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NMR study of virenose and dihydrohydroxystreptose isolated from *Coxiella burnetii* phase I lipopolysaccharide

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Abstract

A lipopolysaccharide (LPS I) isolated from *Coxiella burnetii* in virulent phase I contains in its *O*-polysaccharide chain two unusual sugars, virenose (6-deoxy-3-*C*-methylgulose) and dihydrohydroxystreptose [3-*C*-(hydroxymethyl)lyxose]. The sugars were isolated from LPS I, after acid hydrolysis and removal of lipid A, by a combination of HPLC and preparative paper chromatography. Their enantiomeric forms and ring conformations were established from optical rotation and NMR data. Two-dimensional COSY, HSQC, and HMBC as well as one- and two-dimensional NOEs were used to assign all proton and carbon signals in both monosaccharides. Virenose was found to be the D-*gulo* enantiomer with the $^4C_1$ ring conformation and dihydrohydroxystreptose was shown to be the L-*lyxo* enantiomer also with the $^4C_1$ conformation. The latter sugar was reported [Š. Schramek, J. Radziejewska-Lebrecht, and H. Mayer, *Eur. J. Biochem.*, 148 (1985) 455–461] to be present in LPS I in a furanose form, and it appears that a furanose to pyranose tautomerization took place in the course of the isolation procedure.

Keywords: *Coxiella burnetii*; Lipopolysaccharide; Virenose; Dihydrohydroxystreptose; NMR ring conformations; Tautomerization

1. Introduction

In *Coxiella burnetii*, an obligatory intraphagolysosomal parasiting bacterium, a lipopolysaccharide (LPS I) is present as a structural component of the outer membrane of the cell envelope [1]. It plays an essential role in interaction between the microbe and host, including pathogenicity and immunogenicity of the agent. It is capable of inducing antibody response, and it is considered to be a protective immunogen [2,3]. The compositional analysis of LPS I revealed [4–6], among other sugars, the presence of an appreciable amount of virenose (6-deoxy-3-*C*-methylgulose) and dihydrohydroxystreptose [3-*C*-(hydroxy-

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methyl)lyxose] in its *O*-polysaccharide chain. To our knowledge, neither sugar has been found in other LPSs and they may be considered as important chemotaxonomic markers. Moreover, their release from LPS I, under mildly acidic conditions, led to a considerable decrease [4] in the serological activity of LPS I as shown by the passive hemolysis test. Virenose and dihydrohydroxystreptose isolated from LPS I were characterized for the first time by GC–MS [4] and the latter sugar also by NMR spectroscopy [7]. However, the data concerning both their enantiomeric forms and ring conformations were ambiguous. Thus, the $^1C_4$ conformation and the L form were presented [4,8] for virenose, although insufficient evidence was offered in this respect. Similarly, much uncertainty was connected with dihydrohydroxystreptose. The furanose form of the sugar in LPS I was originally suggested [4]. Later, dihydrohydroxystreptose (the pyranose form) was synthesized [7] and its NMR spectrum, although assigned incompletely, was considered to be identical with that of the sugar isolated from LPS I. However, in a further paper [8], the furanose form of the isolated sugar was presented again.

These ambiguities prompted us to study in more detail by NMR spectroscopy the ring conformations of virenose and dihydrohydroxystreptose as isolated from LPS I.

2. Experimental

Isolation of dihydrohydroxystreptose and virenose.—LPS I from *C. burnetii* strain Priscilla in virulent phase I was isolated as described [6]. The LPS I was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 100 °C. The precipitated lipid A was removed by centrifugation and the supernatant was concentrated. Dihydrohydroxystreptose and virenose were separated from the supernatant by semi-preparative HPLC. The former sugar was obtained pure, as shown by GC–MS, while the latter co-eluted with D-*glycero*-D-*manno*-heptose and a smaller amount of L-rhamnose, and had to be purified by preparative paper chromatography (PC) and re-chromatography on HPLC.

Chromatographic methods.—Semi-preparative HPLC was carried out on an Eurocat Pb column (10 μm/300 × 8 mm, Knauer, Berlin, Germany) using ultra-pure water as the eluent at 75 °C and a flow rate of 0.5 mL/min. The HPLC system used involved an LKB (Bromms, Sweden) 2152 LC controller, an LKB 2150 HPLC programmable pump, a Rheodyne (Lotati, USA) 7125 injector valve equipped with 100 μL loop, an RIDK 102 (Laboratorní přístroje, Praha, Czech Republic) differential refractometer, and a DataApex (Praha, Czech Republic) CSW v. 1.6 integrator.

GC–MS was conducted with a Finnigan MAT SSQ 710 mass spectrometer using helium as the carrier gas. Mass spectra were recorded at an electron energy of 70 eV and ion-source temperature of 150 °C. Sugars were analyzed as the corresponding alditol acetates using an SP-2330 (30 m × 0.25 mm; Supelco, Bellefonte, USA) fused silica capillary column. The column temperature program was 80 (2 min) to 235 °C at 30 °C/min with a 38 min hold.

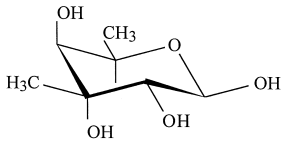
Preparative PC was conducted by the descending method on Whatman No. 3 MM paper employing (60:40:3:30) n-butanol–pyridine–acetic acid–water. The sugars were detected with silver nitrate–sodium hydroxide [9].

NMR spectroscopy.—The NMR spectra were measured at 300 MHz and 500 MHz on Bruker DPX 300 and AMX 500 spectrometers, respectively. The sugars (1–2 mg) were dissolved in deuterium oxide and the measurements were performed at 308 K. Two-dimensional (2D) gradient-enhanced NMR techniques were used for assignment purposes. In gradient-COSY, the data matrix was 256 × 1 K (F1 × F2) and the spectral width 2500 Hz. The spectrum was zero-filled to 1 K × 1 K before Fourier transformation. The ^1H–^{13}C correlated spectrum (HSQC) was recorded in sensitivity-enhanced pure absorption mode with gradient echo for coherence selection [10,11]. The same size of the data matrix (256 × 1 K) gave the spectral resolution of 3.9 and 39 Hz, respectively. A low-pass J-filter was used to suppress one-bond correlations in the gradient-heteronuclear multiple bond correlation (HMBC) sequence. The 2D NOESY spectrum was recorded with mixing time of 1 s. The spectral width and the size of the data matrix were the same as in 2D COSY. The values of ^1H and ^{13}C chemical shifts (δ) are relative to external TSP. WIN-DAISY software was used for simulation of the proton spectrum of dihydrohydroxystreptose.

Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter at 20 °C.

3. Results and discussion

Dihydrohydroxystreptose and virenose were isolated from LPS I of *C. burnetii*, after acid hydrolysis and removal of lipid A, by a combination of HPLC



Scheme 1.

and preparative PC. The sugars were analyzed for purity by GC–MS. Dihydrohydroxystreptose had $[\alpha]_D$ +21.5° in water. For the synthesized compound 3-*C*-(hydroxymethyl)-L-lyxopyranose, $[\alpha]_D^{20}$ +18.7° in water has been reported [7]. It appeared that the isolated and synthesized dihydrohydroxystreptoses could be identical.

The isolated virenose gave $[\alpha]_D$ −9.8° in water which was comparable with $[\alpha]_D^{17}$ −11.6° (water) of the syrupy antiarose (6-deoxy-D-gulose) [12]. In Ref. [4], an $[\alpha]_D^{15}$ value of +8.1° was given for the isolated virenose, although this value was not considered very accurate because of the small amount of sugar available. Glycosidation of virenose (3% methanolic HCl, 80 °C, 24 h) gave a product having $[\alpha]_D$ −30.8° in chloroform. Methyl 6-deoxy-3-*C*-methyl-β-D-gulopyranoside obtained [13] from the antitumor antibiotic virenomycin and the synthesized compound [14] were reported to have $[\alpha]_D^{20}$ −39° and $[\alpha]_D^{29}$ −30° in chloroform, respectively. From these results, we could anticipate that the virenose we isolated from LPS I and virenose as presented in Refs. [13,14] were the same sugars. However, this was not in agreement with the previous findings [4,8] on virenose isolated from the *C. burnetii* LPS I.

In order to support our findings based on optical rotation data, both monosaccharides have been studied in detail by the high-resolution NMR spectroscopy. The NMR analysis of virenose was quite straightforward. The 500 MHz ^{1}H 1D NMR spectrum (not shown) was comparable with those published earlier [13,14] for the corresponding methyl glycosides. The values of chemical shifts and proton–proton coupling constants are listed in Table 1. For the D-*gulo* isomer, the magnitude of three-bond proton–proton coupling constant $^3J_{H\text{-}1-H\text{-}2}$ is in agreement with the β configuration at the anomeric center and with the $^4C_1$ form of the ring (Scheme 1). The abundance of the α anomer was estimated to be about 10%. The assignment of carbon signals was carried out using 2D HSQC and HMBC spectra.

The 500 MHz ^{1}H 1D NMR spectrum of dihydrohydroxystreptose is shown in Fig. 1 and the 2D gradient-COSY spectrum in Fig. 2. There are several well-resolved resonances in the spectra, such as doublets at 4.82 and 3.49 ppm which originate from H-1 and H-2 protons, respectively. For the L-*lyxo* isomer, the magnitude of $^3J_{H1-H2}$ (8.0 Hz) is in agreement with the antiperiplanar position of these protons, that is, the α configuration is the prevailing one (85% α anomer, 15% β anomer). Further evidence support-

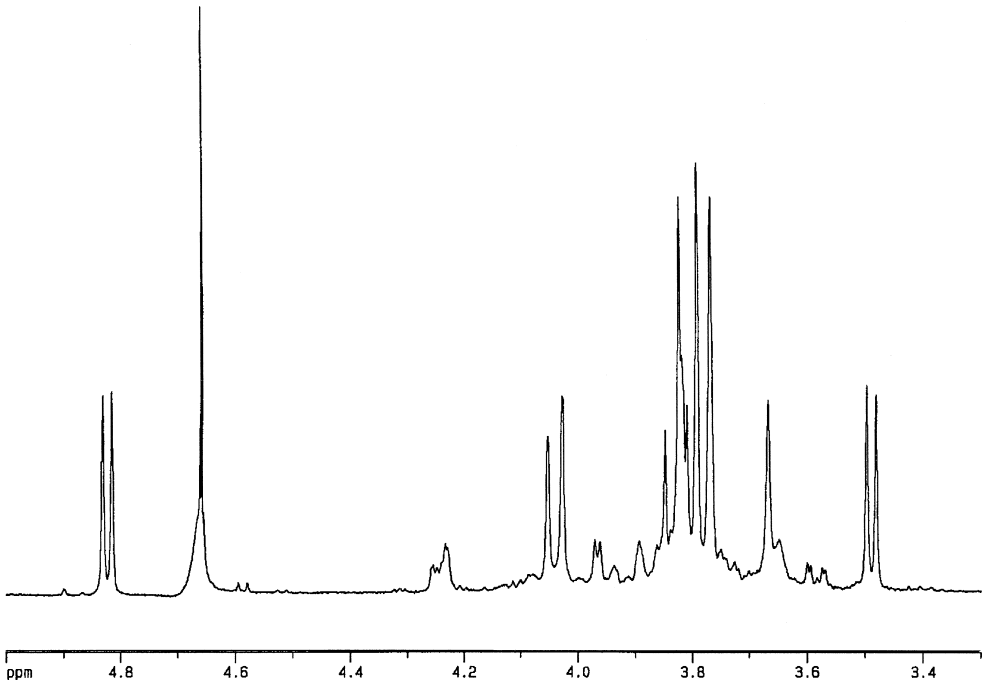


Fig. 1. The 500 MHz ^{1}H spectrum of dihydrohydroxystreptose in deuterium oxide at 308 K.

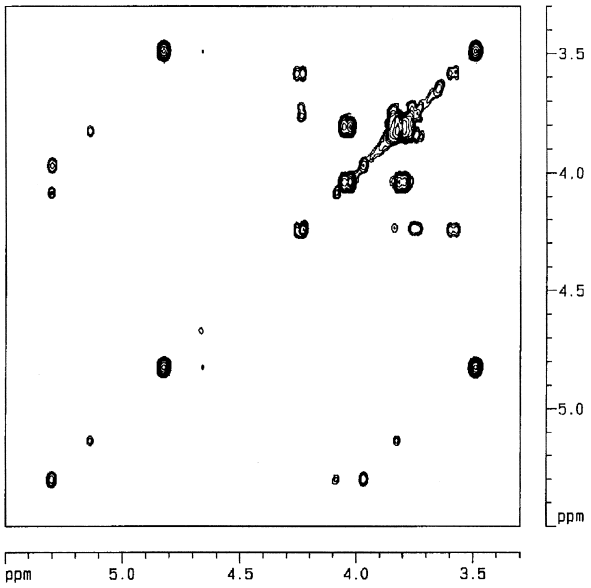


Fig. 2. Part of the 2D gradient-enhanced COSY spectrum of dihydrohydroxystreptose in deuterium oxide at 308 K.

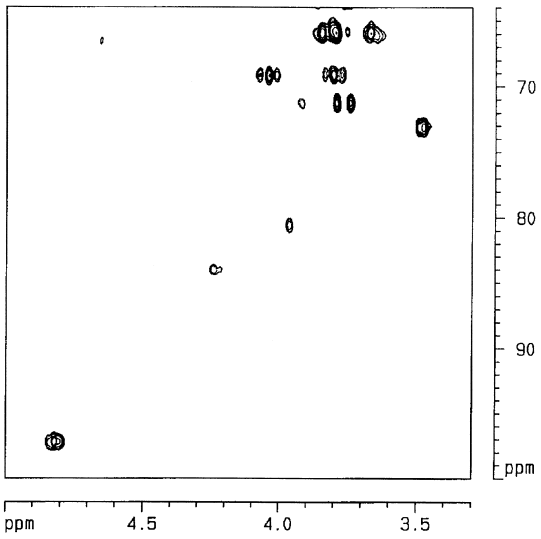


Fig. 3. Part of the 2D gradient-enhanced HSQC spectrum. Two cross-peaks at 80 and 84.5 ppm (^{13}C shifts) as well as the minor peaks at 71.5 and 66 ppm originate from impurities.

ing the antiperiplanar orientation of H-1 and H-2 was based on a 2D NOE experiment. The only NOE between the anomeric and other protons was that seen for H-1 and one of the methylene protons (at 4.04 ppm). Thus, no NOE between H-1 and H-2 was observed. This is possible only when both protons are in antiperiplanar orientation. The remaining signals (between 3.7 and 4.1 ppm) are those of H-4 and of two CH_2 groups. The signal at 3.67 and signals with lower intensities are due to impurities. The assignment of H-4 and the methylene protons, as well as determination of proton–proton coupling constants was carried out by spin simulation. The values of ^{1}H and ^{13}C chemical shifts and ^{1}H–^{1}H coupling constants are given in Table 1. Distinction between the individual protons in the CH_2 groups and assignment of carbon resonances was based on 2D HSQC (Fig. 3) and HMBC (Fig. 4) spectra. The protonated carbons were assigned directly from HSQC and the chemical shift for the quaternary C-3 (78.3 ppm) resulted from HMBC (scalar interaction between C-3 and H-4 and that with the hydroxymethyl protons). One of the methylene groups had its chemical shift at 69.2 ppm (proton signals at 4.04 and 3.80 ppm) and the other at 65.9 ppm (proton shifts at 3.84 and 3.79 ppm).

Table 1
Chemical shifts (δ, referenced to external TSP[a], $\delta_{TSP} = 0$ ppm) and proton–proton coupling constants (J, values in Hz, obtained by spin simulation) for dihydrohydroxystreptose and virenose in aqueous solution at 308 K

Dihydrohydroxystreptose		Virenose	
	δ		δ
H-1	4.82	H-1	3.82
H-2	3.49	H-2	3.34
H-3	–	H-3	–
H-4	3.77	H-4	3.31
H-6(CH_2)	3.84	H-5	4.23
H-6′(CH_2)	3.79	Me(C-3)	1.38
H-5a	4.04	Me(C-5)	1.23
H-5e	3.80		
$J_{1,2}$	8.0	$J_{1,2}$	8.1
$J_{4,5a}$	1.0	$J_{4,5}$	1.0
$J_{4,5e}$	1.0	$J_{5,Me}$	6.5
$J_{5a,5e}$	12.4		
$J_{6,6'}$	12.6		
C-1	97.2	C-1	97.3
C-2	73.1	C-2	75.7
C-3	78.3	C-3	76.5
C-4	71.2	C-4	78.2
C-6(C-3)	65.9	C-5	72.4
C-5	69.2		

[a] Sodium 4,4-dimethyl-4-silapentanoate.

Long-range proton–carbon scalar interactions were crucial for determination of the sugar ring conformation. There are three long-range interactions (Fig. 4) between C-1 (97.2 ppm) and protons at 3.49 ppm, 3.80 ppm, and 4.04 ppm. The first cross-peak corresponds to a two-bond interaction between the anomeric carbon and H-2 (3.49 ppm), the remaining two are between C-1 and the methylene protons at 3.80 and 4.04 ppm. The latter type of interactions is

possible only when the monosaccharide is in a pyranose form rather than in a furanose one. In the pyranose form, the methylene protons are linked to the ring carbon C-5 having a three-bond scalar interaction with C-1. This would not be the case in the furanose form as the CH_2 protons would be as a hydroxymethyl group linked to C-4 and, consequently, the protons would be four bonds apart from the anomeric carbon. In the latter case, the types of cross-peaks in the HMBC spectrum as seen in Fig. 4 would not be observed. The relative intensities of cross-peaks are also in agreement with this premise. The more intense peak at 3.80 ppm corresponds to the interaction between the equatorial proton and C-1 (the dihedral angle between C-1 and H-5e is close to 180° in the pyranose ring), and thus, the long-range coupling constant is relatively large (6–7 Hz) due to the dependence of $^3J_{C-H}$ on dihedral angle [15]. A considerably weaker interaction exists between C-1 and H-5a. The dihedral angle between C-1 and H-5a is ~60°, and thus, the expected coupling constant is small (about 1.5–2.0 Hz). Further support for this assignment and conformation was from the 2D NOE experiment, where the observed NOE is between H-1 (axial in α configuration in the L form) and proton at 4.04 ppm, the latter signal corresponds to axial H-5.

In conclusion, we have demonstrated that virenose isolated from the *C. burnetii* LPS I has the D configuration and the $^4C_1$ ring conformation as it was reported for the sugar isolated [13] from the antitumor antibiotic virenomycin after methanolysis, as well as the synthesized [14] methyl β-D-virenoside. Further, the NMR measurements have shown that the isolated dihydrohydroxystreptose (L form) is in the pyranose form with the $^4C_1$ conformation. Previously, the furanose form of the sugar was reported [4] to be present in native LPS I. From the data presented, it appears that tautomerization of furanose to pyranose (Scheme 2) took place during the isolation procedure. This explains the contradictory results published [4,7,8] on dihydrohydroxystreptose isolated from the *C. burnetii* LPS I.

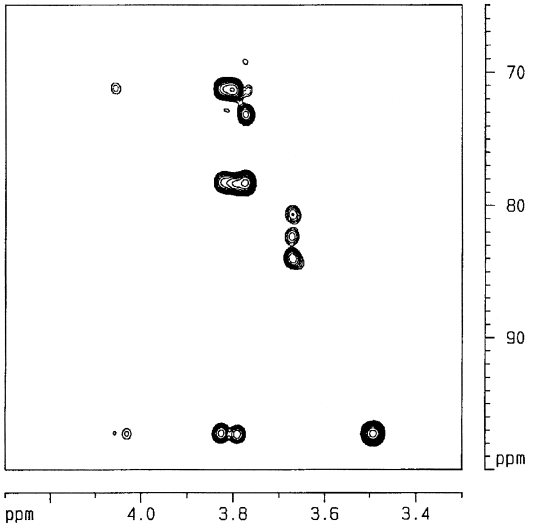
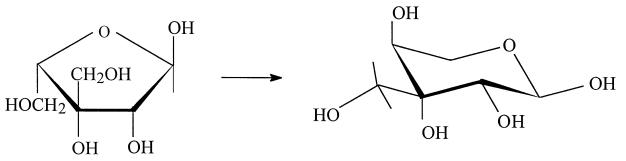


Fig. 4. Part of the 2D gradient-enhanced HMBC spectrum showing the most important scalar interactions within a spin system. Three cross-peaks among C-1 (97.2 ppm) and protons (3.49, 3.80, and 4.04 ppm) are those originating from interactions of the anomeric carbon with H-2, H-5e, and H-5a (lower intensity). Relatively strong cross-peaks at 3.67 ppm (for ^1H) are due to impurities.



Scheme 2.

Acknowledgements

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References

[1] J.C. Williams and D.M. Waag, *Antigens, virulence factors, and biological response modifiers of Coxiella burnetii: Strategies for vaccine development*, in J.C. Williams and H.A. Thompson (Eds.), *Q Fever: The Biology of Coxiella burnetii*, CRC Press, Boca Raton, FL, 1991, pp. 175–223.
[2] J. Kazár, Š. Schramek, and R. Brezina, *Acta Virol.*, 22 (1978) 309–315.
[3] E. Gajdošová, E. Kováčová, R. Toman, L. Škultéty, M. Lukáčová, and J. Kazár, *Acta Virol.*, 38 (1994) 339–344.
[4] Š. Schramek, J. Radziejewska-Lebrecht, and H. Mayer, *Eur. J. Biochem.*, 148 (1985) 455–461.
[5] K. Amano, J.C. Williams, S.R. Missler, and V.N. Reinhold, *J. Biol. Chem.*, 262 (1987) 4740–4747.
[6] L. Škultéty and R. Toman, *Acta Virol.*, 38 (1994) 209–213.
[7] O. Dahlman, P.J. Garegg, H. Mayer, and Š. Schramek, *Acta Chem. Scand.*, B40 (1986) 15–20.
[8] H. Mayer, J. Radziejewska-Lebrecht, and Š. Schramek, *Adv. Exp. Med. Biol.*, 228 (1988) 577–591.

[9] W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature (London)*, 166 (1950) 444–445.
[10] L.E. Kay, P. Keifer, and T. Saarinen, *J. Am. Chem. Soc.*, 114 (1992) 10663–10665.
[11] J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletzky, S.J. Glasser, O.W. Sorensen, and C. Griesinger, *J. Biomol. NMR*, 4 (1994) 301–306.
[12] K. Doebel, E. Schlittler, and T. Reichstein, *Helv. Chim. Acta*, 31 (1948) 688–709.
[13] V.V. Kulyaeva, M.K. Kudinova, N.P. Potapova, L.M. Rubasheva, M.G. Brazhnikova, B.V. Rosynov, and A.R. Bekker, *Bioorg. Khim.*, 4 (1978) 1087–1092.
[14] J. Yoshimura, N. Hong, and K. Sato, *Chem. Lett.*, (1980) 1131–1132.
[15] I. Tvaroška, H. Hricovíni, and E. Petráková, *Carbohydr. Res.*, 189 (1989) 359–362.