ELSEVIER

Carbohydrate Research 269 (1995) 157–166

CARBOHYDRATE RESEARCH

The structure of the O-specific polysaccharide from *Thiobacillus* sp. IFO 14570, with three different diaminopyranoses forming the repeating unit

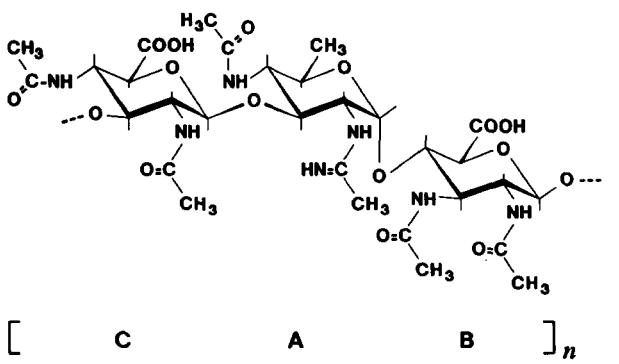
Alexander S. Shashkov [a], Sergio Campos-Portuguez [b], Helga Kochanowski [b], Akira Yokota [c], Hubert Mayer [b,*]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Leninsky Prospect 47, Moscow 117913, Russian Federation*
[b] *Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg i.Br.-Zähringen, Germany*
[c] *Institute for Fermentation, Osaka, 17-85 Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan*

Received 28 July 1994; accepted in revised form 20 October 1994

Abstract

The O-specific polysaccharide, liberated by mild acid hydrolysis of the lipopolysaccharide (LPS-P) from *Thiobacillus* sp. IFO14570 as isolated from the phenol phase after phenol–water extraction, is shown to have a linear trisaccharide repeating-unit containing three different diamino sugars, namely 2,4-diacetamido-2,4-dideoxyglucuronic acid, 2-acetamidino-4-acetamido-2,4,6-trideoxyglucopyranose, and 2,3-diacetamido-2,3-dideoxyglucuronic acid in the molar ratio of 1 : 1 : 1. On the basis of ^{1}H and ^{13}C spectroscopy, including 2D COSY and coherent transfer (RCT)COSY, 1D NOE in the difference mode, and 2D rotating frame NOE(ROESY) the sequence, the type of substitution, and the position of the acetamidino group could be determined. These experiments allowed formulation of the following structure for the O-specific polysaccharide, although the depicted D configuration of the three sugar residues is not yet proven:



* Corresponding author.

SSDI 0008-6215(94)00351-3

Keywords: *Thiobacillus* sp. IFO 14570; Diaminopyranoses; Polysaccharide, O-specific

1. Introduction

The genus *Thiobacillus* does not represent a phylogenetically coherent taxonomic group [1]. Recent phylogenetic studies based on 16S rRNA revealed that, although all the thiobacilli thus far described do belong to the *Proteobacteria* [2], they are spread into the α, β, and γ subdivision [3,4] of this taxon. Previous studies have shown that the composition of the lipid A moiety of the cell-wall lipopolysaccharides of thiobacilli reflects their phylogenetic position [5]. The two species now put together into the α-2 subgroup of *Proteobacteria*, namely *Thiobacillus* sp. IFO 14570 and *T. novellus*, are closely related and have as their nearest relatives two rhizobial species, namely *Rhizobium loti* and *R. huakuii* [6]. These α-2 group thiobacilli share lipid A's with 2,3-diamino-2,3-dideoxy-D-glucose as the backbone sugar (lipid A_{DAG}) [5]. Previous analyses of isolated LPS of *Thiobacillus* sp. IFO 14570 were severely hampered by the fact that LPS was obtained from the water phase, after phenol–water extraction [7], in only extremely low yields ($\sim$ 1–2% of the expected amounts [4]). After recognizing that the bulk of S-type LPS was extracted into the phenol phase and only to a very small amount into the water layer (mainly R-type LPS), a more-detailed investigation of the O-chain polysaccharide (PS) by ^{1}H and ^{13}C NMR analyses became possible. As a result of this NMR study, it was observed that the O-chain of the LPS of *Thiobacillus* sp. IFO 14570 is built up from three different diaminohexoses and does not posses any unsubstituted OH groups. This hydrophobic character of the O-chain explains the unexpected phenol-solubility of the IFO 14570 LPS upon phenol–water extraction. Together with the 2,3-diamino-2,3-dideoxy-D-glucose of the lipid A moiety [4] this unusual LPS contains 4 different diamino sugars, all sharing the *gluco* configuration.

2. Results

LPS extracted from the freeze-dried bacteria by the hot phenol–water procedure [7] into the water phase (LPS-W) amounted only to ca. 0.03%, based on the bacterial dry weight. The bulk of LPS (LPS-P) was detected in the phenol layer of phenol–water extracts and was obtained in a yield of 4.6% (based on bacterial dry weight). It was not possible to extract LPS by phenol–chloroform–light petroleum ether extraction [8].

The LPS material from both phases (LPS-W and LPS-P) was characterized by DOC-PAGE [9,10] (Fig. 1). Only LPS-P afforded the expected pattern, that is, showing an S-type-specific pattern with O-chains being composed of up to 25 repeating units. In contrast, LPS-W showed almost exclusively a fast-migrating R-type LPS [11], as also observed, but in a lower amount, with LPS-P.

The O-specific polysaccharide (PS), liberated by acetic acid hydrolysis in a yield of $\sim$ 60% (based on LPS dry mass), revealed, on examination by ^{13}C NMR (Fig. 2a and Table 1), an unusual composition of its repeating unit. For the three signals of anomeric carbon atoms at 103.0 ppm (double intensity) and 97.9 ppm, six signals of carbon atoms

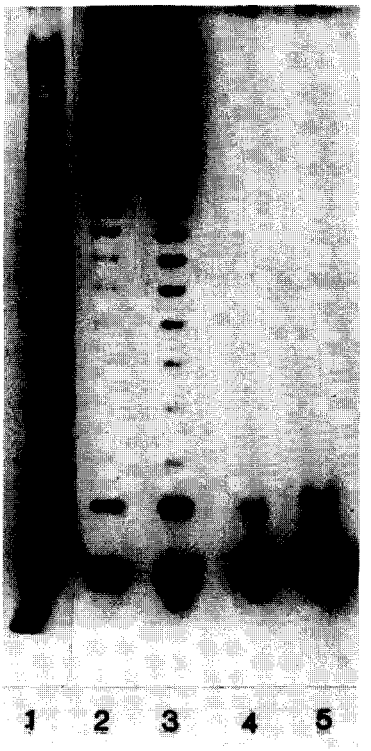


Fig. 1. DOC-PAGE with LPS from *Salmonella montevideo* SH94 (3 μg, lane 1), LPS-P from *Thiobacillus* sp. IFO 14570 (1 and 3 μg, lanes 2 and 3), and LPS-W from *Thiobacillus* sp. IFO 14570 (1 and 3 μg, lanes 4 and 5).

bearing nitrogen (at 53.8–58.3 ppm) were observed. Five peaks (at 23.1–23.5 ppm) together with five peaks in the low-field range (174.1–176.8 ppm) obviously belonged to acetyl amino groups. The sixth pair of signals at 20.4 and 168.5 ppm was identified as belonging to an acetamidino group [12]. One signal, typical for a C-CH_3 group of a 6-deoxypyranose, was present at 17.9 ppm, and there were also two signals at low field (172.4 and 173.0 ppm) characteristic for uronic acids. Carbon atoms bearing one oxygen were represented in the spectrum by five signals at 68.6–79.1 ppm (one of them with double intensity). No signals of CH_2O groups were revealed among the former ones in the APT-spectrum [13]. Thus, the trisaccharide repeating-unit contained three diamino sugars, two of them were uronic acids and the third one a 6-deoxyhexose. Five of the six amino groups were acetylated and the sixth one was substituted by an acetamidino group.

The ^1H NMR spectrum of the polysaccharide (Fig. 2b) was assigned using both 1D homonuclear double resonance in the difference mode [14] and 2D COSY (not shown), and relayed coherence transfer (RCT) COSY (Table 1). Analysis of the coupling constants [15] showed that all sugar residues were in the pyranose form with *α-gluco* (6-deoxy sugar) or *β-gluco* (uronic acids) configurations.

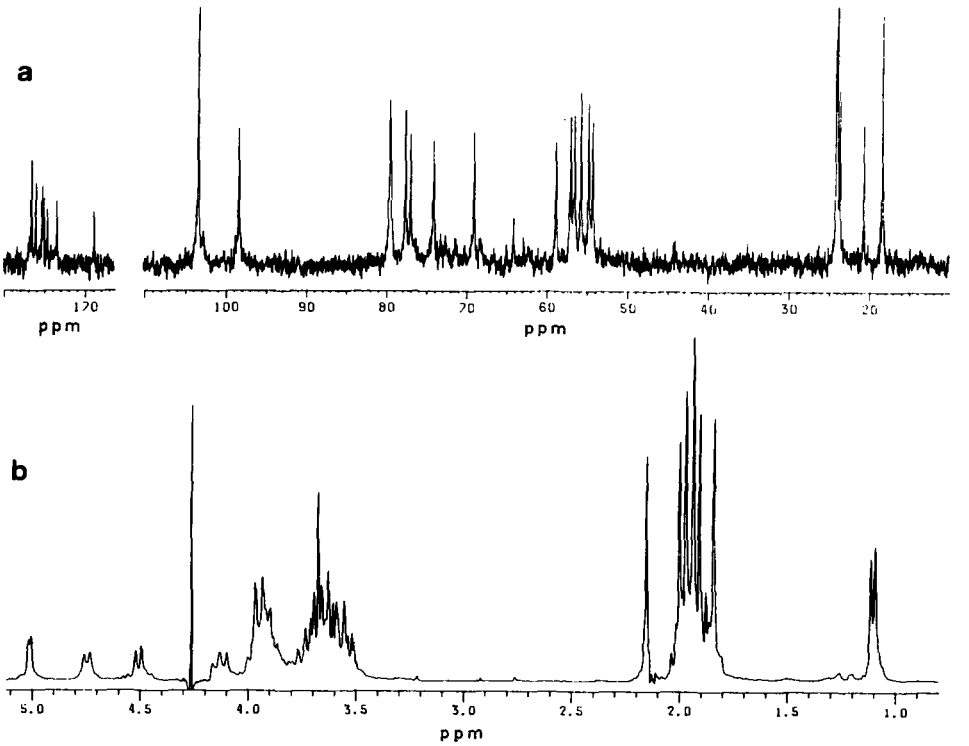


Fig. 2. (a) ^{13}C NMR spectrum and (b) ^{1}H NMR spectrum of the O-specific polysaccharide of the LPS-P from *Thiobacillus* sp, IFO 14570. The signal at 64 ppm in Fig. 2a does not originate from the O-repeating units.

The sequence, the type of substitution, and also the localisation of the acetamidino group, were determined using both 1D NOE experiments in the difference mode (Fig. 3) and 2D rotating frame NOE (ROESY [16], Fig. 4). Pre-irradiation of the anomeric proton of the 6-deoxypyranose (residue A) provided in the 1D difference NOE spectrum an intense peak at 3.9–4.0 ppm (Fig. 3A) and small peaks for H-3 and H-4 of the same residue, as well as H-2 and H-3 of one of the uronic acids (residue **B**). The ROESY spectrum (Fig. 4) showed the only cross-peak on the line of the chemical shift of H-1 of residue **A**, corresponding to the main peak in the 1D spectrum. This observation indicates that the inter-residue NOE peak overlapped with the signal of H-2 of residue **A** and that the aforementioned small signals in the 1D spectrum arose due to spin diffusion, characteristic for polymers. Spin diffusion may occur in the frame of the same residue and the only candidate for explaining the small peaks belonging to protons of residue **B** is the signal of H-4 of unit **B**, which overlaps with the signal H-2 of residue **A** (Table 1). Then residue **B** must be glycosylated by residue **A** at position C-4. The 1D NOE spectrum, under pre-irradiation of H-1 of residue **B** (Fig. 3B), contained intra-residue NOE peaks of H-2, 3, and 5, as usually observed for β-pyranoses, and the only

Table 1
Chemical shifts (^{1}H and ^{13}C) for the repeating unit of *Thiobacillus* sp 14570 degraded PS [a]

Residue	Proton	δ	Coupling $J_{H,H}$ (Hz)	Carbon	δ	$J_{C\text{-}1,H\text{-}1}$ (Hz)
→ 3)-α-D-Pyr1 [c]-1(1 →	H-1	5.01	$J_{1,2}$ 3.5	C-1	97.9	173
	H-2	3.95	$J_{2,3}$ 10	C-2	54.3	
	H-3	3.73	$J_{3,4}$ 10	C-3	76.6	
(A)	H-4	3.55	$J_{4,5}$ 9.5	C-4	58.3	
	H-5	3.68	$J_{5,6}$ 6	C-5	68.6	
	H-6	1.10		C-6	17.9	
→ 4)-β-D-Pyr2 [c]-(1 →	H-1	4.74	$J_{1,2}$ 8	C-1	103.0	164
	H-2	3.66	$J_{2,3}$ 9.5	C-2	53.8	
	H-3	4.13	$J_{3,4}$ 9.5	C-3	56.0	
(B)	H-4	3.93	$J_{4,5}$ 10	C-4	73.6	
	H-5	3.98		C-5	77.1	
				C-6	172.4 [b]	
→ 3)-β-D-Pyr3 [c]-(1 →	H-1	4.50	$J_{1,2}$ 8	C-1	103.0	164
	H-2	3.54	$J_{2,3}$ 10	C-2	55.3	
	H-3	3.90	$J_{3,4}$ 10	C-3	79.1	
(C)	H-4	3.62	$J_{4,5}$ 9	C-4	56.5	
	H-5	3.69		C-5	79.1	
				C-6	173.0 [b]	

[a] Recorded in D_2O (75°C), with acetone (δ_H 2.225; δ_C 31.45) as internal standard.
[b] May be interchanged.
[c] Pyr1: 2-acetamidino-4-acetamido-2,4,6-trideoxy-α-glucopyranose; → 3)-α-6-d-Glc*p*NAmAc-(1 →.
Pyr2: 2,3-diacetamido-2,3-dideoxy-β-glucopyranosid uronic acid; → 4)-β-Glc*p*$(NAc)_2$A-(1 →.
Pyr3: 2,4-diacetamido-2,4-dideoxy-β-glucopyranosid uronic acid; → 3)-β-Glc*p*$(NAc)_2$A-(1 →.
$[\rightarrow 3)\text{-}\alpha\text{-D-Pyr1-}(1 \rightarrow 4)\text{-}\beta\text{-D-Pyr2-}(1 \rightarrow 3)\text{-}\beta\text{-D-Pyr3-}(1 \rightarrow]_n$
(A) (B) (C)

inter-residue peak observed for H-3 of the second uronic acid (residue **C**). The ROESY spectrum showed the same cross-peaks on the line with the chemical shift of H-1 of residue **B** excluding the intra-residue peaks H-1 and H-2 of **B**.

Pre-excitation of the anomeric proton of the remaining residue **C** provided the only inter-residue peak of H-3 of **A** together with the intra-residue signals for H-2, 3, and 5 of **C** (Fig. 3C). Corresponding cross-peaks may also be observed in the ROESY spectrum on the co-ordinates with the chemical shift of H-1 of residue **C** (Fig. 4). Thus, all pyranoses are substituted, as already discussed, and as a consequence should therefore, be diamino sugars. The heteronuclear 2D ^{1}H–^{13}C spectrum [17] confirmed the location of the amino groups and the type of substitution of the residues (Table 1).

1D Selective heteronuclear {^{1}H}–^{13}C double-resonance experiments with saturation of the most downfield protons, including CH_3-C-N (at 2.18 ppm), confirmed that the latter belonged to the acetamidino group. Thus, the downfield shift of the acetamidino methyl signals, in comparison to those of the acetamido groups, proved to be common for sugars with *manno* [12] and *gluco* configurations.

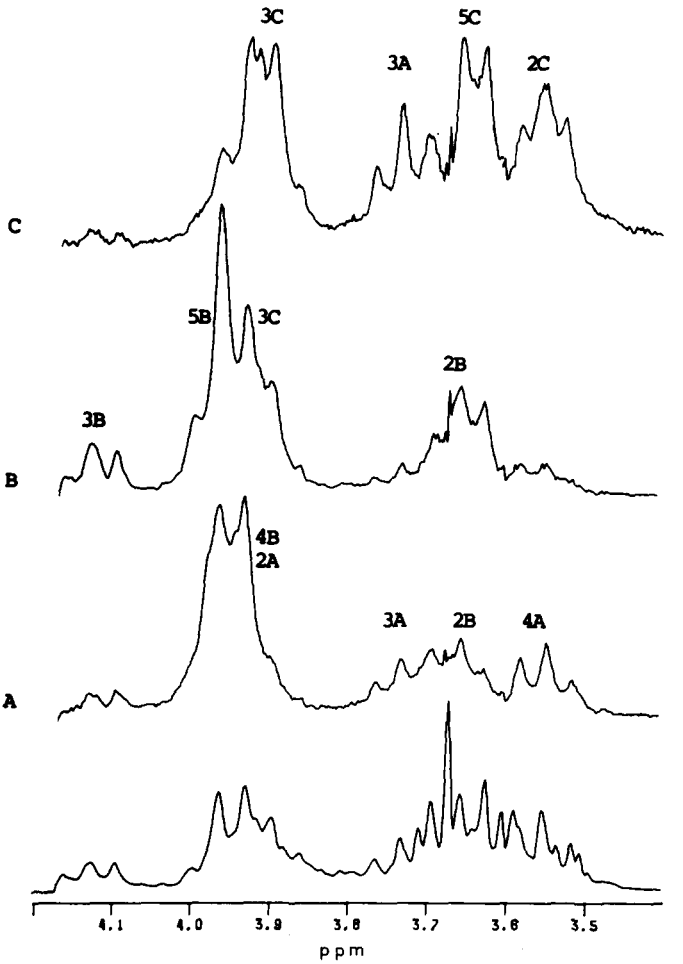


Fig. 3. Part of the ^{1}H NMR spectrum, together with 1D NOE experiments, obtained with pre-irradiation of the anomeric protons of Pyr1 (A), Pyr2 (B), and Pyr3 (C).

The location of the acetamidino group and the relative absolute configuration of the sugar residues **A–C** were determined using long-range NOE, as observed in 1 and 2D NOE spectra. Thus, the inter-residue peaks in the coordinates of the chemical shifts of H-5(**A**) and H-5(**B**) in the ROESY spectrum (Fig. 4) may only be explained on the basis of the same absolute configuration of these two pyranoses involved. The main [18] or the preferred [19] conformation of the glycosidic linkages A1 → 4B, with the angles $\varphi + 60°$ and ψ about 0°, leads to a close proximity of the aforementioned protons, whereas an angle $\varphi - 60°$, which is characteristic for a different absolute configuration of the pyranoses involved, excludes such a proximity.

A 1D NOE experiment with selective pre-irradiation of CH_3 of the acetamidino group (signal at 2.18 ppm) showed in the difference spectrum *inter alia* signals of H-1

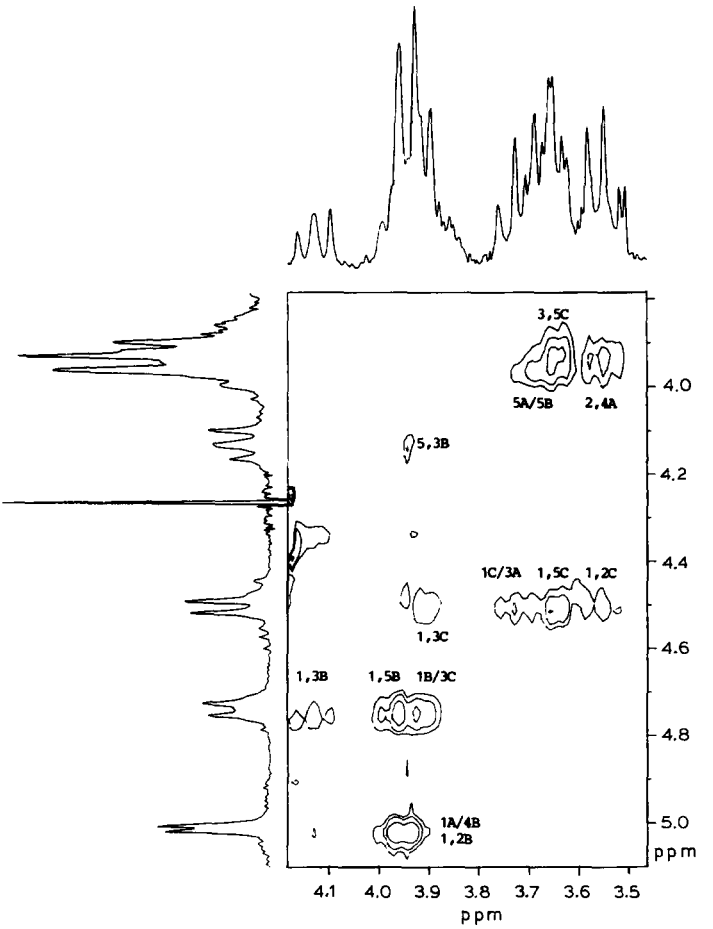


Fig. 4. Part of a ROESY spectrum of the O-specific polysaccharide from LPS-P of *Thiobacillus* sp. IFO 14570. Arabic numbers refer to the protons in the sugar residues denoted by letters. (see Table 1 and formula).

of the two residues **A** and **C** (but not of **B**). Taking the structure of the sugar residues into account, as well as their sequence and their type of substitution, then the only position that provides closeness of the CH_3-C(=ND)ND-group with the two anomeric protons simultaneously, is the position 2 of the 3-substituted residue **A** (Fig. 5). Moreover, the proximity of these protons with the anomeric proton of the glycosylating residue **C** arises only with the same absolute configuration of both the glycosylated and the glycosylating residues. Where there different absolute configurations of these residues in their favored conformations, the anomeric proton of **C** and the protons of a substituent at position 4 of the residue **A** would be observed close to each other. Assuming the absolute D configuration for the residue **A** ("bacillosamine"), the

following tentative structure of the repeating unit of the O-chain of *Thiobacillus* sp, IFO 14570, based solely on the NMR investigation, as shown in Fig. 5, may be proposed:

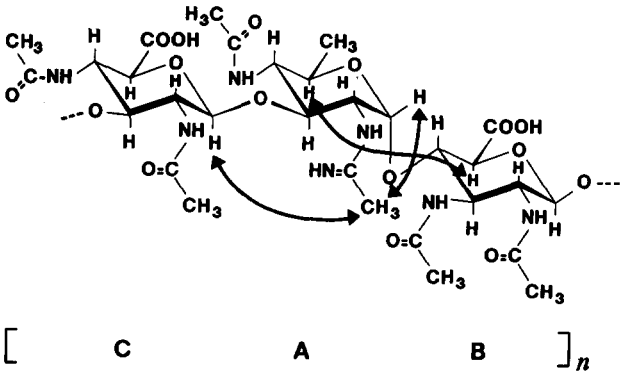


Fig. 5. Structure of the O-repeating unit of the O-chain of *Thiobacillus* sp. IFO 14570 with NOEs proving the location of the acetamidino group (see text for details). The absolute D configuration of the three pyranoses, although very likely, it not yet proven.

3. Discussion

An S-type material (LPS-P) was identified in the phenol phase after phenol–water extraction of *Thiobacillus* sp. IFO 14570, thus indicating a high hydrophobicity of the LPS-P. The chemical structure of the O-chain, being composed of three different diaminohexoses, showed no single unsubstituted OH group, and furthermore the NH_2 groups were found to be quantitatively substituted as acetamido or acetamidino groups. This lack of free hydroxyl groups, in addition to the 6-deoxy group of the bacillosamine derivative, prevails over the hydrophilic groups in the O-chain, namely the two carboxylic groups of the diaminouronic acids and the acetimidoyl group of the bacillosamine derivative. Similar observations were recently reported with the lipopolysaccharide of *Legionella pneumophila* ATCC 33152 [20], which was also obtained from the phenol phase of phenol–water extracts and which likewise showed no free OH-group in its O-chain, being a homopolymer of 2,4-linked 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid.

2,3-Diamino-2,3-dideoxyhexuronic acids having either the D-*gluco* [21], the L-gulo, or the D-*manno* configurations have previously been found in bacterial polysaccharides, especially from *Pseudomonas aeruginosa* (cf. Lindberg [22]), but 2,4-diaminouronic acids have to our knowledge not yet been reported as LPS constituents. 2,4-Diamino-2,4,6-trideoxy-D-glucose ("bacillosamine") in the form of its 4-*N*-acetyl derivative was first reported as a component of *Bacillus licheniformis* [23], whereas the 2,4-diamino-2,4,6-trideoxy-D-galactose is a component of the *Shigella sonnei* O-antigen [24]. In *Pseudomonas aeruginosa* NCTC 8505 the 2,4-diacetamido-2,4,6-trideoxyglucose and in *Pseudomonas aeruginosa* serotypes O3 (Habs) and O1 (Lányi) the 2-acetamido-2,4,6-trideoxy-4-[(*S*)-3-hydroxybutanamido]-D-glucose were encountered as part of their O-

specific polysaccharide chains [25]. The 2-acetamido-4-acetamidino derivative of "bacillosamine", here identified as part of the O-chain of *Thiobacillus* sp. IFO 14570, was hitherto unknown. Acetamidino groups substituting amino groups of aminouronic acids [26], of 6-deoxyhexosamines [26] and of pseudaminic acid [20] have repeatedly been reported.

4. Experimental

General methods.—NMR spectra were recorded with a Bruker AM-300 spectrometer for solutions in D_2O at 50°C using acetone as the internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm) by using standard Bruker software for carrying out NOE and 2D shift-correlated spectroscopy.

Bacterial strain, cultivation, and isolation of lipopolysaccharide and O-specific polysaccharide.—Thiobacillus sp. IFO 14570 (= THI 051, ATCC 12817) [27] was obtained from the culture collection of the Institute for Fermentation, Osaka (Osaka, Japan). The bacteria were cultivated under aerobic conditions at 28°C and pH 7.0 in the medium described previously [4]. After reaching the stationary phase, the cells were harvested by centrifugation, washed with distilled water, and lyophilized. LPS was isolated from lyophilized cells using the hot phenol–water extraction procedure, followed by repeated ultracentrifugations (105 000 *g*, 4 h). The material thus obtained (LPS-W) was further purified by digestion with ribonuclease (Sigma, Deisenhofen, FRG) and proteinase K (Merck, Darmstadt, FRG). Since the LPS yield was only 0.03%, based on bacterial dry weight, the phenol phase was extensively dialyzed and finally lyophilized. The phenol-phase material was then subjected to phenol–chloroform–light petroleum extraction [8]. It was not possible, however, to sediment any material in the usual way, namely by a dropwise addition of water to the phenol phase. The whole phenolic phase was therefore diluted with water, extensively dialyzed against tap water (for 3–5 days and finally against distilled water for 24 h). After lyophilisation, the phenol-phase material was ultracentrifuged (see above) and the sediment treated with proteinase K. The yield of LPS-P was 4.6%, based on bacterial dry weight. LPS was hydrolyzed with 2 M acetic acid (1.5 h, 100°C) and the precipitated lipid A was removed by centrifugation. After washing the material with 9:1 chloroform–methanol, the O-chain material (PS) was dialyzed, freeze-dried, and separated on Sephadex G-50 to give the O-specific polysaccharide in a yield of ~ 40%, based on LPS dry weight.

DOC-PAGE of LPS.— Poly(acrylamide) gel electrophoresis was carried out using DOC as detergent, following the method described previously [9,10]. After pre-electrophoresis at 25 mA without substance, the samples were separated at 20 mA. LPS bands were visualized by silver staining as described by Tsai and Frasch [28].

References

[1] C.R. Wose, *Microbiol. Rev.*, 51 (1987) 221–272.
[2] E. Stackebrandt, R.G.E. Murray, and H.G. Trüper, *Int. J. Syst. Bacteriol.*, 38 (1988) 321–325.

[3] C.R. Woese, E. Stackebrandt, W.G. Weisburg, B.J. Paster, M.T. Madigan, V.J. Fowler, C.M. Hahn, P. Blanz, R. Gupta, K.H. Nealson, and G.E. Fox, *System Appl. Microbiol.*, 5 (1984) 315–326.
[4] A. Yokota, M. Rodriguez, Y. Yamada, K. Imai, D. Borowiak, and H. Mayer, *Arch. Microbiol.*, 149 (1987) 106–111.
[5] H. Mayer, J.H. Krauss, A. Yokota, and J. Weckesser, in H.H. Friedman et al. (Eds.), *Endotoxin*, Plenum, New York 1990, pp. 45–70.
[6] Y. Katayama, A. Hiraishi, and H. Kuraishi, *Annu. Meeting Jpn. Soc. Biosci. Biotechnol., Agrochem.*, Tokyo, April 1994, (in Japanese), *Phylogenetic studies on the strains of* Thiobacillus *species based on 16S rRNA sequences*, 1994, *Abstr.*, p. 336.
[7] O. Westphal, O. Lüderitz, and F. Bister, *Z. Naturforschg.*, 7b (1952) 148–155.
[8] C. Galanos, O. Lüderitz, and O. Westphal, *Eur. J. Biochem.*, 9 (1969) 245–249.
[9] T. Komuro and C. Galanos, *J. Chromatogr.*, 450 (1988) 381–387.
[10] J.H. Krauss, J. Weckesser, and H. Mayer, *Int. J. Syst. Bacteriol.* 38 (1988) 157–163.
[11] E.T. Palva and H.P. Mäkelä, *Eur. J. Biochem.*, 107 (1980) 137–143.
[12] Yu.A. Knirel, N.A. Paramonov, E.V. Vinogradov, A.S. Shashkov, B.A. Dmitriev, N.K. Kochetkov, E.V. Kholodkowa, and E.S. Stanislavsky, *Eur. J. Biochem.*, 167 (1987) 549–561.
[13] S.L. Patt and J.N. Shoolery, *J. Magn. Reson.*, 46 (1982) 535–539.
[14] N.A. Kocharova, Yu.A. Knirel, A.S. Shashkov, N.K. Kochetkov, and G.B. Pier, *J. Biol. Chem.*, 263, 11291–11305.
[15] C. Altona and C.A.G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–429.
[16] D. Morrison and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–970.
[17] A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–569.
[18] R.U. Lemieux, K. Bock, L.T. Delbaere, S. Koto, and V.S. Rao, *Can. J. Chem.*, 58 (1980) 631–663.
[19] G.M. Lipkind, A.S. Shashkov, S.S. Mamyan, and N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
[20] Yu.A. Knirel, E.Th. Rietschel, R. Marre, and U. Zähringer, *Eur. J. Biochem.*, 221 (1994) 239–245.
[21] Yu.A. Knirel, N.A. Kocharova, A.S. Shashkov, B.A. Dmitriev, and N.K. Kochetkov, *Carbohydr. Res.*, 93 (1981) C12–C13.
[22] B. Lindberg, *Adv. Carbohydr. Chem. Biochem.*, 180 (1990) 279–318.
[23] U. Zehavi and N. Sharon, *J. Biol. Chem.*, 248 (1973) 433–438.
[24] L. Kenne, B. Lindberg, K. Petersson, E. Katzenellenbogen, and E. Romanowska, *Carbohydr. Res.*, 78 (1980) 119–126.
[25] Yu.A. Knirel, E.V. Vinogradov, A.S. Shashkov, S.G. Wilkinson, Y. Tahara, B.A. Dmitriev, N.K. Kochetkov, E.S. Stanislavsky, and G.M. Mashilova, *Eur. J. Biochem.*, 155 (1986) 659–669.
[26] L.M. Beynon, J.C. Richards, and M.B. Perry, *Carbohydr. Res.*, 256 (1994) 303–317.
[27] Y. Katayama-Fujimura, N. Enokizono, T. Kaneko, and H. Kuraishi, J. Gen. *Appl. Microbiol.*, 29 (1983) 287–295.
[28] C.M. Tsai, and C.E. Frasch, *Anal. Biochem.*, 119 (1982) 115–119.