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The structure of the homopolymeric *O*-specific chain from the phenol soluble LPS of the *Rhizobium loti* type strain NZP2213

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The *O*-specific polysaccharide of the phenol soluble lipopolysaccharide (LPS) (LPS-P) of the *Rhizobium loti* NZP2213 strain consists of a homopolymeric chain of α-1, 3-linked units of 2-*O*-acetyl-6-deoxy-L-talopyranose, very probably terminated by a single unit of 2-*O*-methyl-6-deoxy-L-talopyranose. The hydrophobic character of the long *O*-chains explains the phenol solubility of LPS-P, in contrast to the water solubility of LPS-W, which is of R-character and accordingly lacks the *O*-acetyl-6d-talose units.

INTRODUCTION

Recent phylogenetic studies, based on sequencing 16S rRNA genes, have revealed that *Rhizobium loti* and *Rhizobium huakuii* form a separate line of descent from all other *Rhizobium* strains (Yanagi & Yamasato, 1993). Our investigation of the type strain NZP2213 of *Rhizobium loti* has revealed that it differs characteristically in the structural composition of its cell wall lipopolysaccharide (LPS) and especially in its lipid A components from all other rhizobial strains so far investigated. Lipid A of *R. loti* has a phosphorylated 2,3-diamino-2,3-dideoxy-D-glucose disaccharide as its backbone sugar (Russa *et al.*, 1995). This carries a number of amide-linked 3-hydroxylated fatty acids, including the rare long chain 3-OH-20:0 and 3-OH-22:0, as well as 4-oxo-20:0 and 4-oxo-22:0. The main ester-linked fatty acids include 16:0, 17:0, 18:0, but also in a relatively large quantity, 27-OH-28:0, a rather rare fatty acid, but characteristic of *Proteobacteria* of the α-2 subgroup (Bhat *et al.*, 1991). A surprising finding, previously encountered also with the phylogenetically closely related species *Thiobacillus sp.* IFO 14570 (Katayama *et al.*, 1994), was the observation, that during phenol–water extraction, the bulk of LPS was extracted into the phenol phase (LPS-P) and only a small amount into the water phase (LPS-W). LPS-P showed on DOC-PAGE the typical banding pattern of *O*-chains, whereas LPS-W had R-type characteristics (Shashkov *et al.*, 1995; Russa *et al.*, 1995). This indicated a highly hydrophobic character of the *O*-chains, which in the case of the phenol-soluble LPS of *Thiobacillus sp.* IFO 14570 could recently be confirmed by showing that the *O*-chain is built up by three different diaminohexoses leaving not a single unsubstituted OH-group in the whole *O*-chain. An investigation of the primary structure of the similar hydrophobic *O*-chain of *R. loti* NZP2213 was, therefore, of interest and the elucidation of its structure will be the topic of this paper.

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MATERIALS AND METHODS

Chemicals and bacterial cultivation

6-deoxy-L-talose was a gift from Dr A. Banaszek, Polish Academy of Sciences, Warsaw, Poland. All other sugars were obtained from E. Merck, Darmstadt, Germany and Sigma Chemie GmbH, Deisenhofen, Germany. The type strain of *Rhizobium loti* (NPZ2213 = HAMBI 1129) was obtained from the collection of the Department of Microbiology, University of Helsinki, Finland, by courtesy of Dr K. Lindström. The cultivation of bacteria was carried out at 28°C, as described recently (Russa *et al.*, 1995).

Preparation of LPS and *O*-specific chains (PS)

The extraction of frozen cells by the hot phenol–water procedure (Westphal & Jann, 1965) and the recovery of

LPS-W, and LPS-P, from the water and the phenol phase have been detailed recently (Russa *et al.*, 1995). The *O*-specific polysaccharide (PS) was removed from LPS-P by hydrolysis with 1% acetic acid (100°C, 3 h). The resulting material was passed through a Sephadex G-50 column (1.5 cm × 90 cm) using water as eluent and the eluent monitored with a Knauer refractometer (Fa. Knauer, Bad Homburg, Germany). Since *O*-acetyl groups were present in PS, part of the material was subsequently de-*O*-acetylated at 50 C with Na_2CO_3 (added in three portions until a pH of 10.5–11.0 was reached). After the final addition of Na_2CO_3, a sediment was formed, the material passed over a small DOWEX 50Wx8 (H^+-form) and the cloudy filtrate concentrated *in vacuo*. After a further passage through a 0.22 μm Millex-GS-filter (Millipore S.A., Molsheim, France) a clear solution of the de-*O*-acetylated material (de-*O*-acPS) was obtained.

PS and de-*O*-acPS in D_2O were then subjected to ^1H- and ^{13}C-NMR analysis on a Bruker AM-300 spectrometer, using acetone at 60°C (δ_H2.25, δ_C31.45) as an internal standard. Homonuclear 2D COSY and 1D N.O.e. were carried out using the standard Bruker software.

Analytical methods

Combined gas chromatography–mass spectrometry (GLC–MS) analyses were performed with a Hewlett Packard 5985 system at 70 eV either using a CB CP-SIL 5 or a DB 5 (25 m × 0.25 mm i.d.) capillary column with helium as the carrier gas. Neutral sugars were analysed and quantified as alditol acetate derivatives (Russa *et al.*, 1995), whereas acidic sugars, liberated by methanolysis, were subjected to carboxyl reduction with $NaBH_4$, then hydrolysed with 1 M trifluoro acetic acid (120°C, 2 h) followed by a second reduction with $NaBH_4$ or $NaBD_4$, respectively, and a final peracetylation (Russa *et al.*, 1991).

The absolute configuration of the individual sugars was determined according to Gerwig *et al.* (1978) by GLC analysis of the trimethylsilylated (TMS)-(-)2-butyl glycoside derivatives of the sugars in the PS hydrolysate and comparison with the retention time of the corresponding derivatives of the authentic standards.

RESULTS

Degraded polysaccharide (PS) was obtained from LPS-P (25.8 mg) in a yield of 68.6%. After passage over a G-50 column, one major peak and three minor ones could be recognised. The major fraction was collected (11.8 mg) and used for sugar analysis and NMR measurements. The quantitative sugar analysis of PS revealed that the *O*-chain was exclusively built of 6-deoxy-talose units, accompanied by small amounts of 2-*O*-Me-6-deoxy-talose (peak ratio = 33:1). Minor amounts of L-rhamnose, D-glucose and LD-heptose were also present (Table 1), which apparently originated from the adjoining core material. The L-configuration of 6-deoxy-talose was proven by co-chromatography of the TMS derivatives of (-)-2-butylglycosides of the PS hydrolysate with that of authentic 6-deoxy-L-talose using a fused silica DB5 column for separation.

Table 1. Sugar composition of the major fraction of PS after Sephadex G-50 column chromatography

Component	% of total sugars
2-*O*-Me-6dHex	2.0
6-dL-Tal	78.3
L-Rha	4.4
D-Man	0.5
D-Glc	12.7
D-GlcA	1.4

Methylation analysis of the polysaccharide (PS)

The methylation analysis of the major fraction obtained by G-50 column chromatography revealed that, by far, the major component was the 2,4-di-*O*-methyl-derivative of 6-deoxy-L-talopyranose with the main fragments at m/z 89, 118, 131, 171, 202 and 234. Besides this, 2,4-di-*O*-Me-Rha, 2,3,4,6-tetra-*O*-Me-Glc and 2,3,6-tri-*O*-Me-Glc could be traced in minor quantities, which most probably originated from the core region. Methylation analysis thus proved that the straight *O*-chain was composed of 1 → 3-linked 6-d-L-talopyranose units.

NMR analysis of PS and de-*O* acPS

The ^{13}C-NMR spectrum of PS showed only eight signals (Fig. 1 and Table 2), including one in the region of the anomeric carbons (97.1 ppm), one in the region of C-CH_3-groups (16.8 ppm) and two in the region of a CH_3CO-group (Me at 21.8 ppm, CO at 174.4 ppm). In addition, four signals of sugar carbons in the region of 68–71 ppm were observed. The spectrum thus corresponded to an unbranched homopolymeric chain built up by an *O*-acetylated 6-deoxyhexose entity in its pyranosidic form (absence of the typical downfield shift of the furanosidic C-4 signal) (Bock & Pedersen, 1983). The observed coupling constant $^1J_{H\text{-}1,C\text{-}1}$ of 175 Hz in the gated decoupled spectrum proved the α-configuration of the glycosidic linkage. The ^1H spectrum of PS showing five signals of ring protons and two signals of CH_3-groups (Table 2) could be assigned using 2D COSY spectroscopy. The coupling constants of the protons proved to be typical for α-linked 6-d-talopyranosyl

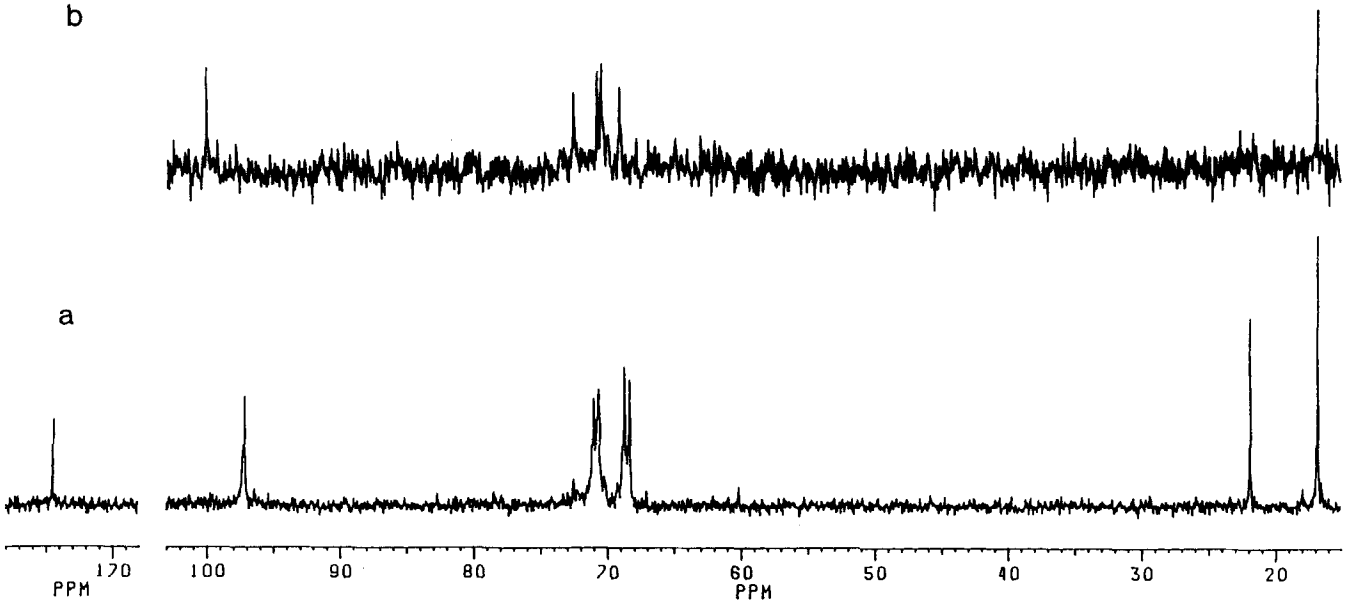

Fig. 1. 75 MHz ^{13}C-NMR spectrum of (a) the genuine (PS); and (b) the de-*O*-acetylated polysaccharide (de-*O*-acPS).

Table 2. NMR data for the genuine (I) and de-*O*-acetylated (II) polysaccharide

Polysaccharide	Proton	δ	$J_{H,H}$	Hz	Carbon	δ	^{J}C-1, H-1
I	H-1	5.11	$J_{1,2}$	2	C-1	97.1	175
	H-2	5.15	$J_{2,3}$	3.5	C-2	71.0	
	H-3	4.09	$J_{3,4}$	3.5	C-3	70.6	
	H-4	3.94	$J_{4,5}$	~1	C-4	68.7	
	H-5	4.07	$J_{5,6}$	6.5	C-5	68.3	
	H-6	1.29			C-6	16.8	
	CH_3CO	2.19			CH_3CO	21.8	
						174.4	
II	H-1	5.20	^{J}1, 2	2	C-1	100.0	
	H-2	4.08	^{J}2, 3	3.5	C-2	70.7	
	H-3	4.02	J3, 4	3.5	C-3	72,5	
	H-4	4.14	J4, 5	~1	C-4	70.4	
	H-5	4.23	^{J}5, 6	6.5	C-5	69.0	
	H-6	1.32			C-6	16.8	

units (Knirel *et al.*, 1992). The characteristic downfield shift of the H-2 protons indicated the location of the *O*-acetyl groups at C-2 and this *O*-acetylation proved to be quantitative.

The 1D N.O.e. experiment with PS and with pre-irradiation of the anomeric protons proved difficult to interpret due to the close proximity of H-1 and H-2 proton signals in the ^{1}H-NMR spectrum (Table 2 and Fig. 1). In the ^{1}H-spectrum of the de-*O*-acetylated polysaccharide, however, all signals were adequately separated for this study (Table 2). As a result, three signals of H-2, H-3 and H-4 were observed in the different N.O.e.-spectrum, a result expected for an α-1->3-linked 6-deoxy-talan chain where the anomeric protons are close to H-2 of the same residue and to H-3 and the equatorial H-4 of the glycosylated residue. In the case of 1->4-linkage, the anomeric protons and H-3 of the glycosylated residue would be remote enough for a N.O.e. interaction due to the axial–axial position of OH-4 and H-3 in the 6d-talopyranose residues. The α-(1->3) linkage of 6d-talose in de-O-acPS was also confirmed by ^{13}C NMR spectroscopy (Table 2) where the signal typical of unsubstituted 6-deoxy-talose at 66.3 ppm (Knirel *et al.*, 1992), was absent.

Together with the knowledge of the absolute L-configuration of the 6-deoxy-talose the complete structure of the O-chain of LPS-P obtained from *Rhizobium loti* can be formulated as shown below.

$$\begin{array}{l}\text{[->3)-}\alpha\text{-6d-L-Tal }p\text{-(1->]}_{n\ 30\text{–}40}\\ \quad\ 2\\ \quad\ |\\ \quad\ \text{OAc}\end{array}$$

DISCUSSION

As a result of the present investigation of the *Rhizobium loti* NZP2213 LPS, its *O*-specific chain was characterised as a homopolymer of 2-*O*-acetylated units of 1,3-linked 6-deoxy-L-talopyranose. Although 6-deoxytalose has previously been described as a constituent of several microbial, mostly capsular polysaccharides and lipopolysaccharides, it is nevertheless a rare sugar in natural products (Lindberg, 1990; Shibuya *et al.*, 1991). *O*-Acetylated 6-deoxy-D-talan and 6-deoxy-L-talan have been described from serotype-specific polysaccharide antigens from *Actinobacillus actinomycetemcomitans* serotype a and c (Shibuya *et al.*, 1991), but these 6-deoxy-talans have alternating α-(1->2)- and (1->3)-linkages, i.e. they do not represent true homopolymers *in sensu stricto*. The *O*-antigen from *Yersinia enterocolitica* serotype 2, however, is such a homopolysaccharide composed of β-(1->2)-linked units of 6-deoxy-L-altropyranose (Lindberg, 1990).

Complete acetylation of distinct and unique positions in sugars of repeating units do certainly require distinct acylating enzyme(s). Since de-*O*-acetylation often abolishes the serological specificity, as shown with the *O*:5-factor of *Salmonella typhimurium*, which corresponds to terminal units of α-linked 2-*O*-acetyl-abequopyranose, in contrast to non-acetylated terminal abequose which corresponds to the *O*:4-factor (Lüderitz *et al.*, 1971; Stellner *et al.*, 1970). A serological investigation of *Rhizobium loti* is not yet available, but the unique structure of its *O*-chains is certainly involved in serological specificity and in the recognition processes leading to an effective nodulation only with *Lotus tenuis* and *Lotus corniculatus* var. *cree* (Hotter & Scott, 1991).

As is often observed with homopolymeric *O*-chains, e.g. with *E. coli O*8 and *Klebsiella O*5, their non-reducing termini often carry single *O*-methyl groups, often localised in position C-3 (Tharanathan & Mayer, 1978). By single ion monitoring, it was possible also with the *R. loti O*-chain to trace small amounts of 2-*O*-methylated 6-deoxy-talose. From the molar ratio between 2-*O*-methyl-6d Tal: 6-dTal in PS, namely 1:30–40, it can be assumed that the average *O*-chain length is approximately 30–40 sugar units. This value also agrees with the DOC–PAGE profile (Russa *et al.*, 1995) which shows a cluster of slow-moving LPS with long *O*-chains and an almost complete lack of shorter chains.

The pathways leading to the biosynthesis of 6-deoxy-L-talose are detailed in Shibuya *et al.* (1991), which indicate that L-Rha and 6-deoxy-L-Tal are both formed by a stereoselective reduction of the carbonyl group at C-4 of the common precursor, dTDP-6-deoxy-hexosyl-4-ulose. The small amount of L-rhamnose presumably originating from the core region of the PS is, therefore, notable.

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