ulosonic acid, of which we now report our preliminary findings.

Since the discovery of the first 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid[6] as a component of the O-chain polysaccharide of lipopolysaccharides (LPSs), other stereoisomeric examples have been found, always from bacterial sources. Nowadays these make up a new class of non-2-ulosonic acids besides that of 5-amino-3,5-dideoxynon-2-ulosonic acids, which are commonly named sialic acids.[7] Structurally correlated to the latter is their deamino analogue, 3-deoxynon-2-ulosonic acid (Kdn).[8] All these non-2-ulosonic acids have, as a common structural feature, the deoxy function at the 3-position, and to our knowledge, no example of a

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3-hydroxylated analogue has been hitherto reported. In the case of oct-2-ulosonic acids, only one 3-hydroxylated analogue has been found, that is the rare Ko,[9] D-*glycero*-D-*talo*-oct-2-ulosonic acid, which can replace Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid, in lipopolysaccharides.

The LPS was obtained from the cell wall by phenol–water extraction. Mild acetic acid treatment led us to isolate the O-chain free of the lipid A moiety. The monosaccharide analysis of the O-chain by methanolysis allowed us to identify by GC–MS, the signals for fucosamine ($t_{\mathrm{R\ inositol}} = 0.613$) and glucose ($t_{\mathrm{R\ inositol}} = 0.805$), in addition to two minor unidentified peaks occurring at high retention times ($t_{\mathrm{R\ inositol}} = 1.258$ and 1.332). On the other hand, the GC–MS analysis of the alditol acetates of the acid-hydrolysed O-chain revealed only the peaks of fucosamine and glucose. More diagnostic evidence was obtained from the preliminary NMR analysis of the O-chain. The ^{1}H NMR spectrum suggested a regular polysaccharide structure containing three acetyl groups, one *O*- and two *N*-acetyls, a 3-hydroxybutyryl residue linked as an amide, and two additional methyl groups as doublets. One of them was assigned to the 6-CH_3 of fucosamine, and the other to the unknown sugar, which must also contain two nitrogen-bearing carbons in order to explain the three amide linkages, two as *N*-acetyls and the third as *N*-hydroxybutyryl, only one of these being on the fucosamine residue. At this stage, a trisaccharide-repeating unit built of glucose, fucosamine, and the unknown sugar was surmised. However, an ^{1}H–^{13}C HSQC spectrum revealed the presence of only two anomeric methine carbons, assignable to glucose and fucosamine residues, suggesting a ketose structure for the third sugar. The ^{13}C NMR spectrum confirmed this hypothesis showing three anomeric signals. In addition, five carboxyl signals were evident, one more than the number expected. Considering the total number of carbon atoms, it could be surmised that a nine-carbon residue was that of the unknown sugar. On this basis, the obvious hypothesis was that the unknown sugar was a 3-hydroxylated non-2-ulosonic acid, because the classical NMR signals of 3-deoxy analogues were missing.

Since any attempts to isolate this sugar both by hydrolysis with triflic acid[10]and methanolysis were unsuccessful, we performed two consecutive Smith degradations of the O-chain, obtaining the novel sugar as the glycoside. The ^{13}C NMR spectrum (Fig. 1) showed signals for 18 carbon atoms, three carboxyl groups (δ 173.5–173.4), one anomeric carbon (δ 101.5), two carbons bearing nitrogen (δ 47.9, 53.7), two methyl doublets (δ 16.1, 21.9), a methylene carbon at δ 45.0, two oxymethylene carbons (δ 58.8, 60.7), one acetyl methyl group (δ 22.1) and six carbinolic methine carbons (δ 73.8–64.7). The ^{1}H NMR spectrum (Fig. 2) showed at low field, two groups of signals in the range of 4.3–4.0 ppm and 3.8–3.6 ppm, which a COSY experiment showed to be uncorrelated by scalar coupling. The HSQC spectrum showed that the protons in the region at δ 3.8–3.6 were correlated to two oxymethylene carbons and at the carbinolic methine signal at δ 74.2, indicating that these signals belong to the aglyconic glycerol moiety. Therefore, in the other region at δ 4.0–4.3, all carbinolic protons of the new sugar, in addition to that of the 3-hydroxybutyryl group, occurred. Accordingly, the integration ratio between the two protonic regions was 7:5. The lack of a cross peak at the carbon chemical shift of 101.5 ppm in the HSQC spectrum showed the quaternary nature of the anomeric carbon atom. Starting from the methyl dou-

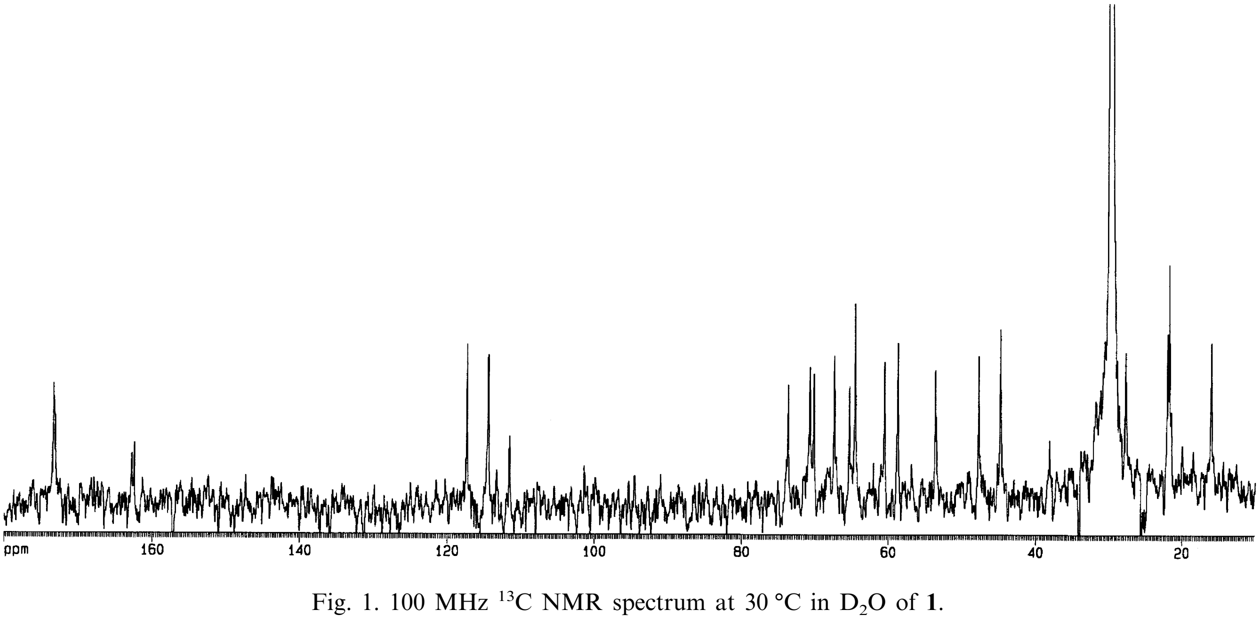


Fig. 1. 100 MHz ^{13}C NMR spectrum at 30 °C in D_2O of **1**.

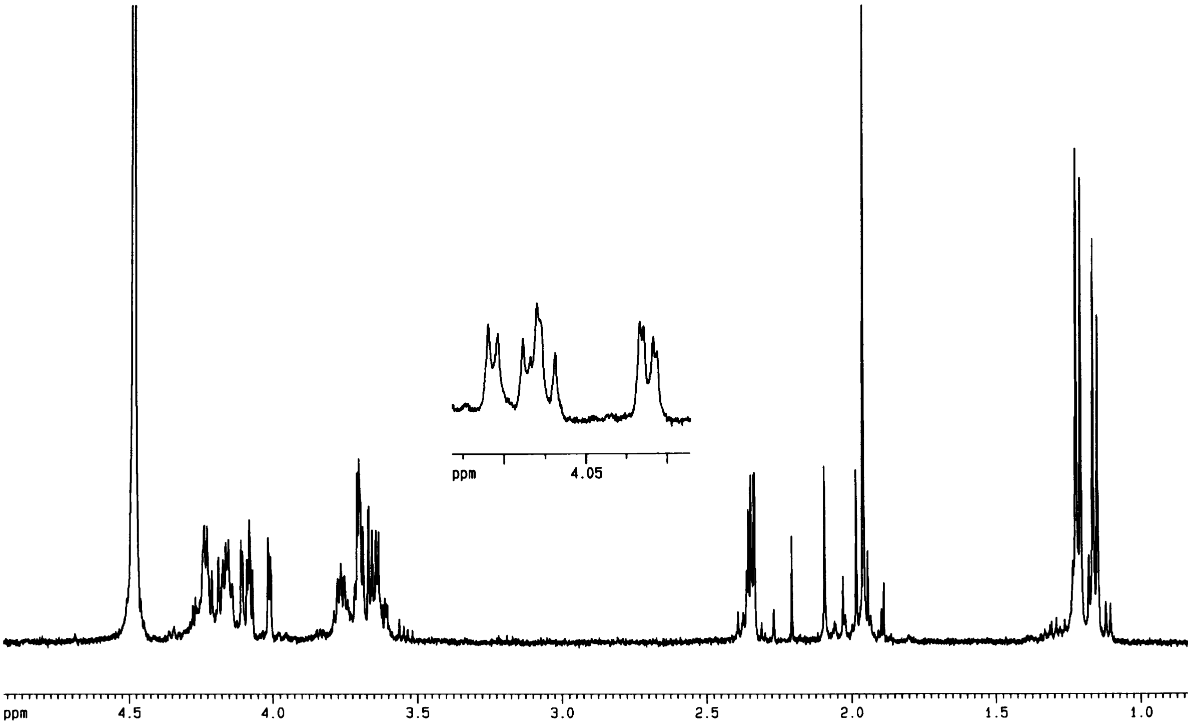


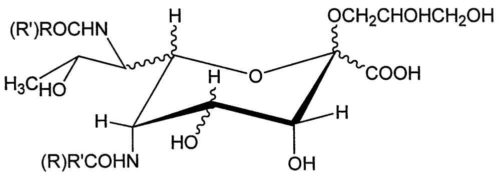
Fig. 2. 400 MHz ^{1}H NMR spectrum at 50 °C in D_2O of **1**.

Table 1
^{1}H and ^{13}C NMR data (δ in ppm), apparent coupling constant in parentheses (Hz) for **1**

	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-7 C-7	H-8 C-8	H-9 C-9
New sugar			4.01 dd	4.08 dd	4.25	4.10 dd	4.22	4.27	1.18 d
			(3.2; 0.9)	(3.2; 4.4)		(2.5; 10.3)			(6.3)
	173.5 [a]	101.5	70.2	65.4	47.9	70.8	53.7	67.5	16.2
$-OCH_2CHOHCH_2OH$	3.6–3.7	3.77	3.6–3.7						
	60.7	73.8	58.8						
$-COCH_2CHOHCH_3$		2.34	4.17	1.22 d (6.3)					
	173.5 [a]	45.0	64.6	21.9					
$-COCH_3$		1.97 s							
	173.9 [a]	22.1							

[a] May be interchanged.

blet at δ 1.18, the complete scalar connectivity of the protons of the unknown sugar was established by combining the information from 1D HOHAHA and COSY spectra. This, together with the HSQC experiment, allowed most of the NMR signals to be assigned (Table 1). All these data were in agreement with a 3-hydroxylated non-2-ulosonic acid structure **1** for the new sugar. MS analysis strongly supported this suggested structure. Actually, the negative-ion ESIMS spectrum of **1** showed a pseudomolecular ion peak at m/z 467, attributable to $[M-H]^-$.



1

R= CH_3

R'= $CH_2CHOHCH_3$

Insofar as the relative configurations of ring protons are concerned, the very small coupling constant (0.9 Hz) of H-3 is particularly diagnostic. It is attributed to a W coupling between H-3 and H-5, suggesting an equatorial orientation for both protons. Unfortunately, both 1D and 2D NOE experiments were not conclusive enough to gain insight into relative configurations of other protons, and the very small amount of material available to us prevented us from using chemical approaches. However, the above data are sufficient to indicate a structure of 3-hydroxylated non-2-ulosonic acid. Work is in progress for the assignment of both the relative and absolute configurations.

1. Experimental

General.—NMR spectra were recorded on a Bruker DRX 400 Avance spectrometer using a 5-mm multinuclear inverse Z-grad probe. ^{13}C and ^{1}H chemical shifts were measured in D_2O using 1,4-dioxane (δ 67.4) and sodium 3-trimethylsilylpropionate-2,2,3,3,-$^{2}H_4$ (δ 0.00), respectively, as internal standards. Two-dimensional experiments (COSY, HSQC) and one-dimensional HOHAHA were performed using standard pulse sequences available in the Bruker software. Electrospray-ionisation mass spectra (ESIMS) were performed on a Micromass ZQ instrument (Waters). The sample (100 pmol) was deionised on Dowex-50 H^+ resin (Fluka) and dissolved in 2% triethylamine in 50% MeCN and injected into the ion source at a flow rate of 5 μL min^{-1}. The spectrum was acquired in negative-ion mode. All the GC–MS analyses were performed on a Hewlett–Packard 5890 instrument equipped with an RTX-5 capillary column (Restek, 30 m × 0.25 mm i.d., flow rate, 1 mL min^{-1}, He as carrier gas). Acetylated methyl glycosides analysis was performed with the following temperature program: 150 °C for 5 min, 150 → 250 °C at 3 °C min^{-1}, 250 °C for 10 min. The alditol acetate mixture was analysed with the following temperature program: 150 °C for 5 min, 150 → 300 °C at 3 °C min^{-1}.

Preparation of cellular lipopolysaccharide.—*P. corrugata* strain 2445, obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, UK, was grown in 1-L flasks containing 400 mL of PDB (Difco) supplemented with 0.4% (w/v) Casamino acids (Difco) at 27 °C with shaking (120 rpm) for 5 days. The culture (12 L) was centrifuged (10,000 × 15 min), and the harvested cells were washed twice with 0.85% (w/v) NaCl and lyophilised. The dry cells (17.3 g) obtained were suspended in 280 mL of ultrapure Milli-Q water and extracted with phenol according to the conventional procedure.[11] The resulting aqueous phases were combined and dialysed (cut-off 3500 Da) for 2 days. Contents of the tubes were lyophilised; the residue was dissolved in ultrapure Milli-Q water (60 mL) and brought to 1.5% (w/v) NaCl and 1.5% (w/v) Cetavlon (hexadecyltrimethylammonium bromide) while stirring at rt for 15 min. The mixture was centrifuged (5000*g* for 45 min), and the supernatant solution was mixed with 10 vols of cold (−20 °C) EtOH and left overnight at −20 °C. The resulting precipitate was collected by centrifugation (5000*g* at 5 °C for 1 h), dissolved in ultrapure Milli-Q water (65 mL), and dialysed as described above. The contents were lyophilised to yield "crude" LPS (307 mg).

Hydrolysis of LPS.—A sample (300 mg) of the LPS fraction was hydrolysed with 1% AcOH at 100 °C for 90 min. After cooling, the sample was centrifuged at 3000 rpm for 15 min. The supernatant (240 mg) was applied to a Sephacryl S-300 HR (Pharmacia) column and eluted with 50 mM NH_4HCO_3 buffer, obtaining the O-chain in the void volume together with a glucan polymer (17 mg). The O-chain was further purified by a cold precipitation with EtOH. In brief, the mixture of the two polysaccharides was dissolved in Milli-Q water and treated with cold EtOH (up to 4 vols) at −20 °C for 16 h. The pellet containing the glucan was separated from the liquid phase by centrifugation, and the supernatant was dried (O-chain, 6 mg).

Glycosyl analysis.—A sample of O-chain (1 mg) was dried under vacuum over P_2O_5 for 16 h and then subjected to methanolysis by adding 1 M HCl–MeOH (1 mL) for 20 h at 80 °C. The methyl glycosides obtained were acetylated with pyridine (200 μL) and Ac_2O (100 μL) for 30 min at 100 °C and analysed by GC–MS. Another sample of O-chain (1 mg) was hydrolysed with 9:1 2 M H_2SO_4–AcOH (2 mL) for 9 h at 100 °C, reduced with $NaBD_4$, and acetylated and analysed by GC–MS.

Smith degradation.—The O-chain (4 mg) was treated with 0.1 M $NaIO_4$ (0.3 mL) at 4 °C for 96 h, as previously described. After the usual workup, the sample was treated with 2.0 mL 0.5 M TFA at rt for 72 h. After TFA evaporation, the crude mixture was purified on a Bio-Gel P-2 (Bio-Rad) column using ultrapure Milli-Q water as eluent. The run was monitored with a differential refractomer (Waters). The main fraction obtained was again Smith degraded as above. The product of the second degradation was further purified by HPLC using a C_{18} column (Macherey–Nagel), eluted with 4:1 CH_3OH–water giving mainly the glycoside **1**.

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