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# Lipooligosaccharidic antigens from *Mycobacterium kansasii* and *Mycobacterium gastri*

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A set of Lipooligosaccharides (LOSs) has previously been characterized in *M. gastri* W471. The structure of the highly antigenic LOS (LOS-III) was elucidated and this molecule can unambiguously distinguish *M. gastri* from the opportunistic pathogen *M. kansasii*. In the present study, the structures of three other *M. gastri* W471 LOSs were determined by one-dimensional $^1$H-NMR spectroscopy and gas liquid chromatography. They differ by the number of Xyl*p* units and by the structure of the distal monosaccharide. The two dimensional (2D) NMR approach was successfully applied to the LOS antigen of *M. kansasii* to locate the acetyl and acyl substituents and to determine the anomeric configuration of the α-D-Fuc*p* unit.

The molecular specificity of anti-LOS-III antibodies was investigated and the LOS-III epitope was defined as the distal disaccharide: 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*hex*p*-(1→3)-β-L-Xyl*p*.

*Keywords*: *Mycobacterium*, lipooligosaccharide (LOS), *Mycobacterium gastri* LOS epitope, ELISA, 2D NMR spectroscopy

*Abbreviations*: CI, Chemical Ionisation; COSY, $^1$H-$^1$H COrrelated SpectroscopY; COSY LR, $^1$H-$^1$H COrrelated SpectroscopY Long Range; DQF-COSY, Double Quantum Filtered $^1$H-$^1$H COrrelated SpectroscopY; EI, Electronic Impact; GC, Gas Chromatography; HMBC, Heteronuclear Multiple Bond Correlation spectroscopy; HMQC, Heteronuclear Multiple Quantum Correlation spectroscopy; HOHAHA, HOmonuclear HArtmann-HAhn spectroscopy; HPLC, High Performance Liquid Chromatography; $^3J_{H,H'}$, vicinal spin-spin coupling constants; LAM, LipoArabinoMannan; LOS, LipoOligoSaccharide; MS, Mass Spectrometry; FAB/MS, Fast Atom Bombardment-Mass Spetrometry; $^1$H- or $^{13}$C- NMR, proton or carbon Nuclear Magnetic Resonance; PBS, Phosphate Buffered Saline; PheG1 K-I, Major phenolic glycolipids from *M. kansasii*; RCT-1, -2, -3, relayed coherence transfer 1 step, 2 steps, 3 steps; ROESY, Rotative frame Overhauser Effect SpectroscopY; Sugars, Fuc*p*: fucopyranose, Gal*p*: galactopyranose, Glc*p*:glucopyranose, hex*p*: hexopyranose, Rha*p*: rhamnopyranose, Xyl*p*: xylopyranose; TLC, Thin Layer Chromatography; TMS, Tri Methyl Silyl.

## Introduction

The serological differentiation of many species within the *Mycobacterium* genus is based on immunoreactive species-specific glycolipids. They are classified into three types according to their structure: the phenolic glycolipids (Phe Gls), the glycopeptidolipids (GPLs) and the trehalose-containing lipooligosaccharides (LOSs). In the latter glycolipids, an acyltrehalose residue is invariably found, glycosylated in distinct fashion, to confer species-specific antigenicity.

*Mycobacterium kansasii*, a pathogenic strain, has been proven to be very closely related to another mycobacterial strain, *Mycobacterium gastri*, considered to be a non-pathogen. The *M. kansasii* and *M. gastri* cell walls share the same phenolic glycolipid antigens [1] despite the unique structure of the immunodominant monosaccharide (2,6-dideoxy-4-*O*-Me-α-D-*arabino*hexopyranose) [2–4]. The presence and the structures of the LOSs were established for the *M. kansasii* smooth but not rough strains [5–7]. In an attempt to differen tiate these two species on the basis of species-specific glycolipids, a set of four previously unknown LOSs was isolated from the cell wall of *M. gastri* W471. The structure of the highly antigenic lipooligosaccharide, LOS-III, was determined and it was established that LOS-III is a permanent marker for *M. gastri* [8]. Moreover, by ELISA and a chro-

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matographic approach, it was found that LOS-III unambiguously distinguished *M. gastri* from *M. kansasii*. The LOS-III structure was essentially elucidated from its native [8] and peracetylated [9] forms using homonuclear and heteronuclear two dimensional (2D) NMR spectroscopy and FAB/MS spectrometry. An interesting feature of the LOS-III structure is the presence of 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*hexopyranose, a new type of mono saccharide located at the oligoxylose non-reducing end.

We now report the structures of the remaining lipooligosaccharides purified from the cell wall of *M. gastri* W471 (LOS-I, LOS-II, LOS-IV) which were established from comparison of the gas chromatography (GC) profiles and one dimensional (1D) $^1$H-NMR anomeric region. Furthermore, a 2D NMR based strategy applied to the LOS isolated from *M. kansasii* allowed us to complete the structure previously established by Brennan [5–7]. The binding of the LOS-III epitope by anti-LOS-III antibodies was studied in order to develop a specific probe for the differentiation of *M. kansasii* from *M. gastri*.

## Materials and methods

### *Culture conditions*

*M. gastri* strains W471, HB4362 and HB4389 and *M. kansasii* strains ATCC12478, S890175 and S890370 were grown on the surface of Sauton's medium at 37°C for 2 months.

### *LOS purification*

The purification and characterization of lipooligosaccharides from *M. gastri* W471 have been described in a previous work [8]. The purification of the LOS of *M. kansasii* was carried out in the same way with a HPLC mobile phase $CHCl_3$ : $CH_3OH$ : $H_2O$, 68 : 32 : 4, by vol.

### *Analytical methods*

TLC was run on commercial silica gel plates (DC Alurolle, Kieselgel 60 $pF_{254}$ Merck, Darmstadt, Germany) using $CHCl_3$:$CH_3OH$:$H_2O$, 60 : 35 : 8, by vol, as migration solvent. A spray composed of 0.1% orcinol in 40% $H_2SO_4$ was used to locate the LOSs.

HPLC was conducted with a Gilson apparatus composed of a model 303 pump, an 802 manometric module, an 811 dynamic mixer and a Waters model 450 variable wavelength detector. Elution and data acquisition were controlled with Gilson GME 714 Software and a Gilson model 621 data module interface, connected to an OPAT Normerel computer.

Infrared analysis was conducted on a Perkin-Elmer 1600 series FT-IR spectrophotometer.

Routine gas chromatography (GC) was performed on a Girdel series 30 equipped with an OV1 wall-coated open tubular, 25 m capillary column, inner diameter 0.3 mm, using nitrogen gas at a flow rate of 2.5 ml min$^{-1}$ with a flame ionization detector. A temperature programme from 100 to 250°C at a speed of 2°C per min and an injector temperature of 260°C were used for TMS methyl glycoside analysis. The temperature of the detector was 310°C. The identification of derivatized products was performed by CI and EI mass spectra analysis and GC co-chromatography with authentic standards purchased from Sigma Chemical Company, St Louis, MO (Xyl, Gal, Fuc, Glc and palmitic acid) and synthesized in the laboratory (3-O-Me-Rha).

GC/MS was performed on a Nermag model R 10/10 quadrupole mass spectrometer connected to a PDP-8M computer.

### *LOS methanolysis procedure*

LOS (100 μg) were methanolysed with 2 M acetylchloride in dry methanol at 80°C for 2 h. $CH_3OH$/HCl was evaporated under nitrogen.

### *Nuclear magnetic resonance spectroscopy*

Spectra were recorded on a 500 MHz Bruker AMX spectrometer equipped with an Aspect X32 computer. $^1$H and $^{13}$C chemical shifts are expressed in ppm downfield from internal tetramethylsilane (0 ppm) and internal chloroform (77 ppm) respectively. The sample was dried under vacuum, dissolved in $CDCl_3$:$CD_3OD$ (Spin et Techniques, Paris, France) (1 : 1, by vol) at a concentration of 20 mg ml$^{-1}$ (3.7 μmol of LOS-k) and analysed in a 200 × 5 mm 535-PP NMR tube. All 2D NMR data sets were recorded at 303 K without sample spinning for HOHAHA, ROESY and heteronuclear experiments. Data were acquired in the phase-sensitive mode using the TPPI method [10] or the method of States *et al.* [11]. DQF-COSY [12] and HOHAHA [13] were recorded with standard pulse sequences. $^1$H-$^{13}$C correlation spectra were recorded in the proton-detected mode with a Bruker 5 mm $^1$H–broadband tunable probe with reversed geometry. The pulse sequence used for single-bond correlation spectra (HMQC) was that of Bax [14]. GARP sequence [15] at the carbon frequency was used as a composite pulse decoupling during the acquisition. Multiple-bond correlation spectra (HMBC) [16] were recorded and processed in the magnitude mode. The pulse sequence used for $^1$H detected heteronuclear relayed spectra (HMQC-HOHAHA) was that of Lerner and Bax [17]. Experimental details and processing parameters of the spectra are given in the figure captions.

### *FAB mass spectrometry*

FAB/MS spectra were recorded in the positive mode on a VG Analytical 70–250 SEQ spectrometer using a 35 kV cesium beam, in the 3500–700 mass per charge range. Samples of LOS (2 μg) dissolved in 1 μl $CHCl_3$ were added to 10 μl of matrix of triethylene glycol monobutylether:glycerol, 1 : 1.

### *Antiserum preparation*

Two 2-month-old 'fauve de Bourgogne' rabbits were inoculated intradermally with 3 mg of *M. kansasii* (ATCC 12478) sonicated and emulsified in incomplete Freund's adjuvant

(Difco). Three weeks later, the antigens (3 mg) were injected subcutaneously. The same procedure was applied to two rabbits inoculated with *M. gastri* (W 471). Antibody titres were determined from test bleeds using the ELISA procedure.

*Competition test using ELISA*

ELISA was carried out as previously described [18]. LOS (50 µl), dissolved in ethanol at a concentration of 1 mg $ml^{-1}$, was coated by evaporation at 37°C overnight onto wells of microtitre plates (Nunc-Immuno Plate I). The ELISA competition test was done by incubating antisera at the indicated dilutions in microtitre plate wells with the potential inhibitor. The inhibitory glycolipids were suspended at various concentrations in PBS containing sodium deoxycholate (1 mg $ml^{-1}$) 0.4% (w/v) powdered defatted milk. The inhibitory sugars (L- and D-Xyl*p*) were suspended at various concentrations in PBS/0.4% (w/v) powdered defatted milk. A 90 µl aliquot of a serial dilution of test antigen or test methyl glycoside and 10 µl of a 1 : 10 dilution of antiserum were added to each well and incubated for 2 h at 37°C. After four washings with a solution of PBS/1% (w/v) powdered defatted milk, 50 µl of a 1 : 1000 dilution of anti-rabbit Ig alkaline phosphatase conjugate (Sigma Chemical Company, St Louis, MO), diluted in PBS/0.4% (w/v) powdered defatted milk, was added followed by a 2 h incubation at 37°C. After additional washings, 100 µl of a solution of paranitrophenyl phosphate (Sigma Chemical Company, St Louis, MO) at a concentration of 1 mg $ml^{-1}$ in phosphate buffer were added to each well. Absorbance was read at 405 nm with a multiscan apparatus (Flow Laboratories Inc., McLean, VA, USA). The remaining activity was calculated as previously described [18].

## Results

*Purification of a set of* M. gastri *W471 antigenic LOSs*

From a $CHCl_3/CH_3OH$ extract of *M. gastri*, the crude acetone-insoluble fraction contained the LOSs and phospholipids. Most of the phospholipids were $CH_3OH$-precipitated while the LOSs were solubilized. This methanol-soluble fraction, which was still contaminated by phospholipids, was then chromatographed on either a DEAE or QMA ion-exchange column. The QMA column (acetate) was first washed with $CHCl_3$ to eliminate the more apolar neutral compounds. Then the LOSs were eluted by $CHCl_3$:$CH_3OH$, 1 : 2 by vol. An homogeneous family of glycolipids was revealed by anthrone on silicic acid TLC. The fractionation of these glycolipids was successfully accomplished using an HPLC device equipped with a 5 mm spherisorb semi-preparative column monitored by a refractory detector. The mobile phase consisted of a tertiary solvent: $CHCl_3$:$CH_3OH$:$H_2O$, 60 : 30 : 4, by vol. In this way, the two quantitatively major LOSs, LOS-III and LOS-IV, were purified until homogeneous on silicic acid TLC while two other apolar compounds, LOS-I and LOS-II, were partially purified [8]. The typical features of the Fourier Transform infrared spectrum of carboxylic ester (absorption at 1740 $cm^{-1}$) and alcohol function (absorption at 3000–3500 $cm^{-1}$) confirmed that all these glycolipids were indeed structurally related to the LOS class.

Conventional plate ELISA using rabbit polyclonal antiserum raised against *M. gastri* W471 showed that only LOS-III and LOS-IV were antigenic among the LOSs [8].

*Structural elucidation of LOS-I, -II, -IV*

The monosaccharide composition was established after methanolysis of the native glycolipids, pertrimethylsilylation

**Table 1.** $^1H$ NMR data for *M. gastri* LOSs.

| | | *α-Glc*p *H1* | *α-Glc*p *H1* | *α-Rha*p *H1* | *X*p *H1* | *α-Glc*p *H4* | *α-Glc*p *H2* | *β-Gal*p *H1* | *β-Glc*p *H1* | *β-Xyl*p *H1* | *β-Xyl*p *H1* | *β-Xyl*p *H1* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **LOS-III** | δ(ppm) | 5.18 | 5.05 | 4.98 | 4.96 | 4.78 | 4.73 | 4.45 | 4.42 | 4.34 | 4.31 | 4.26 |
| | *m* | *d* | *d* | *d* | *d* | *t* | *dd* | *d* | *d* | *d* | *d* | *d* |
| | $^3J_{1,2}$ (Hz) | *3.6* | *3.5* | *2.4* | *3.8* | *9.8* | *10.3/3.6* | *7.8* | *7.7* | *7.1* | *7.2* | *7.4* |
| | Int | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 4 |
| **LOS-I** | δ(ppm) | 5.29 | 5.18 | 5.12 | | 4.95 | 4.87 | 4.55 | 4.54 | 4.48 | | |
| | *m* | *d* | *d* | *d* | | *t* | *dd* | *d* | *d* | *d* | | |
| | $^3J_{1,2}$ (Hz) | *3.7* | *3.6* | *2.8* | | *9.6* | *3.7/10.1* | *7.9* | *7.9* | *6.9* | | |
| | Int | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | | |
| **LOS-II** | δ(ppm) | 5.28 | 5.18 | 5.11 | | 4.95 | 4.87 | 4.54 | | 4.47 | 4.38 | |
| | *m* | *d* | *d* | *d* | | *t* | *dd* | *d* | | *d* | *d* | |
| | $^3J_{1,2}$ (Hz) | *3.7* | *3.6* | *2.8* | | *9.2* | *3.3/9.9* | *7.6* | | *7.1* | *6.8* | |
| | Int | *1* | *1* | *1* | | 1 | 1 | 2 | | 1 | 1 | |

*m*: multiplicity; Int: integration

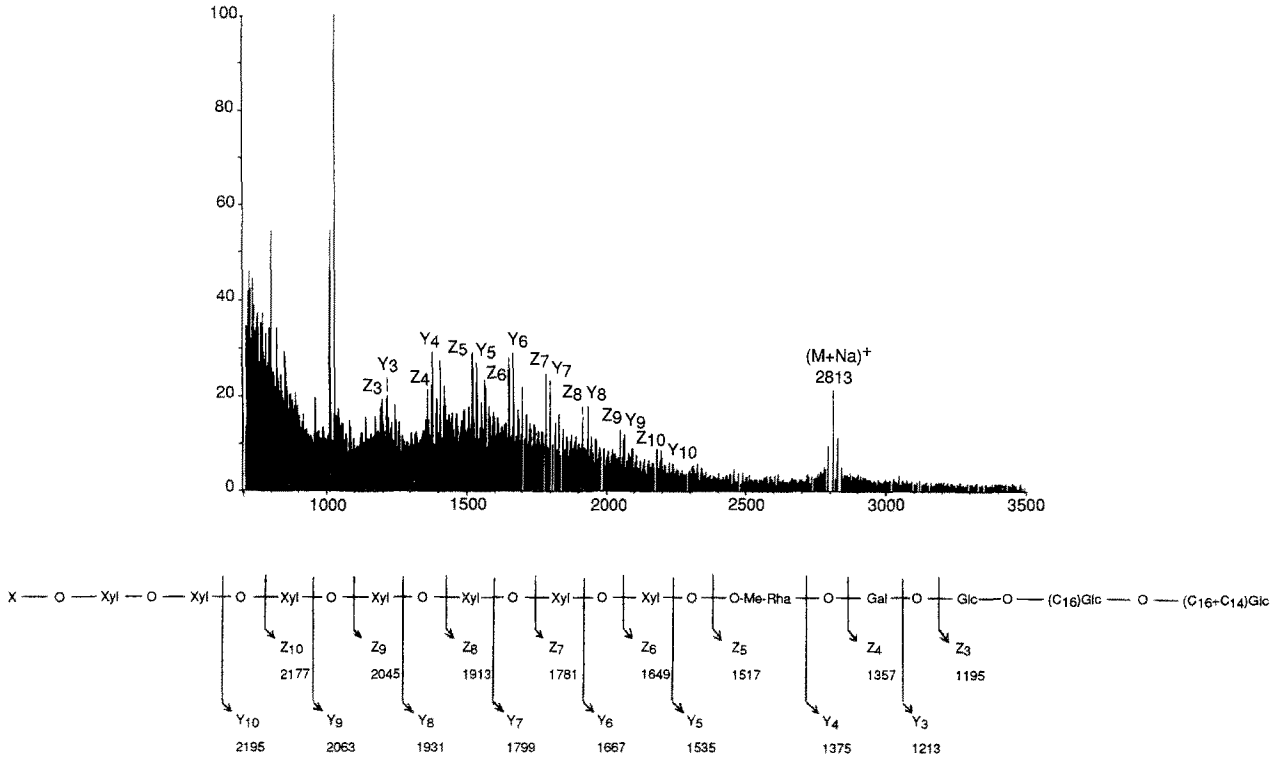


**Figure 1.** FAB/MS spectrum of the native LOS-IV.

**Table 2.** *M. gastri* LOS structures.

| M. gastri *LOS* | *Oligosaccharide structure* |
|---|---|
| LOS-I | β-L-Xyl*p*-(1 → 4)-3-*O*-Me-Rha*p*-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1↔1)-α-D-Glc*p* |
| LOS-II | [β-L-Xyl*p*-(1 → 4)]$_2$-3-*O*-Me-Rha*p*-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔ 1)-α-D-Glc*p* |
| LOS-III | 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-xylo-hex*p*-(1 → 3)-[β-L-Xyl*p*-(1 → 4)]$_6$-3-*O*-Me-Rha*p*-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔1)-α-D-Glc*p* |
| LOS-IV | 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-xylo-hex*p*-(1 → 3)-[β-L-Xyl*p*-(1 → 4)]$_7$-3-*O*-Me-Rha*p*-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔ 1)-α-D-Glc*p* |

and analysis by EI- and CI-GC/MS [19]. Both CI and EI mass spectra analysis and GC co-chromatography with authentic standards proved the presence of 3-*O*-Me-Rha, Xyl, Gal and Glc. Two remaining peaks were assigned to 2,4-dimethyltetradecanoic methyl ester and palmitic methyl ester respectively, supporting the conclusion that these glycolipids belong to the LOS group [5]. Quantitative GC analysis revealed the following sugar ratio for the LOS family: LOS-1 (Rha : Xyl : Gal : Glc; 1 : 1 : 1 :3; mol ratio), LOS-II (Rha : Xyl : Gal : Glc; 1 : 2 :1 : 3; mol ratio) and LOS-IV (Rha:Xyl:Gal :Glc; 1: 7 : 1 : 3; mol ratio).

The 500 MHz $^1$H-NMR spectra of native LOS-I and -II solubilized in $CDCl_3$:$CD_3OD$, 1 : 1, by vol, showed in the anomeric region 8 and 9 signals respectively (Table 1). Most of these signals were assigned (Table 1) by analogy to the NMR studies of LOS-III [8]. From the chemical shifts and coupling constant values, two of these resonances at δ 5.29 ($J_{1,2}$ 3.7 Hz) and δ 5.18 ($J_{1,2}$ 3.6 Hz) for LOS-I and at δ 5.28 ($J_{1,2}$ 3.7 Hz) and δ 5.18 ($J_{1,2}$ 3.6 Hz) for LOS-II were assigned to H1 of the trehalose α-Glc*p*s. The doublet at δ 5.12 (LOS-I) and δ 5.11 (LOS-II) with a weak $J_{1,2}$ coupling constant value (2.8 Hz) corresponded to 3-*O*-Me-Rha*p* anomeric proton. The

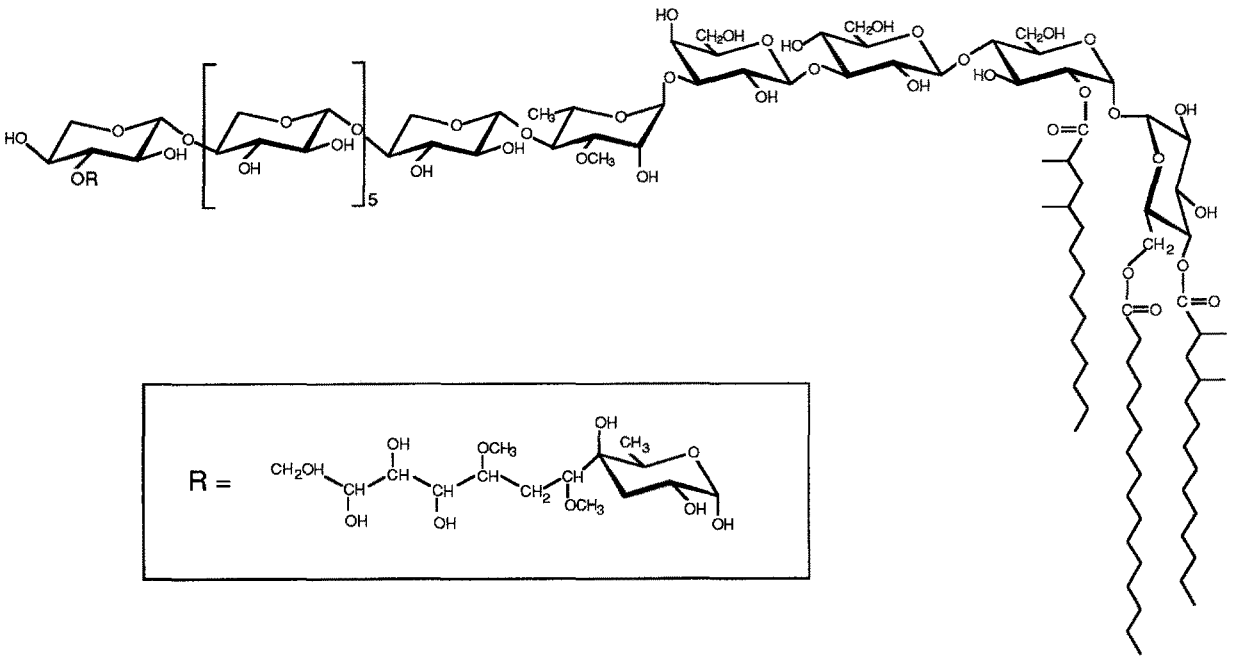


**Figure 2.** *M. gastri* LOS-IV structure.

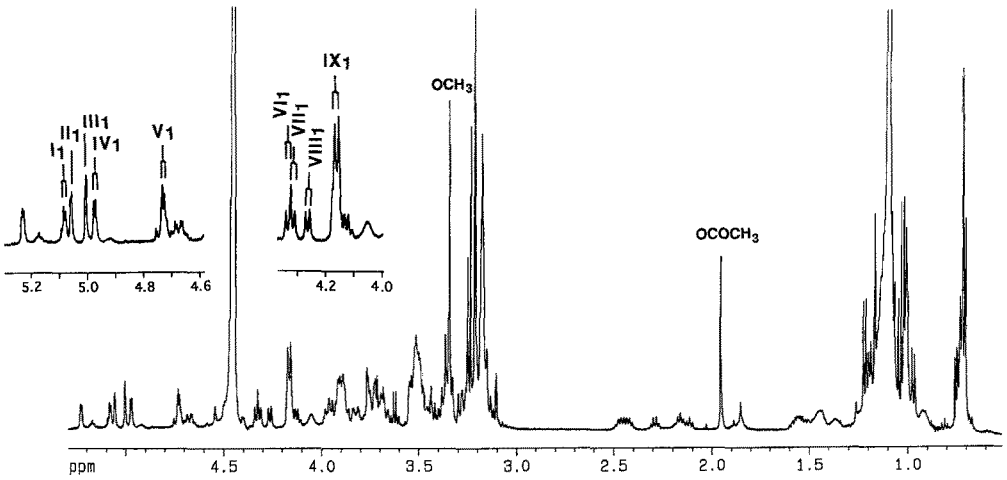


**Figure 3.** 500 MHz $^1$H-NMR spectrum of native LOS-k in $CDCl_3$:$CD_3OD$, 1 : 1, by vol. The one-dimensional $^1$H spectrum was measured using a 90° tipping angle for the pulse and 1.5 s as a recycle delay between each of the 128 acquisitions of 2.23 s. The spectral width of 3700 Hz was digitized on 32 K complex points that were multiplied by a Lorentz-Gauss function (LB–1.3 Hz; GB 0.3) prior to processing to 32 K real points in the frequency domain.

non-anomeric proton resonances at δ 4.95 and δ 4.87, which appeared as triplet and doublet of doublets, respectively, were assigned to the gem-acyl protons. $^1$H-NMR spectra of LOS-I and -II showed three and four β-anomeric signals, respectively, which by comparison with $^1$H chemical shift values of LOS-III were assigned to one residue each of Gal, Glc and Xyl. $^1$H-NMR spectra and GC data identified the presence of an additional β-configurated Xyl residue in LOS-II. These data revealed that LOS-I and -II differed from each other by one β-Xyl*p* unit and were devoid of the terminal C4-branched 3,6-dideoxy-hex*p* present in LOS-III and LOS-IV.

The LOS-IV contained the same monosaccharidic units as LOS-III from GC and $^1$H 1D anomeric region analysis. They differed from each other by the number of Xyl*p* units. Indeed, the $^1$H 1D spectra of LOS-III and LOS-IV were similar except for the Xyl*p* anomeric proton resonance at 4.25 ppm of LOS-

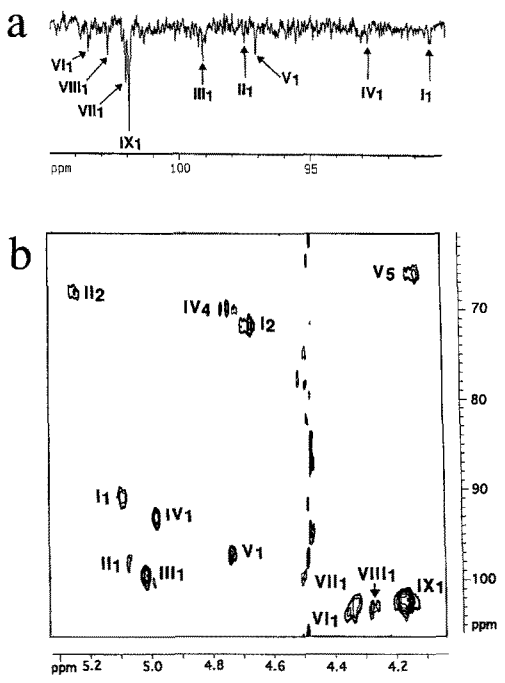


**Figure 4.** (**a**) Expansion of the δ 90 to δ 105 region of the $^{13}$C-NMR spectrum of native LOS-k in $CDCl_3$:$CD_3OD$ 1:1 by vol . The $^1$H-decoupled $^{13}$C-modulated spectrum was recorded with a recycle delay of 2.5 s and 38198 scans accumulated. The spectral width used was 27700 Hz, the data were digitized on 32 K real points, giving a digital resolution of 0.42 Hz per point. The data were multiplied by a line-broadening function (LB 2 Hz) prior to Fourier transformation. (b) Expanded area of the phase-sensitive, $^{13}$C-decoupled, $^1$H-detected multiple quantum correlation spectrum ($^1$H{$^{13}$C} HMQC) of the LOS-k at 500 MHz. The data matrix was 2 K × 256 (TPPI) complex points with 48 scans per $t_1$ value. The spectral window was 27670 Hz in the $F_1$ dimension ($^{13}$C) and 4108 Hz in $F_2$ dimension ($^1$H). The relaxation delay was 1.5 s and the delay after BIRD pulse was 380 ms. For processing, a sine bell window shifted by π 2 was applied in both dimensions, followed by expansion of the data matrix to 4 K × 512 real matrix. Peaks are labelled with a Roman numeral identifying the residue followed by the number or the letter assigning the carbon atom.

IV which integrated for five protons instead of four in the case of LOS-III. This assignment was supported by the FAB/MS spectrum of LOS-IV (Fig. 1) which showed a cationized molecular ion at *m/z* 2813 ($M + Na^+$). Thus, the LOS-IV molecular mass was 132 higher than that of LOS-III in agreement with an additional Xyl*p* unit. The FAB/MS spectrum of the native LOS supported the sequence showing two sets of fragment ions Y and Z assigned to oligosaccharidic and anhydro-oligosaccharidic ions. The assignment of these fragment ions is summarized in Fig. 1. Moreover, the mass values of the fragmentations Y3 (*m/z* 1213) and Z3 (*m/z* 1195) agreed with two C16 and one C14 fatty acids esterifying the trehalose core. Moreover, the localization of C16 and C14 on the distal α-D-Glc*p* was established from the EI-mass spectrum of the per-acetylated LOS showing an oxonium ion at *m/z* 695. LOS-I, -II and -IV differ from each other as illustrated in Table 2 and the structure of LOS-IV is depicted on Fig. 2.

*Structural NMR investigation of the* M. kansasii *LOS*

The *M. kansasii* LOS structures were established by Brennan [5–7]. They are composed of variable residues of β-D-Xyl*p*, 3-*O*-Me-α-L-Rha*p* and Fuc*p* linked to a common tetraglucose core (β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔ 1)-α-D-Glc*p*). Only the polar LOSs which contain, at the other extremity, the 4,6-dideoxy-2-*O*-Me-3-*C*-Me-4-(2′-methoxypropionamido)-α-L-*manno*-hexopyranosyl-(1 → 3)-fucopyranosyl disaccharide unit, were found to be species-specific antigens. The structure of this LOS was established as: KanNacyl-(1 → 3)-Fuc*p*-(1 → 4)-[β-D-Xyl*p*-(1 → 4)]$_6$-3-*O*-Me-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔ 1)-tri-*O*-acyl-α-D-Glc*p*. However, some structural features were missing, i.e. the anomeric configuration of the Fuc*p* unit involved in the antigenic epitope and the localization and number of the acetate [5] and formiate [20] groups described for this molecule. The aim of this investigation was to complete the structure previously proposed using the analytical strategy based on 2DNMR analysis (Figs 3–5) successfully applied for the structural elucidation of LOS-III [8].

LOSs isolated from *M. kansasii* (ATCC 12478) were purified as described by Brennan [5]. The DEAE fractions containing the LOSs, but still contaminated with phospholipids, were then chromatographed by direct-phase HPLC eluted by $CHCl_3$:$CH_3OH$:$H_2O$ 68 : 32 : 4 solvent. By this approach, 30 mg of a polar antigenic LOS (LOS-k) containing the kansosamine (deduced by the presence of an exchangeable proton at δ 6.368 in the $^1$H-NMR spectrum recorded in $CDCl_3$) was recovered. The absolute configurations of the glycosyl residues were determined according to the Gerwig method [21]. The LOS-k was solvolysed in the presence of (+)-2-butanol to yield butyl glycoside diastereoisomers. GC analysis of the trimethylsilylated derivatives showed, as expected, that the glucosyl and the fucosyl residues are 'D'. However, it was found that the xylosyl residues belong to the 'L' series instead of 'D' as previously described.

The anomeric proton area showed 11 signals (Fig. 3). From the HMQC spectrum analysis (Fig. 4), the multiplets at 5.234, 4.749 and 4.679 ppm which correlated with non-anomeric carbon resonances at δ 68.23, δ 69.83, δ 71.69, were assigned to gem acyl protons. The remaining resonances [$I_1$ δ 5.078 ($J_{1,2}$ 3.4 Hz), $II_1$ δ 5.043 ($J_{1,2}$ 1.5 Hz), $III_1$ δ 5.030 ($J_{1,2}$ < 1 Hz), $IV_1$ δ 4.975 ($J_{1,2}$ 3.4 Hz), $V_1$ δ 4.750 ($J_{1,2}$ 3.6 Hz), $VI_1$ and $VII_1$ δ 4.326 ($J_{1,2}$ 7.9 Hz), $VIII_1$ δ 4.290 ($J_{1,2}$ 7.6 Hz), $IX_1$ δ 4.185 ($J_{1,2}$ 7.2 Hz)] were attributed to anomeric protons and were the starting point for the elucidation of the different spin systems on the COSY contour map (not shown) with the help of the HOHAHA (Fig. 5) and ROESY (not shown) spectra (Table 4). Coupling constant values, determined either from the 1D spec-

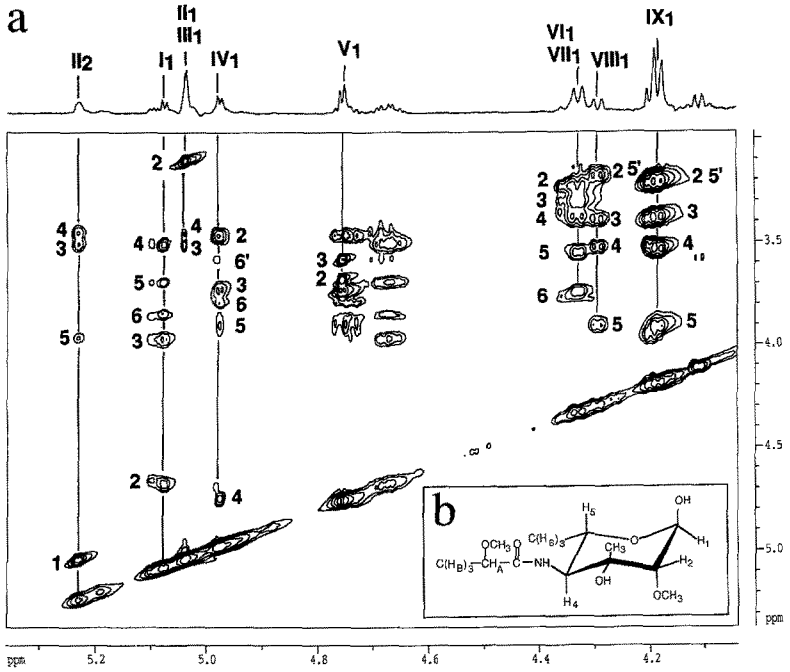


**Figure 5.** (a) Expanded area of the 2D ($^1$H, $^1$H) HOHAHA spectrum of LOS-k (10 mg) in $CDCl_3/CD_3OD$ 1:1 by vol is realized with a spectral width of 4108 Hz, a relaxation delay of 1 s and a spin lock time of 120 ms; the data matrix was 4 K × 512 points, with 72 scans per $t_1$ value. For processing, a sine bell window shifted by $\pi$ 2 was applied in both dimensions, followed by expansion of the data matrix to 4 K × 1 K data points and transformation. (b) *N*-acylkansosamine structure.

**Table 3.** Proton chemical shift values (in ppm) characterizing the *M. kansasii* LOS monosaccharides.

| *Sugar unit* | *H-1* | *H-2* | *H-3* | *H-4* | *H-5* | *H-6* | *Others* |
|---|---|---|---|---|---|---|---|
| NacylKan*p* (III) 1 → 3 | 5.030 | 3.100 | – | 3.76[a] | 3.769 | 1.033 | $OCH_3$ (C2) 3.353<br>$OCH_3$ (ch) 3.247<br>$CH_3$ (C3) 1.186<br>$C(H_B)_3$ (ch) 1.239<br>$H_A$(ch) 3.628 |
| α-Fuc*p* (V) 1 → 4 | 4.750 | 3.676 | 3.577 | ND | 4.111 | 1.101 | |
| (β-Xyl*p*(IX)1 → 4)$_5$ | 4.185 | 3.209 | 3.380 | 3.529 | 3.909/3.209<br>3.955/3.209 | – | |
| β-Xyl*p*(VIII) 1 → 4 | 4.290 | 3.171 | 3.386 | 3.520 | 3.904/3.171 | – | |
| 2-*O*-Ac-3-*O*-Me-Rha*p*(II) 1 → 3 | 5.043 | 5.234 | 3.498 | 3.447 | 3.963 | 1.207 | $OCH_3$(C3)3.255 |
| β-Glc*p*(VI) 1 → 3 | 4.326 | 3.230 | 3.300 | 3.378 | 3.549 | 3.745 | |
| β-Glc*p*(VII) 1 → 4 | 4.326 | 3.230 | 3.300 | 3.378 | 3.549 | 3.745 | |
| α-Glc*p*(I) 1↔1 | 5.078 | 4.679 | 3.968 | 3.500 | 3.692 | 3.857 | |
| α-Glc*p*(IV) | 4.975 | 3.459 | 3.716 | 4.749 | 3.892 | 3.785 | |

[a]The assignment is obtained from the HMQC spectrum.
ND: not determined; ch: propionamido chain

trum or from the cross-peaks in the phase-sensitive DQF-COSY spectrum, allowed the determination of the anomeric configuration [22] as well as the relative stereochemical configuration of the ring protons. Only the chemical shifts of the reporter carbons (anomeric, glycosylated, methoxylated, acetylated or acylated) were determined by the analysis of the HMQC spectrum (Table 4).

The upfield $^{13}$C resonances at δ 90.61 and 92.82 typified a trehalose unit. The anomeric carbon at δ 90.61 correlated on the HMQC spectrum with the proton $I_1$ at δ 5.078 (Fig. 4).

**Table 4.** Carbon chemical shift values (in ppm) characterizing the *M. kansasii* LOS monosaccharides.

| *Sugar unit* | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *Others* |
|---|---|---|---|---|---|---|---|
| NacylKan*p* (III) 1 → 3 | 99.26 | 83.13 | 70.85 | 55.67 | 65.99 | ND | $OCH_3$(C2) 58.32<br>$CH_3$(C3)18.19<br>CO (C4) 175.43<br>$OCH_3$(C4)56.59<br>$C(H_B)_3$(C4)17.39 |
| α-Fuc*p* (V) 1 → 4 | 97.11 | ND | 77.67 | ND | 66.19 | ND | |
| [β-Xyl*p*(IX) 1 → 4]$_5$ | 101.93 | 71.88 | 73.62 | 76.07 | 63.05 | – | |
| β-Xyl*p*(VIII) 1 → 4 | 102.76 | ND | ND | ND | ND | – | |
| 2-*O*-Ac-3-*O*-Me-Rha*p*(II) 1 → 3 | 97.53 | 68.23 | ND | 78.91 | ND | ND | $OCH_3$(C3) 57.01<br>acetate (C2) 20.05;171.02 |
| β-Glc*p*(VI) 1 → 3 | 103.52 | ND | 81.70 | ND | ND | ND | |
| β-Glc*p*(VII) 1 → 4 | 102.06 | ND | 86.60 | ND | ND | ND | |
| α-Glc*p*(I) 1 ↔ 1 | 90.61 | 71.69 | ND | 79.19 | ND | ND | |
| α-Glc*p*(IV) | 92.82 | ND | ND | 69.83 | ND | ND | |

ND, not determined.

This spin system characterized a 2-*O*-acyl-α-Glc*p* since the non-anomeric deshielded proton which resonated at δ 4.679 was identified as $I_2$. The second trehalose anomeric carbon at δ 92.82 correlated on the HMQC spectrum with the proton $IV_1$ at δ 4.975 ($J_{1,2}$ = 3.4 Hz) (Fig. 4). The system IV was attributed to a 4-*O*-acyl-α-Glc*p* based on the resonance of H4 at δ 4.749. This proton resonated independently as a triplet ($J_{3,4}$ = $J_{4,5}$ = 9.2 Hz) supporting the glucosyl structure of the unit. Thanks to the 2D HOHAHA spectrum (Fig. 5), all the proton resonances up to the H6 were assigned.

The coupling constants of the anomeric protons $II_1$ at δ 5.043 ($J_{1,2}$ 1.5 Hz) and $III_1$ at δ 5.030 ($J_{1,2}$ 1.0 Hz) was indicative of an equatorial H2 configuration, in agreement with the Rha*p* and kansosamine structures. The H3 resonance at 3.498 ppm established that the spin system corresponds to the 3-*O*-Me-Rha*p* since the kansosamine is devoid of H3 protons. The downfield shift of the H2 resonance agreed either with a *O*-acyl or a *O*-acetyl group on C2 (δ 68.23). The correlation between this H2 and the carbonyl of the acetyl group which resonated at 171.02 ppm was missing. This chemical shift was determined from HMBC spectrum, showing a correlation between the carbonyl carbon and the methyl at 1.967 ppm (C : 20.05 ppm). In agreement with the GC/MS data and by analogy to the LOS structures, it was assumed that the acetyl group revealed by the singlet at 1.967 ppm (Fig. 3) is linked to the C2 leading to the structure 2-*O*-Ac-3-*O*-Me-Rha*p*.

The anomeric proton $III_1$ which resonated independently as a doublet ($J_{1,2}$ 1.0 Hz) at δ 5.030 assigned to the *N*-acyl kansosamine H1 correlated with a H2 at 3.100 ppm which also appeared as a doublet ($J_{1,2}$ 1.0 Hz). As expected no connectivity with an H3 was observed by the COSY, HOHAHA, RCT-COSY experiments. The presence of a methyl group in C3 was deduced from the HMBC spectrum, showing a correlation between the C3 (δ 70.85) and the methyl group (δ 1.186), and from the ROESY spectrum by the observation of intra-residue correlations between the proton of the C3 C-methyl group (1.186 ppm) and the H1, H2 and H5 (not shown). The structure of the methoxypropionic acid chain linked to the C4-amine function was revealed by the HMBC correlations between $H_A$ (δ 3.628) and the methyl carbon (δ 17.39), the methoxy carbon (δ 56.59) and the carboxy carbon (δ 175.43) of the propionamido substituent. The downfield chemical shift of the C4 (δ 55.67) indicated the location of the methoxypropionamido chain on the C4 (23) (Fig. 5b).

The system V was attributed to the α-Fuc*p* ($J_{1,2}$ 3.6 Hz) from the characteristic multiplicity and chemical shift (q, δ 4.111) of its H5. It was not possible to characterize the H4 from the COSY and the HOHAHA spectra. This was coherent with the *manno* configuration ($J_{3,4}$ 3.0 Hz ; $J_{4,5}$ < 1.0 Hz). The spin systems VI and VII, starting from the degenerated anomeric protons at δ 4.326 ($J_{1,2}$ 7.9 Hz) showed identical cross-peak pathways. They corresponded to 2 β-Glc*p*s. From the COSY spectrum, the cross-peaks were hindered by those of system IX, but were highlighted by the HOHAHA spectrum, by a cross-section through the anomeric proton resonances (Table 3).

At this point, all the expected units identified by GC/MS have been characterized except β-Xylose which corresponds to the VIII and IX, integrating respectively for 1 and 5 protons. The entire proton and carbon spin system IX, starting from its anomeric proton resonance at δ 4.185 was easily determined due to the intensities of the cross-peaks until one H5 proton (Table 3). The other H5′ proton resonated at the same chemical shift as the H2 as proved by correlation on the HMQC spectrum (not shown) between C2 (δ 71.88) and C5 (δ 63.05) and degenerated H2/H5 at 3.209 ppm. The remaining anomeric proton $VIII_1$ δ 4.290 correlated with H2

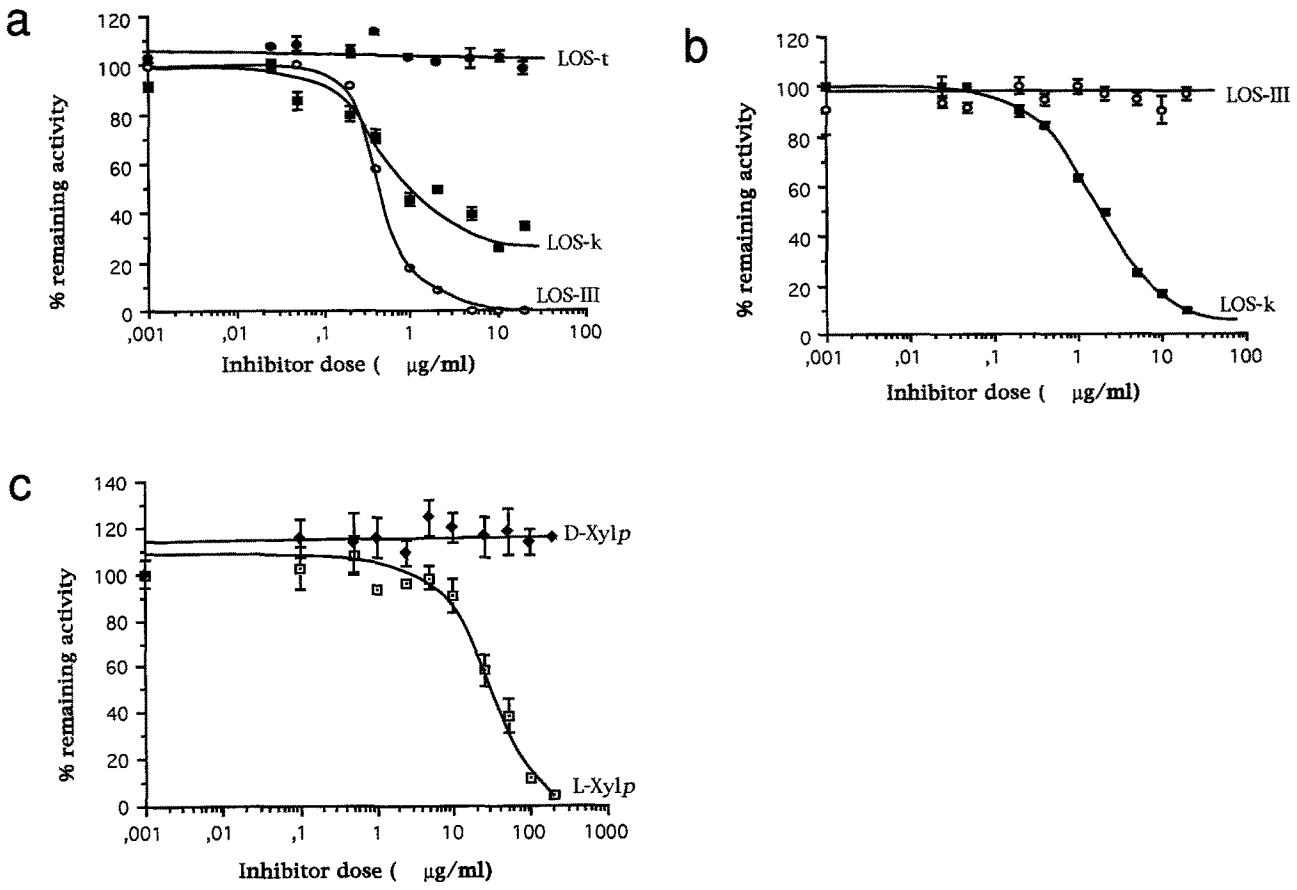


**Figure 6.** Inhibition ELISA of: (a) anti-*M. gastri* serum binding to coated LOS-III by LOS-III, LOS-k, LOS-t; (b) anti-*M. kansasii* serum binding to coated LOS-k by LOS-III and LOS-k; and (c) anti-*M. gastri* serum binding to coated LOS-III by L- and D-methyl xylopyranosides.

**Table 5.** Structure of the oligosaccharidic part of the major LOS isolated from *M. kansasii*, *M. gastri* W471, *M. tuberculosis* strain Canetti.

*M. kansasii*:

4,6-dideoxy-2-*O*-Me-3-*C*-Me-4-(2′-methoxypropionamido)-L-*manno*-hex*p*-(1 → 3)-Fuc*p*-(1 → 4)- [{β-L-Xyl*p*-(1 → 4)}$_6$] 2-*O*-Ac-3-*O*-Me-Rha*p*-(1 → 3)-β-D-Glc*p*-(1 → 4)- [β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔1)-α-D-Glc*p*]

*M. gastri* W471:

3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*hex*p*-(1 → 3)- [{β-L-Xyl*p*-(1 → 4)}$_6$] 3-*O*-Me-Rha*p*-(1 → 3)-β-D-Gal*p*-(1 → 3) [-β-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 ↔ 1)-α–D-Glc*p*]

*M. tuberculosis* (strain Canetti):

4-amino-4,6-dideoxy-Gal*p*-(1 → 3)-2-*O*-Me-α-L-Fuc*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)-2-*O*-Me-α-L-Rha*p*-(1 → 3)-2-*O*-Me-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)-4-*O*-Me-α-L-Rha*p*-(1 → 3)-6-*O*-Me-α-D-Glc*p*-(1 ↔ 1)-α-D-Glc*p*

at 3.171 ppm from the 2D COSY spectrum. The entire spin system was defined by the HOHAHA spectrum with a cross-section through the H1 (Fig. 5) revealing that the protons of system VIII resonate very closely with those of system IX (Table 3).

The sequence of the glycosyl residues was deduced by HMBC experiments from scalar couplings $^3J_{C,H}$ between $^{13}C$ and $^1H$ involved in the glycosidic linkage and/or by ROESY experiment from dipolar couplings between space proximal protons of two distinct units. As a resonance of a carbon either

involved in an interglycosidic linkage or linked to a methoxy group is shifted downfield by 4 to 10 ppm, the glycosylated carbons are easily characterized from their chemical shifts (between 75 and 87 ppm for C3 and C4). This study supported the sequence proposed by Brennan and leads us to propose the LOS-k structure: KanNacyl-(1→3)-α-D-Fuc*p*-(1→4)-[β-L-Xyl*p*-(1→4)]$_6$-2-*O*-Ac-3-*O*-Me-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-2-*O*-acyl-α-D-Glc*p*-(1↔1)-4-*O*-acyl-α-D-Glc*p*.

*LOS-III epitope*

As previously described for the LOS from *M. kansasii* [5–7], ELISA binding studies revealed the presence of anti-LOS-III antibodies in the rabbit polyclonal anti-*M. gastri* serum used, supporting the LOS-III antigenicity. Unexpectedly, it was observed that anti-LOS-III antibodies also bind, but with a lower affinity, the LOS from *M. kansasii* (LOS-k) (Fig. 6a). By means of the serological approach, but using an anti-*M. kansasii* serum, it was established that LOS-III do not bind the anti-LOS-k antibodies (Fig. 6b). The lack of LOS-III binding can be explained by the structure of the LOS-k epitope assigned to 4,6-dideoxy-2-*O*-Me-3-*C*-Me-4-(2′-methoxypropionamido)-α-L-*manno*hexopyranosyl-(1→3)-Fuc*p* (Table 3). In order to determine the molecular basis of LOS-k binding to anti-LOS-III antibodies, two extra ligands were used, the LOS from *M. tuberculosis* strain Canetti (LOS-t) (Table 5) and methyl xylopyranoside. The LOS-t epitope can be delineated to the distal disaccharide with the following incomplete structure 4-amino-4,6-dideoxy-Gal*p*-(1 → 3)-2-*O*-Me-α-D-Fuc*p* [23]. This LOS-t, which was also characterized by the absence of a xylane core, was unable to bind the anti-LOS-III antibodies. Moreover, methyl L-xylopyranosides, but not methyl D-xylopyranosides, bound the LOS-III antibodies (Fig. 6c).

## Discussion

Mycobacterial lipooligosaccharides contain an invariant trehalose backbone glycosylated by species-specific oligosaccharide appendages. In the case of the *M. kansasii* LOS, it was reported that upon deacylation, the LOSs yield non-reducing oligosaccharides of different sizes. But all have in common a trehalose 'core' β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔1)-α-D-Glc*p* and 3-*O*-Me-Rha*p*. They differ by the number of xyloses while fucose and *N*-acylkansosamine are only present in the more polar *M. kansasii* LOSs which are the antigens. Similarly the *M. gastri* LOSs are composed of a common core 3-*O*-Me-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→ 4)-α-D-Glc*p*-(1↔1)-α-D-Glc*p* glycosylated by one β-L-Xyl*p* (LOS-I) or by two β-L-Xyl*p* (LOS-II) in a similar manner to *M. kansasii* LOSs, only the more polar LOSs (LOS-III and LOS-IV), containing the distal 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-D-*xylo*hex*p*, are antigenic (Table 2).

The NMR approach developed for the structural elucidation of *M. gastri* LOS-III was applied to the native LOS of *M. kansasii*. This strategy allowed us to determine more precisely the structure previously established by Brennan [5–7, 20]. One acetyl group was identified and localized on the C2 of the 3-*O*-Me-Rha*p*. Two fatty acyls were found linked to the C2 of the penultimate α-Glc*p* and on the C4 of the terminal one. The presence of a third acyl group located on the C6 of the 4-*O*-acyl-α-Glc*p*, as for *M. gastri* LOS, was established from MS data. The α-anomeric configuration was found for the D-Fuc*p* unit. The xylose appeared to belong to the L-series, instead of 'D' as previously reported.

By analogy to the LPS dideoxyhexoses, the distal LOS-III monosaccharide 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-D-*xylo*hex*p* must typify the *M. gastri* species since it has previously been shown that LOS-III is present in all the *M. gastri* strains so far investigated. Thus, a molecular marker has now been identified allowing the unambiguous differentiation of *M. gastri* from *M. kansasii*, two strains considered by some bacteriologists as synonymous. Moreover, *M. kansasii* is known to be the etiological agent of pulmonary disease while *M. gastri* is considered to be non-pathogenic. Thus, in order to characterize *M. gastri* by a serological probe, the specificity of the anti-LOS-III antibodies was investigated. Using ELISA, it was established that the LOS-III epitope is composed of the distal disaccharide: 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*hex*p*-(1→3)-β-L-Xyl*p*. The principle that the terminal mono- or disaccharide of an assortment of glycolipids is the primary antibody binding site and responsible for much of the exquisite specificity of mycobacterial species and subspecies is well documented. For example, the use of murine monoclonal antibodies has been shown to implicate the unique terminal 4,6-dideoxy-2-*O*-Me-3-*C*-Me-4-(2′-methoxypropionamido)-L-*manno*hexopyranosyl-(1→3)-Fuc*p* as the specific antigenic determinant of the *M. kansasii* LOS [7]. Moreover, the oligosaccharidic moiety of the major phenolic glycolipid described in the *M. kansasii* cell wall (PheGl K-I) contains a monosaccharide unique in nature, i.e. a 2,6-dideoxy-4-*O*-Me-α-D-*arabino*hexopyranose [2, 3]. This monosaccharide, which is found at the extremity of the molecule, has been demonstrated to behave as the immunodominant monosaccharide of the PheGl K-I antigen [4], the epitope being defined as the terminal acetylated disaccharide [1]. Similarly, the LOS-III epitope was delineated to the distal disaccharide containing at the penultimate position the β-Xyl*p* unit. Surprisingly, this study revealed that LOS-k, which contains an epitope structurally different from the LOS-III epitope, was able to significantly bind the LOS-III antibodies. Thus, this binding does not involve the LOS-k epitope but a structure common to both LOSs, i.e. either the xylane core or the acylated trehalose unit. The LOS-t which contains the trehalose core but where the xylane core is missing (Table 5) did not bind the LOS-III antibodies. Thus, it can be proposed that LOS-III antibodies bind LOS-k through the xylane core.

In a previous study [24], it was established that antibodies against immunoreactive glycolipids bound optimally two sequential glycosyl residues located at the non-reducing end suggesting that two sub-sites compose the paratope cavity. The deeper one, with the stronger affinity, bound the terminal glycosyl residue. It was assumed that the stereospecific binding occurred via a two step process: (i) ligand penetration into the paratope cavity; and (ii) stereospecific linkage through hydrogen bonds [18]. From the binding studies and the literature data, two sub-sites were assumed to compose the LOS-III cavity paratope. One sub-site optimally bound the 3,6-dideoxy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*hex*p* and the second one β-L-Xyl*p*. Due to the steric hindrance of the distal glycosidic unit, its sub-site cavity size must fit with a disaccharide composed of ubiquitous monosaccharides. Thus, it can be advanced that the LOS-III paratope could fit with the volume size of trisaccharide. Indeed, it can be proposed that the distal trisaccharidic unit 4,6-dideoxy-2-*O*-Me-3-*C*-Me-4-(2′-methodypropionamido)-L-*manno*hexopyranosyl-(1→3)-Fuc*p*-(1→4)-Xyl*p* from the LOS of *M. kansasii* can penetrate into the LOS-III paratope cavity. From this model, it emerges that the Xyl*p* located in the antepenultimate position could bind the Xyl*p* sub-site of the LOS-III paratope. These assumptions are in complete agreement with the binding of LOS-k by the anti-LOS-III antibody through Xyl*p* but also with the lack of binding of LOS-III by the *M. kansasii* anti-LOS antibody. Thus, a monoclonal antibody restricted to the recognition of 3,6-dideo xy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*-hex*p* needs to be prepared with the aim of differentiating the pathogenic strain *M. kansasii* from the non-pathogenic one *M. gastri*.

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