Structure Elucidation of the Highly Heterogeneous Lipid A from the Lipopolysaccharide of the Gram-Negative Extremophile Bacterium *Halomonas Magadiensis* Strain 21 M1

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Keywords: *Halomonas magadiensis* / Extremophilic bacteria / Lipid A / NMR spectroscopy / Mass spectrometry

Halomonas magadiensis (formerly named *Halomonas magadii*) is a Gram-negative extremophilic and alkaliphilic bacterium isolated from Lake Magadi, which is located in the East African Rift Valley. Several members of the halomonad group of bacteria have been shown to inhabit the alkaline brines, including a new member, *Halomonas magadiensis* strain 21 M1 (NCIMB 13595), an organism that grows at high pH and relatively high salt concentration. The unusual structure of the lipid A family derived from the lipopolysaccharide of *Halomonas magadiensis* is reported herein. The structure was determined using chemical analysis, NMR spectroscopy and MALDI-TOF mass spectrometry. Lipid A was also analysed after either de-*O*-acylation or dephosphorylation. The resultant mixture was very heterogeneous in fatty acid substitution, from heptaacyl to triacyl species. The various lipid A molecules obtained by the removal of one or more acyl substituents from the heptaacyl species are described below.

Introduction

Lipopolysaccharides (LPSs) are the major component of the outer membrane of almost all Gram-negative bacteria. They are amphiphilic macromolecules composed of a hydrophilic polysaccharide (formed from a core oligosaccharide and an O-specific polysaccharide) covalently linked to a lipophilic moiety called lipid A, which anchors these macromolecules to the external membrane.

LPSs are also known as endotoxins because of their role in the pathogenesis of Gram-negative infection. In fact, they are responsible for septic shock. The biological activity of LPS is due principally to the lipid A constituent while the toxicity of lipid A is strictly dependent on its primary structure.

Several nontoxic lipid A compounds, such as that from *Rhodobacter capsulatus,*[1] and some synthetic analogues, act as antagonists to the action of LPS in human cells. Thus, the study of lipid A molecules from bacteria non-pathogenic to humans is extremely important for identification of lipid A molecules that,in human cells, display inhibitory activity on effects such as cytokine production stimulated by LPS/lipid A from toxic Gram-negative bacteria.

Generally, lipid A has a highly conservative structure. It is composed of a 2-amino-2-deoxy-glucopyranose (glucosamine, GlcN) disaccharide backbone, phosphorylated at positions O-1 and O-4′ and acylated at positions N-2 and O-3, of both proximal and distal glucosamines (GlcN I and GlcN II, respectively) with 3-hydroxy-substituted fatty acids (primary acylation). These fatty acids are themselves esterified by fatty acids (secondary acylation).[2,3]

Herein, we report the determination of the unusual structure of the lipid A family derived from the LPS of *Halomonas magadiensis,* which is a Gram-negative extremophilic and alkaliphilic bacterium isolated from Lake Magadi, which is located in the East African Rift Valley.

Several members of the halomonads group of bacteria were found inhabiting the alkaline brines, including *Halomonas magadiensis* strain 21 M1 (NCIMB 13595), an organism that grows at high pH and relatively high salt concentration. Studies on the unusual structure of the O-specific chain of the LPS fraction have been reported previously.[4]

The structure determination was achieved using chemical analysis, NMR spectroscopy and MALDI-TOF mass spectrometry

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Results

Isolation and Compositional Analysis of Lipid A

The LPS was recovered in the water phase after the hot phenol/water extraction, then dialyzed and lyophilized. The LPS produced by *Halomonas magadiensis* is a smooth type LPS, as confirmed by the ladder-like pattern shown by SDS-PAGE.[4b] Lipid A was obtained by mild hydrolysis of the LPS.

The compositional and methylation analyses of lipid A indicated organic bound phosphate, 6-D-GlcN and *t*-D-GlcN.

Fatty acid analysis revealed the presence of (*R*)-3-hydroxydodecanoic acid [(*R*)-12:0 (3OH)] both as amide and ester, while tetradecanoic acid (14:0), decanoic acid (10:0), esadecanoic (16:0) and ottadecenoic acid (18:1) were found exclusively as their esters.

Traces of dodecanoic acid (12:0) were also found. A molar ratio 1:1:3.3 was found for GlcN/phosphate/fatty acid.

The negative-ion MALDI-TOF MS of intact lipid A was analysed with reference to the chemical analysis and revealed a remarkable heterogeneity. Actually, the spectrum (Figure 1) contained several molecular ion peaks indicating differences in the number, type and distribution of the acyl moieties on the disaccharide backbone. On the basis of the compositional data obtained, it was possible to give an initial structural assignment of the main ion peaks of the spectrum.

In particular, several groups of ion peaks, from m/z = 1026.1 to 1921.9 (**1**−**8**, Table 1), corresponding to tri-, tetra-, penta-, hexa-, and heptaacyl bis-phosphorylated lipid A species, could be identified.

The highest mass ion peaks, **1a** and **1b** at m/z = 1921.9 and 1895.2 are consistent with a heptaacylated bis-phosphorylated disaccharide glucosamine backbone possessing four 12:0 (3-OH) residues and a 14:0, a 10:0 and a 18:1 or 16:0 residue, respectively. In order of decreasing mass, the hexaacylated species **2a**, **2b** at m/z = 1767.4 and 1740.5

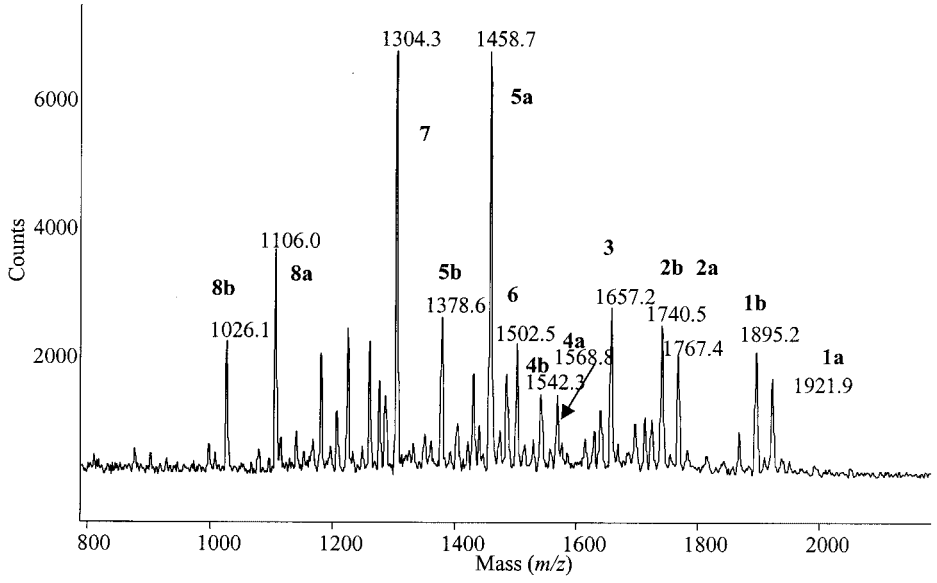


Figure 1. Negative-ion MALDI-TOF mass spectrum of lipid A of LPS from *H. magadiensis*. All the relevant ion peaks are described in the text and in Table 1

Table 1. The main MALDI-TOF MS negative-ion peaks from Figure 1 and the proposed interpretation of the substituting fatty acids on the bis-phosphorylated lipid A backbone

Species	Observed ion peaks (m/z)	Acyl substitution	Proposed fatty acid composition
1a	1921.9	heptaacyl	4 × 12:0 (3-OH), 10:0, 14:0, 18:1
1b	1895.2	heptaacyl	4 × 12:0 (3-OH), 10:0, 14:0, 16:0
2a	1767.4	hexaacyl	4 × 12:0 (3-OH), 14:0, 18:1
2b	1740.5	hexaacyl	4 × 12:0 (3-OH), 14:0, 16:0
3	1657.2	hexaacyl	4 × 12:0 (3-OH), 14:0, 10:0
4a	1568.8	pentaacyl	3 × 12:0 (3-OH), 14:0, 18:1
4b	1542.3	pentaacyl	3 × 12:0 (3-OH), 14:0, 16:0
6	1502.5	pentaacyl	4 × 12:0 (3-OH), 14:0
5a	1458.7	pentaacyl	3 × 12:0 (3-OH), 10:0, 14:0
7	1304.3	tetraacyl	3 × 12:0 (3-OH), 14:0
8a	1106.0	triacyl	2 × 12:0 (3-OH), 14:0

lacked a 10:0 residue ($\Delta m/z$ = 154) whereas the other hexaacylated species, **3**, at m/z = 1657.2, carried four 12:0 (3-OH), one 10:0, and one 14:0 residue. The pentaacyl ion **6** at m/z = 1502.5 lacked a 10:0 residue ($\Delta m/z$ = 154) when compared with species **3**. Pentaacyl species **4a** and **4b** differed from **2a** and **2b**, respectively, by a 12:0 (3-OH) residue ($\Delta m/z$ = 198).

The most abundant peaks in the MS spectrum were penta- and tetraacylated species at m/z = 1458.7 and 1304.3. The species **5a**, at m/z = 1458.7, is consistent with a lipid A molecule carrying three 12:0 (3-OH), one 14:0, one 10:0 residue. The species **7** at m/z = 1304.3 lacks a 10:0 residue($\Delta m/z$ = 154). In order of decreasing mass, the ion peaks **8** at m/z = 1106.0 can be assigned to a triacyl species minus a 12:0(3-OH) residue.

Other ion peaks with $\Delta m/z$ = 80 (**8b** and **5b**) suggest the presence of mono-phosphorylated species. Other peaks with $\Delta m/z$ = 28 indicate the presence of minor species with 12:0 instead of 14:0 residues.

Analysis of De-O-acylated Lipid A

An aliquot of lipid A from *Halomonas magadiensis* was completely de-*O*-acylated with anhydrous hydrazine and the product analysed by MALDI-TOF MS and NMR spectroscopy.

The MALDI-TOF mass spectrum showed, as expected, an ion peak at m/z = 896.3 (spectrum not shown) diagnostic of a bis-phosphorylated lipid A species possessing two amide-linked 12:0(3-OH) residues. A minor peak at m/z = 816.1 ($\Delta m/z$ = 80) corresponds to a mono-phosphorylated species.

In order to elucidate the fine structure of the carbohydrate backbone, the de-*O*-acylated lipid A was analysed by 2D-NMR spectroscopy in [D_6]DMSO at 373 K (COSY, TOCSY, ROESY, ^{13}C-^{1}H HSQC, ^{31}P-^{1}H HSQC).

Two major signals were detected in the anomeric region of the HSQC spectrum at δ = 5.23 and 4.45 ppm, while the carbon signals resonated at 90.2 and 101.2 ppm, respectively. These two cross peaks are diagnostic of the α-configuration of GlcN I and the β-configuration of GlcN II, respectively. Two cross peaks were observed in the ^{31}P-^{1}H HSQC spectrum, representative of mono-phosphate monoester groups. The phosphate signal at δ = −1.8 ppm correlates with H-1 of α-D-GlcpN and the one at 4.0 ppm correlates with a proton at δ = 3.55 ppm, identified as H-4 from GlcN II. A complete 2D-NMR analysis allowed assignment of all the proton and carbon resonances of the two GlcN spin systems (Figure 2 and Table 2). Moreover, it was possible to distinguish most of the distinctive resonances of the acyl chains at position 2 and 2′ of the carbohydrate backbone, as proven by the acylation shift of the H-2 and H-2′ signals.

The ^{13}C downfield shift of C-6 of GlcN I and, in the ROESY spectrum, the *intra* residue NOE contact of H-1′ to H-3′ and H-5′, together with the *inter* residue NOE contact of H-1′ with H-6, are diagnostic of a β-(1→6) linkage between the GlcN residues.

On the basis the above data, it can be concluded that the structure contains a P→4-D-GlcpN-β-(1→6)- D-GlcpN-α-1→P backbone bearing acyl chains at position 2 and 2′.

In order to determine the identity of the amide-bound acyloxyacyl moieties, an aliquot of the sample was treated with 32% NH_4OH. This mild hydrolysis cleaves selectively acyl- and acyloxyacyl esters leaving acyl- and acyloxyacylamide moieties unaltered.[5] The partially degraded lipid A was analysed by MALDI-TOF MS. The negative-ion mass

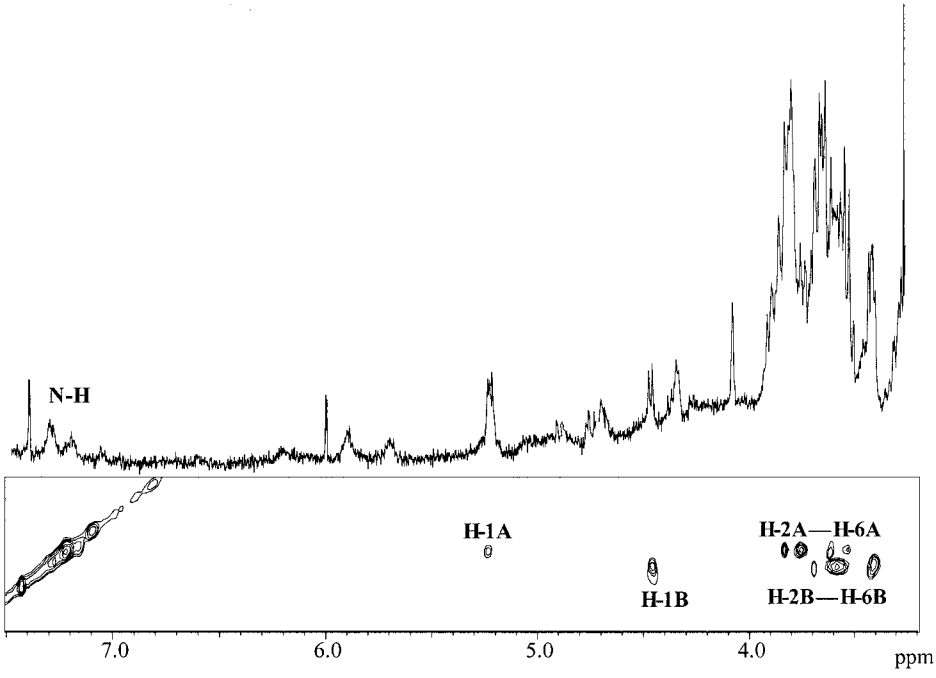


Figure 2. Sections of TOCSY and ^{1}H NMR spectra of de-*O*-acylated lipid A from *H. magadiensis* in which several spin system correlations of the disaccharide backbone are visible. In this figure, Letters **A** and **B** refer to GlcN I and GlcN II, respectively

Table 2. ^{1}H-, ^{13}C- and ^{31}P NMR resonances of de-*O*-acylated lipid A from *Halomonas magadiensis*; the spectra were obtained in $[D_6]$DMSO at 400, 100 and 162 MHz, respectively, at 373 K

δ ($^{1}H/^{13}C/^{31}P$)	1	2/N−H	3	4	5	6
A	5.23	3.61/7.17	3.77	3.15	3.52	3.85/3.79
α-GlcpN	90.2	54.1	71.3	70.2	70.8	67.1
	−1.8					
B	4.45	3.42/7.27	3.52	3.55	3.13	3.73/3.55
β-GlcpN	101.2	55.7	72.6	70.9	76.5	60.8
				4.0		
C12:0 (3-OH)	$(CH_2)_\alpha$	$(CH_2)_\beta$	$(CH_2)_\gamma$	$(CH_2)_n$	CH_3	
	2.18	3.81	1.38	1.26	0.85	
	43.1	70.9	36.2	28.2	13.1	

spectrum (Figure 3) revealed tetraacylated species at m/z = 1261.3 and 1181.2 and triacylated species at m/z = 1106.9 and 1027.1. These ion peaks indicate the presence of acyloxyacyl amide moieties, the nature of which was apparent on the basis of the previous data. The most important ion, at m/z = 1261.3, is consistent with a tetraacyl bis-phosphorylated lipid A species with two amide-linked 12:0 (3-OH) residues, one of which is esterified by a 10:0 residue and the other esterified by a 14:0 residue. The triacyl ion peak at m/z = 1106.9 lacked a 10:0 residue ($\Delta m/z$ = 154). The ions at m/z = 1181.2 and 1027.1 were attributed to the same species bearing only one phosphate group.

The ions at m/z = 1232.7 and 1079.4 were diagnostic of minor amounts of tetra- and triacylated species, respectively, carrying a 12:0 instead of a 14:0 acyl residue.

Thus, the ammonium hydrolysis provided further information about the structure: the secondary fatty acids 14:0 and 10:0 are carried by amide bound primary fatty acids (Figure 3).

Analysis of Dephosphorylated Lipid A

The accurate distribution of fatty acids on the two GlcNs can be determined by the study of the oxonium ions of GlcNs in the positive-ion MALDI-TOF mass spectrum. These ions originate from the cleavage of glycoside linkages between the two GlcN residues.

For this purpose, an aliquot of lipid A was fully dephosphorylated and analysed. The positive-ion MALDI-TOF MS showed pseudomolecular adduct ions $[M + Na]^+$ and the mass difference between the molecular species in the negative-ion MALDI-TOF MS of intact lipid A could be assigned only to phosphate groups, thus confirming that no other groups were linked to the polar heads (data not shown).

Interestingly, the spectrum contained numerous and informative oxonium ions derived from the non-reducing GlcN (Figure 4). The oxonium ions at m/z = 1007.3 and 1033.6 were identified as tetraacyl ions containing two 12:0(3-OH), one 14:0 and one 16:0 or 18:1 residues, respectively. According to the above ammonium hydrolysis data, the amide-linked 12:0 (3-OH) residue is esterified by a 14:0 residue and, consequently, the ester-linked 12:0 (3-OH) residue is substituted by a 16:0 or a 18:1 residue, furnishing a [GlcN-12:0(3-OH)-12:0(3-OH)-14:0−16:0/18:1]$^+$ structure.

The presence of a 14:0 residue on the non-reducing GlcN indirectly demonstrates that on the reducing unit a 10:0 residue substitutes the primary *N*-linked 12:0(3-OH) residue.

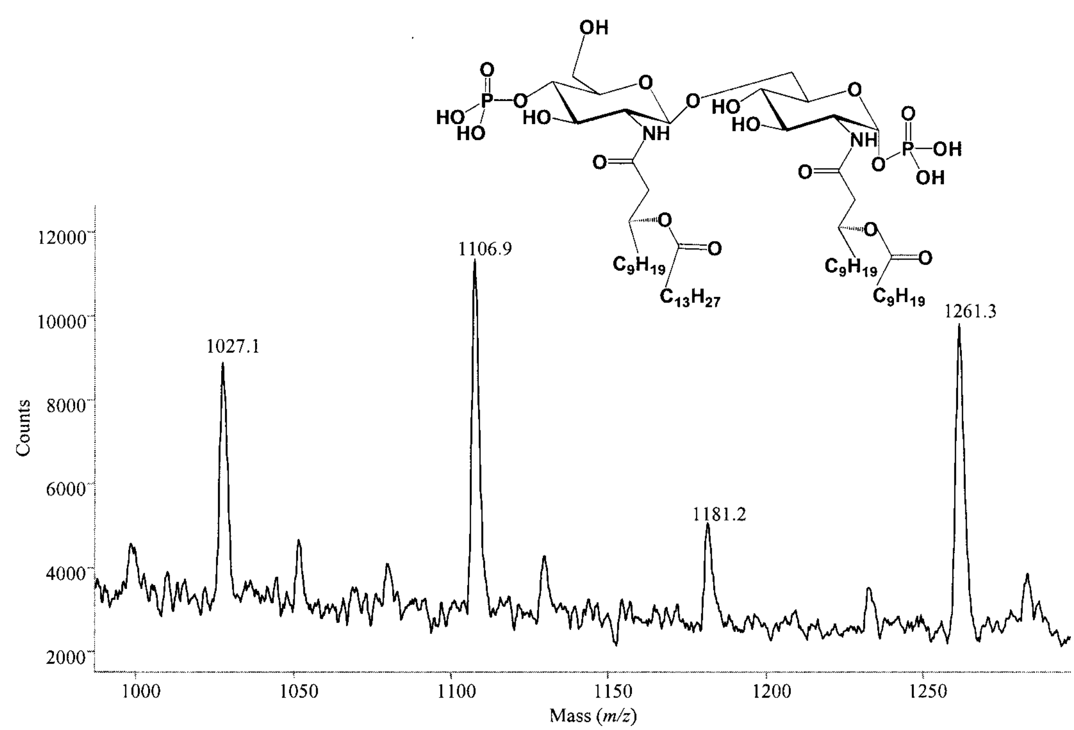
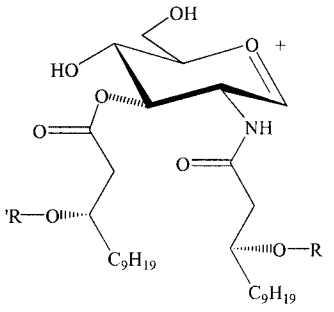


Figure 3. Negative-ion MALDI-TOF mass spectrum of de-*O*-acylated lipid A of *H. magadiensis.* The sketched formula refers to m/z = 1261. Other peaks derive from the lack of 10:0 residue and a phosphate group ($\Delta\ m/z$ = 154 and $\Delta\ m/z$ = 80, respectively)

	1033.6	1007.3	823.2	797.0	769.1	570.8	559.8
R	C14:0	C14:0	H	H	C14:0	C14:0	H
R'	C18:1	C16:0	C18:1	C16:0	H		H



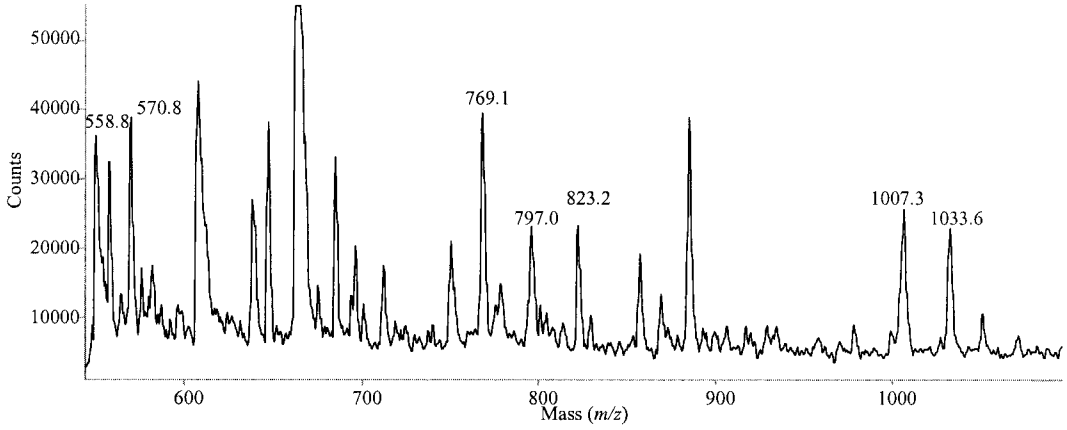


Figure 4. Positive-ion MALDI mass spectrum of dephosphorylated lipid A of *H. magadiensis,* with relevant ion peaks indicated. At the top of the spectrum, a general formula of the GlcN II oxonium ion is shown together with a small table indicating the secondary fatty acid substitution on oxonium ions. The ion peak at m/z = 570.8 does not possess a primary C12:0 (3-OH) (dotted bond)

The oxonium ions at m/z = 797.0 and 823.2 were consistent with a triacylated species lacking a 14:0 residue.

The oxonium ion at m/z = 769.1 could be assigned to a different kind of triacyl species possessing two 12:0 (3-OH) and a 14:0 residue. The diacyl species at m/z = 570.8 lacked a 12:0(3-OH) residue, indicating that the O-3 position on GlcN II is non-stoichiometrically substituted.

The diacyl species at m/z = 558.8 carries two 12:0 (3-OH) residues.

NMR Spectroscopy of Lipid A

All the collected data were studied and confirmed by a combination of homo- and heteronuclear NMR spectra performed on intact lipid A. The ^{1}H, ^{13}C and ^{31}P resonances were assigned by COSY, TOCSY, ROESY, ^{13}C-^{1}H and ^{31}P-^{1}H HSQC. As shown in the MS analysis (see below), the lipid A fraction of *Halomonas magadiensis* represents a complex family of compounds differing in their degree of acylation.

The chemical shifts and the coupling constants indicate the presence of two GlcN residues as pyranose rings and in $^{4}C_{1}$ conformation. Several cross peaks were clearly observed in the anomeric region of the HSQC spectrum and are attributable to different α- and β-GlcN spin systems with varying degrees of acylation and phosphorylation (Table 3, species **X**, **Y**, **W**, **Z**). Starting from the amide and/or the anomeric proton signals in the COSY and TOCSY spectra (Figure 5), it was possible to assign the chemical shift values of each sugar residue. The carbon resonances at 92.9 and 101.2 ppm correlate with two proton resonances at 5.32 (**X**) and 4.56 ppm (**Y**), respectively. These values are in accordance with the α-configuration of GlcN I and β-configuration of GlcN II. The H-2 proton signals of **X** (3.92 ppm) and **Y** (3.58 ppm) correlate with carbon resonances at 51.2 and 54.2 ppm; these values are typical of C−N linkages at position 2. The down-field shift of proton resonances of H-3**X** (4.98 ppm) and H-3**Y** (4.96 ppm) are indicative of *O*-acylation at the C-3 positions of **X** and **Y** and, likewise, the chemical shift values of H-2**X** (3.92 ppm) and H-2**Y** (3.58 ppm) are diagnostic of *N*-acylation at both C-2 positions. The inter-residual NOE between H-1**Y** and H-6**X**, together with the downfield resonance of C-6**X** (66.4 ppm) are indicative of the β-(1→6) glycoside linkage between the residues **X** and **Y**. A ^{31}P-^{1}H HSQC spectrum (Figure 5) allowed assignment of the phosphorylation sites. The phosphorus resonances observed are all typical of monophosphate monoester groups. The phosphorus resonance at −1.85 ppm correlates with a proton resonance at 5.32 ppm identified as H-1**X**. The resonance at 0.82 ppm correlates with a proton at 4.03 ppm attributed to H-4**Y**. Thus, the two phosphate groups are linked to O-1 of **X** and O-4 of **Y**. Many of the proton resonances of these two spin systems can be observed clearly starting from the amide proton in the TOCSY spectrum (Figure 5, N−H **X** 7.22, N−H **Y** 7.55). These data allowed the identification of the carbohydrate backbone P→4-D-GlcpN-(1→6)- β-D-GlcpN-α-1→P acylated at position 2 and 3 of both GlcN residues

(**X** and **Y**), as one of the main species. Alternative spin systems for GlcN I and GlcN II were also identified (Table 3, **W** and **Z**). In the anomeric region of the HSQC spectrum, the cross peak at 4.91/99.2 ppm was attributed to H-1/C-1 of an α-GlcN (**W**), whereas the cross peak at 4.44/101.2 could be assigned to a β-GlcN (**Z**). The chemical shift values of the remaining proton for both species **W** and **Z** were found in the region 3.33−3.87 ppm. The proton resonances of H-3**W** (δ =3.50 ppm) and of H-3**Z** (δ =3.77 ppm) testified to the lack of acylation at these positions, confirming the MS data. For these two spin systems, the ^{31}P-^{1}H HSQC spectrum revealed a single correlation for a phosphate group (^{31}P 3.6 ppm) at O-4**Z**. Thus, the anomeric position of **W** is not phosphorylated, as was already indicated by its unusual H-1 and C-1 chemical shifts. As above, many of the proton resonances of these two spin systems can be observed clearly starting from the amide proton in the TOCSY spectrum (N−H **W** 7.24, N−H **Z** 7.44). Thus, another species present was composed of a P→4-β-D-GlcpN-(1→6)-α-D-GlcpN sequence formed by **W** and **Z** residues, acylated only at the 2 position of non-reducing GlcN (**Z**) and of distal GlcN (**W**).

Other minor molecular species formed by combinations of **X**−Z species were present, as confirmed by the MS analyses, but these were not detectable by NMR spectroscopy.

Moreover, in the 2D spectra, all the distinctive resonances of the fatty acids were assigned. Two cross peaks at 66.8/3.81 and 66.8/3.71 identified two β-protons of the 12:0 (3-OH) fatty acids since in the TOCSY and COSY spectrum they correlated with the α- and the γ- methylene protons (Table 3). The cross peak at 70.3/5.07 was correlated with the α- and γ- methylene protons and was assigned to the β-proton of 12:0 (3-OR) moieties as proven by its ^{1}H downfield displacement following acylation. In addition, the cross peak at 129.3/5.32 ppm identified the olefin moiety of 18:1 residues (species **1b**, **2b** and **4b** of Table 1).

MALDI-TOF Mass Spectrometry of Intact Lipid A

The results obtained above allowed unequivocal assignment of all ion peaks in the negative-ion MALDI-TOF mass spectrum of the intact lipid A.

This spectrum (Figure 1) contains a complex pattern of peaks because of differing degrees of acylation. There are five main groups of ion peaks representing hepta-, hexa-, penta-, tetra- and triacyl species with a β-D-GlcpN-(1→6)-α-D-GlcpN-1→P backbone (Table 1, compounds **1**−**8**, Figure 6). The members of each group differ in fatty acid composition.

The highest mass ions at *m*/*z* = 1921.9 and 1895.2 (**1a** and **1b**) are consistent with a *bis*-phosphorylated heptaacyl species possessing four 12:0(3-OH) residues directly linked to the sugar backbone. Two of these residues are substituted at the O-3 position. Two acyloxyacyl residues [(*R*)-12:0(3-O-R)] are present on the non-reducing unit (GlcN II): the amide-linked acyloxyacyl moiety consists of the 12:0[(3-*O*-(14:0)] group, while the ester-linked acyloxyacyl moiety consists of a 12:0[3-*O*-(16:0/18:1)] group. Finally, one 10:0 residue substitutes the β-position of the amide-linked 12:0(3-OR) residue on the reducing-unit (GlcN I). In order of decreasing mass, the hexaacyl species lacking a 10:0 residue at the non-reducing unit were present (**2a** and **2b**, *m*/*z* = 1740.5 and 1767.4).

The ion at *m*/*z* = 1657.2 (compound **3**) can be assigned to a different hexaacyl species, with two N-linked acyloxyacyl moieties: 12:0[3-*O*-(14:0)] on GlcN II and 12:0[3-*O*-(10:0)] on GlcN I. The pentaacylated species **6** lacks the 10:0 residue at the O-3 position of GlcN I.

Table 3. The distinctive ^{1}H-, ^{13}C- and ^{31}P NMR resonances of the major species of lipid A from *Halomonas magadiensis*; the spectra were obtained in [D_6]DMSO at 400, 100 and 162 MHz, respectively, at 373 K

δ (^{1}H/^{13}C/^{31}P)	**1**	**2**/N−H	**3**	**4**	**5**	**6**
X	5.32	3.92/7.22	4.98	3.65	3.88	3.70/3.80
α-GlcpN	92.9	51.2	73.6	66.0	68.8	66.4
	−1.85					
Y	4.56	3.58/7.55	4.96	4.03	3.17	3.70/3.62
β-GlcpN	101.2	54.2	70.1	70.0	75.2	60.3
				0.82		
W	4.91	3.58/7.24	3.50	3.05	3.87	3.75
α-GlcpN	99.2	53.9	72.9	70.7	68.8	66.4
Z	4.44	3.33/ 7.44	3.77	3.74	3.15	3.51
β-GlcpN	101.2	56.0	70.1	72.4	74.0	61.0
				3.6		
Fatty acids	$(CH_2)_\alpha$	$(CH_2)_\beta$	$(CH_2)_\gamma$		$(CH_2)_n$	CH_3
12:0 (3-OH) O-linked	2.39/2.22	3.81	1.30		1.26	0.82
	42.6	66.8	36.8		28.4	13.2
12:0 (3-OH) N-linked	2.09	3.71	1.29			
	43.3	66.8	36.8			
12:0 (3-OR)	2.44	5.07	1.56/1.48			
	39.9	70.3	33.2			
18:1	$(CH_2)_\alpha$	(CH=CH)				
	1.97	5.32				
	26.3	129.3				

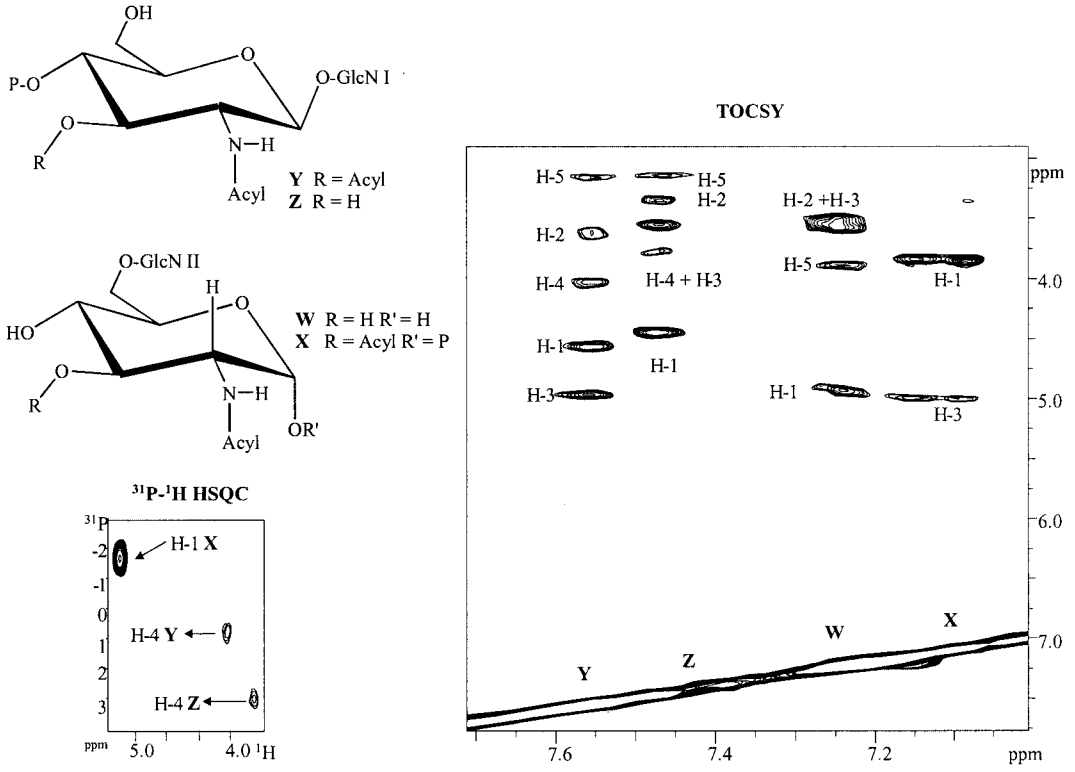


Figure 5. Sections of TOCSY and ^{1}H-^{31}P HSQC NMR spectra of the intact lipid A from *H. magadiensis.* In the TOCSY spectrum, most of the spin system correlations of the disaccharide backbone are observable. Letters **X**−**Z** in this figure refer to the main GlcN I and GlcN II species as drawn at the left. In the ^{1}H-^{31}P HSQC, the sole anomeric correlation, O-1**X**, and the two different O-4**Y** and O-4**Z** correlations are observable

	1a	1b	2a	2b	3	4a	4b	5a	5b	6	7	8a	8b
R	C10:0	C10:0	H	H	C10:0	H	H	C10:0	C10:0	H	H	H	H
R'	C16:0	C18:1	C16:0	C18:1	H	C16:0	C18:1			H			
R''	PO_3^{--}	PO_3^{--}	PO_3^{--}	PO_3^{--}	PO_3^{--}	PO_3^{--}	PO_3^{--}	PO_3^{--}	H	PO_3^{--}	PO_3^{--}	PO_3^{--}	H

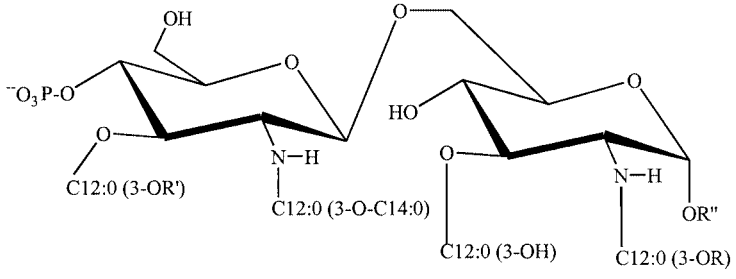


Figure 6. All components of lipid A from *H. magadiensis* are described. Arabic numbers refer to ions in Figure 1 and Table 1. Blank spaces in the table indicate that a fatty acid is absent from that position

The pentaacyl species **4a** and **4b** lacked a 12:0(3-OH) residue at the O-3 position of GlcN II.

In the spectrum, the most abundant ions are penta- and tetraacyl lipid A at m/z = 1458.7 and 1304.3 (**5** and **7**). The species **5**, at m/z = 1458.7, carries three 12:0(3-OH), a 14:0 and a 10:0 residue. The species **7** at m/z = 1304.3 lacks a 10:0 residue. The triacyl species **8a** at m/z = 1106.0 lacks the other *O*-linked 12:0(3-OH) residue. Minor species with a 12:0 residue instead of a 14:0 residue were also observed.

Monophosphorylated species ($\Delta m/z$ = 80, **8b** and **5b**, Figure 1) lack the phosphate group at the anomeric position of GlcN I. This was confirmed directly by NMR analysis and indirectly by the absence, in the positive-ion MALDI-TOF, of an oxonium ion for GlcN II lacking the phosphate group (data not shown).

In conclusion, the lipid A fraction isolated from the LPS of alkaliphilic bacterium *Halomonas magadiensis* has been completely elucidated. This is one of the few lipid A mol-

ecules derived from extremophile bacteria [6] which has been analysed and it is characterised by a peculiar acylation pattern.

Lipid A from *Halomonas magadiensis* consists of a complex and heterogeneous mixture of species in which the carbohydrate backbone is acylated in various patterns (species **1**−**8**, Figure 6). The main species **5** and **7** are penta- and tetraacylated lipid A, respectively, and both lack a primary 12:0 (3-OH) residue at the reducing GlcN. A considerable amount of triacylated species (compounds **8a** and **8b**) and of heptaacylated species (compounds **1a** and **1b**) were also found. In addition, the lipid A family contained hexa- and pentaacylated species (**2**−**4**).

Discussion

It is known that the exterior environment of the bacteria can strongly influence lipid A structure and biosynthesis, i.e. it can induce changes in the length and in the nature of the fatty acids. The reason for the peculiar acylation pattern of the lipid A from *Halomonas magadiensis* might be a consequence of a different physical structure of the external membrane as a response to the alkaline environment and this could help the bacterium to survive in its extreme habitat. Thus, the search for new lipid A structures from extremophile bacteria may give new insights into the biosynthetic and adaptive response of these organisms to their external environments.

LPSs, also known as endotoxins, play a key role in the pathogenesis and manifestation of Gram-negative infection.[7,8] The endotoxic properties of LPS result principally from the lipid A component, which is the primary immunostimulator center of Gram-negative bacteria. Lipid A interacts with Toll-like receptors triggering the activation of the innate immune system.[8,9] The induction of an excessive and uncontrolled immune response leads to septic shock in mammalian hosts.

The biological activity of lipid A strongly depends on its primary structure, especially the fatty acid composition and distribution.[10,11] Thus, fine structural variations can strongly influence its toxicity and, subsequently, the immune response of the host. Lipid A induction of inflammatory cytokine released by human cells is strongly influenced by structure. Maximal endotoxic activity is expressed by bis-phosphorylated hexaacylated lipid A species. The cytokine-inducing capacity decreases with lower or higher acyl-substitution patterns. Non-cytokine-inducing species could operate as antagonists and reduce or, in a dose-dependent manner, completely inhibit cytokine production induced by toxic lipid A, such as that from *Escherichia coli*. This is the case for the bis-phosphorylated pentacyl lipid A from *Rhodobacter capsulatus* and for the *Salmonella*-type heptaacylated lipid A, both of which are antagonists.[1,12]

Hence, the study of lipid A molecules from bacteria non-pathogenic in humans is extremely important for the identification of lipid A molecules that can interfere with cytokine induction in human cells. Therefore, it will be very interesting to observe the biological activity of lipid A from *Halomonas magadiensis* as a potential antagonist.

Work is currently in progress to ascertain the agonist/antagonist behaviour of lipid A from *Halomonas magadiensis*.

Experimental Section

Bacteria and Bacterial LPSs: *Halomonas magadiensis*, originally isolated from Lake Magadi, Kenya, was grown at 37 °C in liquid shake culture (200 rpm) in the alkaline medium already described.[4] Cells were pelleted at 2000 g, washed with the salts component of the medium, and freeze dried. Dried cells (10 g) were extracted by the phenol−water method.[13] Both phases were separately dialyzed against distilled water, freeze-dried and screened by discontinuous SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis),[14] with a 12% gel on a miniprotean gel system from Bio-Rad. The samples were run at constant voltage (150 V) and stained with silver nitrate. The lipopolysaccharide material was recovered in the water phase (yield: 6% of the bacterial dry mass).

Preparation of Lipid A, De-O-acylated Lipid A and Dephosphorylated Lipid A: Free lipid A was obtained after hydrolysis of the LPS by treatment with 1% acetic acid (100 °C, 2 h). The solution was then centrifuged (10.000 rpm, 4 °C, 2 h), and the resulting precipitate (free lipid A) was washed with water (yield of 5% of LPS). Lipid A was de-*O*-acylated with anhydrous hydrazine in THF at 37 °C for 1.5 h, followed by precipitation with cold acetone and centrifugation (5000 rpm, 15 min, 4 °C). The resulting sediment was the de-*O*-acylated lipid A. The mild de-*O*-acylation was performed by treatment of lipid A (200−500 μg) with 32% ammonium hydroxide (200 μL, 20 °C, 16 h). The samples were simply dried under nitrogen and directly analyzed by mass spectrometry.[5] Lipid A was dephosphorylated with 48% HF (4 °C, 48 h) followed by neutralization, under a stream of nitrogen, and lyophilization.

MALDI-TOF Analysis: MALDI-TOF analyses were conducted using a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology and a reflectron. Ions were formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) and accelerated through 24 kV. Mass spectra reported are the result of 256 laser shots. The dried samples were dissolved in $CHCl_3/CH_3OH$ (50:50 v/v) at a concentration of 25 pmol·μL^{-1}. The matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) in CH_3OH/0.1% trifluoroacetic acid/CH_3CN (7:2:1 by volume) at a concentration of 75 mg·mL^{-1}. A sample/matrix solution mixture (1:1 v/v) was deposited (1μL) onto a stainless steel gold-plated 100-sample MALDI probe tip, and allowed to dry at room temperature.

NMR Spectroscopy: ^{1}H, ^{13}C and ^{31}P NMR spectra of lipid A were obtained in [D$_6$]DMSO at 400, 100 and 162 MHz, respectively, in FT mode at 373 K, using a Bruker DRX 400 spectrometer equipped with a reverse probe. ^{13}C and ^{1}H chemical shifts are expressed in δ relative to dimethyl sulfoxide (δ_H = 2.49 ppm, δ_C = 39.7 ppm). Aqueous 85% phosphoric acid in [D$_6$]DMSO was used as reference (δ = 0.00 ppm) for ^{31}P NMR spectroscopy. Two-dimensional spectra (COSY, TOCSY, ROESY, HSQC) were measured using standard Bruker software.

The homonuclear experiments were performed with 4096 data points in the F2 dimension and 512 experiments in F1. The data

matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. The TOCSY and ROESY experiments were performed with a mixing time of 80 ms and 300 ms, respectively. The heteronuclear experiment was performed using a pulse field gradient program as gHSQC.

Phosphate and Monosaccharides Analysis: Total and inorganic phosphorus were determined according to the published method.[15] Monosaccharides were identified as their respective acetylated *O*-methyl glycoside derivatives. After methanolysis with HCl/MeOH 2 M (85 °C, 24 h) and acetylation with acetic anhydride in pyridine (85 °C, 30 min), the sample was analysed by GC-MS. The absolute configuration of the monosaccharides was obtained according to the published method.[16]

Fatty Acid Analysis: The total fatty acid content was obtained by acid hydrolysis: lipid A was treated first with HCl 4 M, (4 h, 100 °C), then with NaOH 5 M (30 min, 100 °C); the product was acidified and the fatty acids were extracted in $CHCl_3$, methylated with diazomethane and analysed by GLC-MS. The ester-bound fatty acids were selectively liberated by alkaline hydrolysis with NaOH 0.5 M/MeOH (1:1 v/v, 85 °C, 2 h). The product was acidified, extracted in $CHCl_3$, methylated with diazomethane and analysed by GLC-MS. The absolute configuration of 3-hydroxy fatty acids was determined as described previously.[16]

GLC Analysis: All GLC analyses were performed on a Hewlett−Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco). For monosaccharide analysis, the temperature program was 150 °C for 5 min, then 5 °C min^{-1} to 300 °C. For monosaccharide absolute configuration analysis the temperature program was 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min. For all fatty acids analysis, the temperature program was 150 °C for 3 min, then 10 °C min^{-1} to 280 °C over 20 min.

Acknowledgments

A. M. thanks Dr. V. Piscopo from C.I.M.C.F. of the University of Naples Federico II for ^{31}P NMR spectroscopy. This work was financially supported by MIUR − Roma − (Progetto di Ricerca di Interesse Nazionale, 2002, Roma) (M. P.).

[1] W. J. Christ, O. Asano, A. L. Robidoux, M. Perez, Y. Wang, G. R. Dubuc, W. E. Gavin, L. D. Hawkins, P. D. McGuinness, M. A. Mullarkey, M. D. Lewis, Y. Kishi, T. Kawata, J. R. Bristol, J. R. Rose, D. P. Rossignol, S. Kobayashi, I. Hishinuma, A. Kimura, N. Asakawa, K. Katayama, I. Yamatsu, *Science* **1995**, *268*, 80−83.
[2] U. Zähringer, B. Lindner, E. Th. Rietschel, *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 211−276, and references cited therein.
[3] U. Zähringer, B. Lindner, E. Th. Rietschel, in *Endotoxin in Health and Disease* (Eds.: H. Brade, D. C. Morrison, S. Opal, S. N. Vogel), Marcel Dekker, New York, **1999**, pp. 93−114, and references cited therein.
[4] [4a] A. W. Duckworth, W. D. Grant, B. E. Jones, D. Meijer, M. C. Marquez, A. Ventosa, *Extremophiles* **2000**, *4*, 53−60. [4b] C. De Castro, A. Molinaro, W. D. Grant, A. Wallace, M. Parrilli, *Carbohyd. Res.* **2003**, *338*, 567−570. [4c] C. De Castro, A. Molinaro, W. D. Grant, A. Wallace, M. Parrilli, *Eur. J. Org. Chem.* **2003**, 1029−1034.
[5] A. Silipo, R. Lanzetta, A. Amoresano, M. Parrilli, A. Molinaro, *J. Lipid Res.* **2002**, *43*, 2188−2195.
[6] B. M. Plötz, B. Lindner, K. O. Stetter, O. Holst, *J. Biol. Chem.* **2002**, *275*, 11222−11228 and references cited therein.
[7] C. Alexander, E. T. Rietschel, *J. Endotoxin Res.* **2000**, *7*, 167−202, and references cited therein.
[8] B. Beutler, E. T. Rietschel, *Nature Rev. Immunol.* **2003**, *3*, 169−176, and references cited therein.
[9] R. Medzhitov, *Nature Rev. Immunol* **2001**, *1*, 135−145.
[10] O. Holst, A. Ulmer, H. Brade, H. D. Flad, E. T. Rietschel, *FEMS Immunol. Medical Microbiol.* **1996**, *16*, 83−104.
[11] U. Seydel, M. Oikava, K. Fukase, S. Kusumoto, K. Brandenburg, *Eur. J. Biochem.* **2000**, *267*, 3032−3039.
[12] K. Tanamoto, S. Azumi, *J. Immunol.* **2000**, *164*, 3149−3156.
[13] O. Westphal, K. Jann, *Methods Carbohydr. Chem.* **1965**, *5*, 83−91.
[14] C. M Tsai, C. E. Frasch, *Anal. Biochem.* **1982**, *119*, 115−119.
[15] W. Kaca, J. de Jongh-Leuvenink, U. Zähringer, E. T. Rietschel, H. Brade, J. Verhoef, V. Sinnwell, *Carbohydr. Res.* **1988**, *179*, 289−299.
[16] K. Leontein, J. Lönngren, *Methods Carbohydr. Chem.* **1978**, *62*, 359−362.
[17] E. T. Rietschel, *Eur. J. Biochem.* **1976**, *64*, 423−428.

Received January 26, 2004

