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The structure of the carbohydrate backbone of the rough type lipopolysaccharides from *Proteus penneri* strains 12, 13, 37 and 44

Evgeny Vinogradov,[a,*] Zygmunt Sidorczyk[b]

[a]*Institute for Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, Ont., Canada K1A 0R6*
[b]*Department of General Microbiology, Institute of Microbiology, Immunology, University of Łodz, 90-237 Łodz, Banacha 12/16, Poland*

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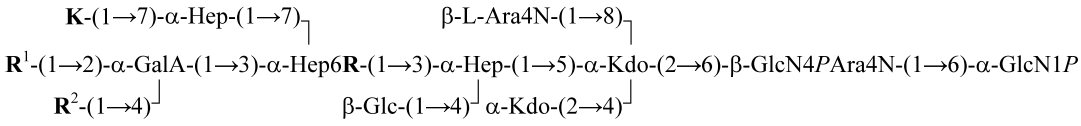
Abstract

The following structure of the lipid A–core backbone of the rough type lipopolysaccharides (LPS) from *Proteus penneri* strains 12, 13, 37, and 44 was determined using NMR and mass spectroscopy and chemical analysis of the oligosaccharides obtained by mild-acid hydrolysis, alkaline *O*,*N*-deacylation, *O*-deacylation with hydrazine, and deamination of the LPSs:



where **K** = H, **R** = *P*EtN, $\mathbf{R}^1$ = α-Hep-(1→2)-α-DDHep, and $\mathbf{R}^2$ = α-GalN (strains 12 and 13) or β-GlcNAc-(1→4)-α-GlcN (strains 37 and 44). LPS from each strain contained several structural variants. LPS from strain 12 contained a variant with $\mathbf{R}^1$ = α-DDHep, whereas LPS from strains 13, 37, and 44 contained structures with **K** = amide of β-GalA with putrescine or spermidine. The phosphate group at O-1 of the α-GlcN residue in the lipid part was partially substituted with Ara4N.

Keywords: Lipopolysaccharide; Proteus; *Proteus penneri*; Core

1. Introduction

Gram negative bacteria of the genus *Proteus* from the family *Enterobacteriaceae* are opportunistic pathogens causing nosocomial and urinary tract infections, which lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones.[1] Lipopolysaccharides (LPS) have been identified as an important virulence factor of *Proteus* bacilli.[2] Analysis of the *Proteus* LPS core part revealed that it has a variable structure, differing between strains and having a number of variants within the LPS from each strain.[3–11] Here we present the results of structural analysis of four naturally occurring rough strains of *Proteus penneri*, having the smallest core oligosaccharide in LPS molecule found until now.

Abbreviations: LPS, lipopolysaccharide; Hep, L-*glycero*-D-*manno*-heptose; DD-Hep, D-*glycero*-D-*manno*-heptose; GalA, galacturonic acid; Kdo, 3-deoxy-D-*manno*-octulosonic acid; *P*, phosphate; *P*EtN, 2-aminoethylphosphate; Ara4N, 4-amino-4-deoxy-L-arabinose; anh-Man, 2,5-anhydromannose; ΔGalA, 4-deoxy-β-L-*threo*-hex-4-enopyranosyl; GalAPu, amide of β-GalA with putrescine $-HN(CH_2)_4NH_2$; GalASp, amide of β-GalA with spermidine $-HN(CH_2)_3NH(CH_2)_4NH_2$; HPAEC, high-performance anion-exchange chromatography.

* Corresponding author. Tel.: +1-613-9900397; fax: +1-613-95290 92.
E-mail address: evguenii.vinogradov@nrc.ca (E. Vinogradov).

2. Results and discussion

LPSs from P. *penneri* strains 12, 13, 37, and 44 were treated with 4 M KOH for the deacylation, and the

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resulting products **1a**–**d** (Table 1) isolated using gel chromatography and preparative HPAEC. Oligosaccharides **1a** and **1b** coeluted on HPAEC and were isolated as a mixture from strains 13, 37, 44; **1a** was isolated in the pure form from the LPS of strain 12 which did not produce compound **1b**. Structural analysis and NMR data for oligosaccharides **1** have been described earlier.[3,6,8–10]

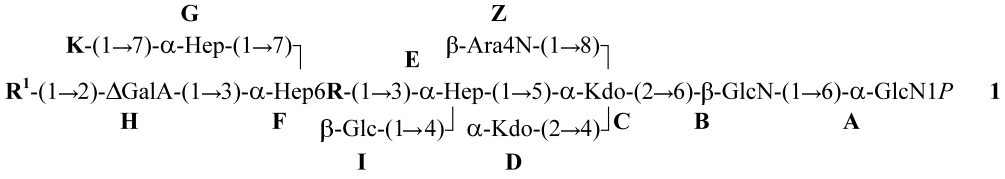
Mild-acid hydrolysis (AcOH, 2%) of the LPSs with subsequent fractionation of the products using gel chromatography followed by anion- and then cation-exchange chromatography gave products **2a**–**2i** (Table 2). Oligosaccharides **2a** (from strain 13), **2g**–**2i** were isolated as individual compounds, having various Kdo degradation products at the reducing end. Oligosaccharide pairs **2b**,**2d**; **2c**,**2e**; and **2a**,**2f** were obtained as mixtures.

Deamination of LPSs from strains 37 and 44 afforded the disaccharide **3**, which was purified by reverse-phase HPLC.

β-GlcNAc-(1→4)-anh-Man **3**
(L) (M)

Oligosaccharides **2** and **3** were analyzed using NMR spectroscopy (Table 3). The identities of monosaccharides and non-sugar components were established on the basis of ^{1}H and ^{13}C NMR chemical shifts and $^{3}J_{H,H}$ coupling constants. The sequence of monomers was determined using NOESY and HMBC data. NOESY experiments with the usual mixing time of 200 ms did not show the expected correlation between H-1 of β-GalA **K** and H-7 of heptose **G**; therefore a mixing time of 400 ms was used. ^{1}H,^{13}C HMBC spectra showed intraresidual long-range correlations from H-1

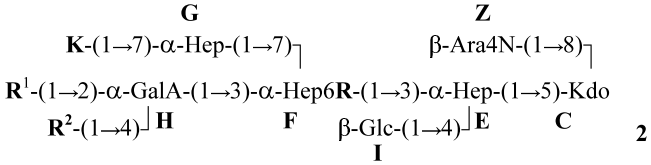
Table 1
Substituents of compounds **1a**–**d**



1

Compound	Derived from *P. penneri* strain	K	R	R^1
1a	12, 13, 37, 44	H	*P*EtN	α-Hep-(1→2)-α-DDHep-
1b	13, 37, 44	β-GalA	H	α-Hep-(1→2)-α-DDHep-
1c	13	β-GalA	*P*EtN	H
1d	12	H	*P*EtN	α-DDHep-

Table 2
Substituents of compounds **2a**–**2i**



2

Compound	Derived from *P. penneri* strain	R	R^1	R^2	K
2a	12, 13	*P*EtN	α-Hep-(1→2)-α-DDHep- (X) (T)	α-GalN- (M)	H
2b	13	H	α-Hep-(1→2)-α-DDHep-	α-GalN-	GalASp
2c	13	H	α-Hep-(1→2)-α-DDHep-	α-GalN-	GalAPu
2d	13	*P*EtN	H	α-GalN-	GalASp
2e	13	*P*EtN	H	α-GalN-	GalAPu
2f	12	*P*EtN	α-DDHep-	α-GalN-	H
2g	37, 44	*P*EtN	α-Hep-(1→2)-α-DDHep-	β-GlcN-Ac-(1→4)-α-GLcN- (L) (M)	H
2h	37, 44	H	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	GalASp
2i	37, 44	H	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	GalAPu

Table 3
^{1}H and ^{13}C NMR data for the products **2** and **3**

Unit, compound	Nucleus	1	2	3	4	5	6a	6b
H, **2a–c,g–i**	^{1}H	5.45	4.06	4.20	4.46	4.46		
	^{13}C	99.3	73.0	67.8	78.9	71.9	176.3	
H, **2d,e**	^{1}H	5.37	3.90	4.11	4.42	4.46		
	^{13}C	101.6	69.4	69.7	79.4	72.2	176.1	
H, **2f**	^{1}H	5.60	4.08	4.20	4.46	4.44		
	^{13}C	98.3	72.0	67.9	79.2	71.9		
M, **2a–f**	^{1}H	5.22	3.50	4.10	4.04	4.48	3.73	3.73
	^{13}C	96.7	51.7	67.2	68.8	71.9	61.3	
M, **2g–i**	^{1}H	5.20	3.34	4.05	3.68	4.28	3.67	3.79
	^{13}C	95.9	54.7	69.6	79.8	71.6	60.3	
M, **3**	^{1}H	4.99	3.75	4.34	4.12	3.97	3.62	3.71
	^{13}C	90.6	86.7	77.8	86.7	83.5	62.2	
K, **2b–e,h,i**	^{1}H	4.52	3.56	3.73	4.20	4.26		
	^{13}C	103.9	71.3	73.3	70.3	75.7	171.4	
L, **2g–i**	^{1}H	4.62	3.78	3.59	3.46	3.50	3.75	3.95
	^{13}C	102.5	56.6	74.5	70.8	76.9	61.6	
L, **3**	^{1}H	4.54	3.71	3.55	3.43	3.47	3.73	3.94
	^{13}C	102.3	56.8	74.8	71.3	77.2	62.1	
T, **2a–c,g–i**	^{1}H	5.25	3.95	3.98	3.78	3.95	4.08	3.68
	^{13}C	96.4	70.8	70.9	68.6	74.4	72.5	63.0
T, **2f**	^{1}H	5.11	3.97	3.85	3.73	3.95	4.02	3.70
	^{13}C	97.8	70.8	71.5	68.7	74.2	72.5	62.7
X, **2a–c,g–i**	^{1}H	5.06	4.04	3.85	3.85	3.68	4.02	3.73
	^{13}C	103.2	71.0	71.3	67.1	73.5	70.4	64.3
NAc, **2g–i**	^{1}H		2.06					
	^{13}C	175.7	23.2					

of α-pyranoses to C-2 (weak), C-3 and C-5 (strong), and to the transglycosidic carbon atom. For β-pyranoses, intraresidual correlations between H-1 and C-2, C-3 (both weak), as well as between H-1 and transglycosidic carbon (strong) were observed. HMBC data were used for the localization of spermidine and putrescine residues, which gave correlations between H-1 of the amine to C-6 of the uronic acid residue **K**. NMR structural determination of the fragment containing residues **C** to **H**, **M**, **T**, **X**, **Z** has been described for similar structures.[3,6,8]

In addition to the residues **C** to **H**, **T**, **X**, **Z**, oligosaccharide **2a** contained an unsubstituted α-GalN residue **M** linked to O-4 of the α-GalA residue **H**. Oligosaccharide **2f** differed from **2a** by the absence of Hep residue **X**. Compound **2g** differed from **2a** only in the substituent at O-4 of α-GalA **H**, which in **2g** was the disaccharide β-GlcNAc-(1→4)-α-GlcN-. The structure of this fragment was confirmed by the isolation of the disaccharide **3** after nitrous acid deamination of the LPS from strains 37 and 44.

Basic fractions **2b**–**2e** contained amides of β-GalA (residue **K**) with putrescine $-HN(CH_2)_4NH_2$ or spermidine $-HN(CH_2)_3NH(CH_2)_4NH_2$. NMR spectroscopic data for **2b**–**2e** closely resembled published data for similar products.[9] These compounds contained either an α-Hep-(1→2)-α-DDHep- (**X**–**T**-) fragment and no *P*EtN (**R** = H) on O-6 of Hep **F**, or no **X**–**T**- fragment with *P*EtN at O-6 of Hep **F** (**R** = *P*EtN). In contrast to the products from strain 13, basic core fractions **2h** and **2i** from strains 37 and 44 contained only one structural variant with the diheptosyl **X**–**T**- fragment but without *P*EtN on Hep **F**.

The charge-deconvoluted ESI mass spectrum of the oligosaccharide mixture obtained after alkaline deacylation of the LPS from strain 12 contained peaks with masses of 2203.0 and 2395.1 Da, corresponding to compounds **1a** and **1d** (Table 1). The ESI mass spectrum of the mixture of *O*,*N*-deacylation products from strain 13 LPS contained peaks at 2395.0, 2447.8, and 2186.9 Da, corresponding to compounds **1a**–**1c**. These compounds are expected deacylation products of the LPS variants, containing structures **2a**–**2e**. Mass spectra of the *O*,*N*-deacylated LPSs from strains 37 and 44 contained peaks at 2395.7 and 2449.2 Da, belonging to oligosaccharides **1a**,**1b**.

The ESI mass spectrum of the products of mild-acid hydrolysis of the LPS from strain 12 contained peaks corresponding to the compounds **2a**,**2f**; each product gave two peaks due to partial loss of water from the

Kdo residue (Fig. 1, Table 2). The mass spectrum of the core fraction of strain 13 LPS revealed the presence of five major products with masses corresponding to structures **2a**–**2e**. the transformed ESI mass spectrum of the core fractions of the LPSs from strains 37 and 44 contained peaks at 2138.3, 2261.0, and 2318.5, corresponding to the anhydro form of the structures **2g**–**2i**.

LPSs from all strains were *O*-deacylated with anhydrous hydrazine and the resulting mixtures were analyzed by ESI MS (Figs. 2 and 3). Most of the observed peaks belonged to compounds **4** (Table 4). The number of their variants was higher in comparison with the mild-acid degradation products due to the presence of a variable number of 4-amino-4-deoxy-aminoarabinose residues. The position of these residues could be inferred as follows: formation of the oligosaccharides of type **1** with unsubstituted O-4 of GlcN residue B after alkaline deacylation of LPS is known to result from the removal of the Ara4N*P* residue from this position in alkaline conditions,[12] hence oligosaccharides **1** originate from LPS variants with Ara4N*P* at **B**-4. Similar alkaline elimination of Ara4N*P* from O-1 of GlcN **A** resulted in the subsequent destruction of the molecule, which took place mostly in strains 37 and 44, containing three Ara4N residues in the majority of LPS variants. Mass spectra of the *O*-deacylated LPS from strains 37 and 44 also contained peaks of unknown compounds (Fig. 3).

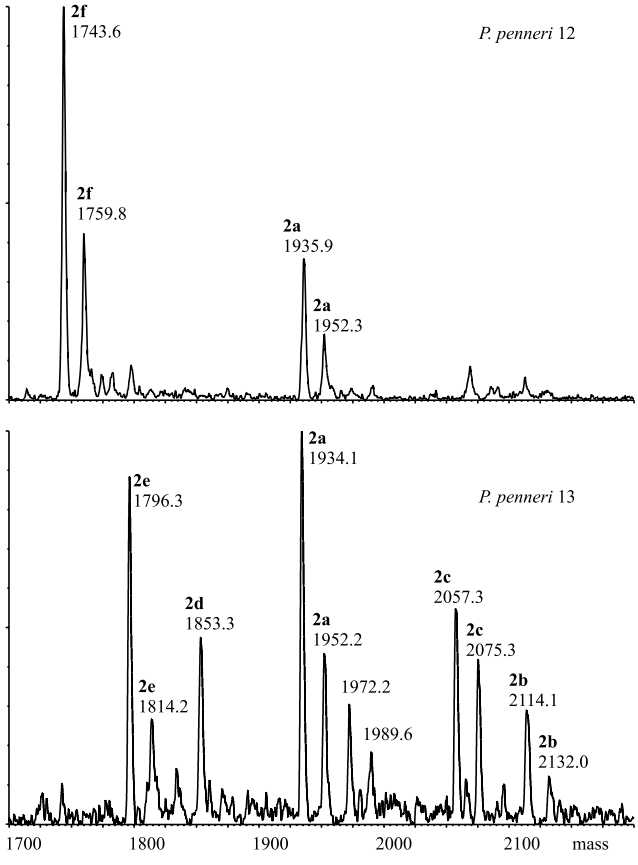


Fig. 1. Charge deconvoluted ESI MS spectra of the core fractions from LPS of *P. penneri* strains 12 and 13.

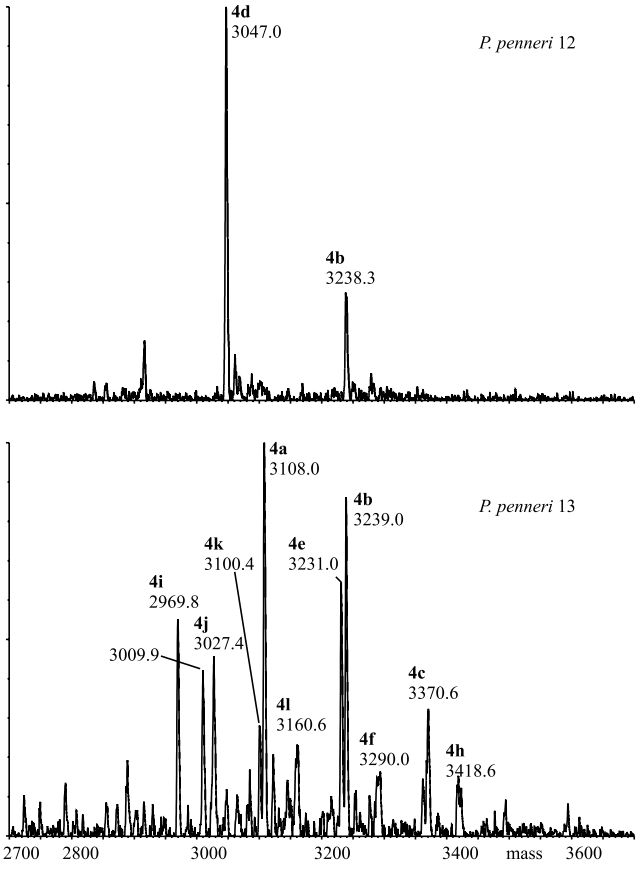


Fig. 2. Charge deconvoluted ESI MS spectra of the *O*-deacylated LPS from *P. penneri* strains 12 and 13.

The composition and structures of oligosaccharides **1**–**3** were confirmed by sugar analysis (GLC of the alditol acetates) and methylation analysis. The absolute D configuration of the GlcNAc residue in oligosaccharide **3** was determined by GLC of the acetylated glycosides with chiral 2-butanol. Spermidine and putrescine were identified in LPS hydrolyzates by HPLC.[13]

The analyzed LPS have different degrees of structural heterogeneity of the core–lipid A backbone, varying from two variants in *P. penneri* strain 12 to six variants in strains 37 and 44 and ten variants in strain 13. It is worth emphasizing that the presence of some substituents is mutually dependent. Thus, compounds with amides of β-GalA lack either *P*EtN substituent at O-6 of Hep residue F (strains 13, 37, 44, the same feature was observed in other *Proteus* strains[7,9]) or the α-Hep-(1→2)-α-DD-Hep fragment $\mathbf{R}^1$ (strain 13). The presence of additional Ara4N substituents in the lipid A portion seems to be independent of other structural features.

3. Experimental

Bacteria.—These were cultivated and LPS isolated as described.[6]

NMR spectroscopy, ESI MS, chemical analyses, general methods, and preparation of oligosaccharides **1**–**3**.—These were performed as described.[9] Oligosaccharide **3** was purified by reversed-phase HPLC on

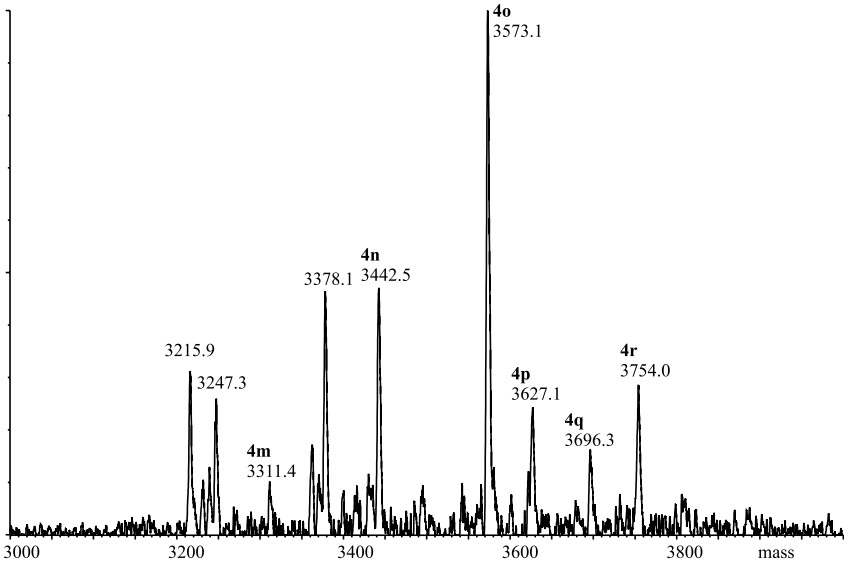
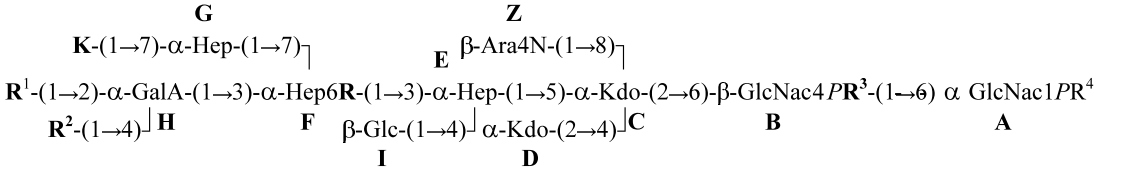


Fig. 3. Charge deconvoluted ESI MS spectrum of the *O*-deacylated LPS from *P. penneri* strain 44.

Table 4
Substituents of the products of structure **4**



Compound	Derived from *P. penneri* strain	**K**	**R**	**R**^{1}	**R**^{2}	**R**^{3}	**R**^{4}
4a	13	H	*P*EtN	α-Hep-(1→2)-α-DDHep-	α-GalN-	H	H
4b*	12, 13	H	*P*EtN	α-Hep-(1→2)-α-DDHep-	α-GalN-	β-Ara4N-	H
4c	13	H	*P*EtN	α-Hep-(1→2)-α-DDHep-	α-GalN-	β-Ara4N-	β-Ara4N-
4d	12	H	*P*EtN	α-DDHep-	α-GalN-	β-Ara4N-	H
4e	13	β-GalAPu	H	α-Hep-(1→2)-α-DDHep-	α-GalN-	H	H
4f	13	β-GalASp	H	α-Hep-(1→2)-α-DDHep-	α-GalN-	H	H
4g*	13	β-GalAPu	H	α-Hep-(1→2)-α-DDHep-	α-GalN-	β-Ara4N-	H
4h*	13	β-GalASp	H	α-Hep-(1→2)-α-DDHep-	α-GalN-	β-Ara4N-	H
4i	13	β-GalAPu	*P*EtN	H	α-GalN-	H	H
4j	13	β-GalASp	*P*EtN	H	α-GalN-	H	H
4k*	13	β-GalAPu	*P*EtN	H	α-GalN-	β-Ara4N-	H
4l*	13	β-GalASp	*P*EtN	H	α-GalN-	β-Ara4N-	H
4m	37, 44	H	*P*EtN	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	H	H
4n*	37, 44	H	*P*EtN	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	β-Ara4N-	H
4o	37, 44	H	*P*EtN	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	β-Ara4N-	β-Ara4N-
4p	37, 44	β-GalA	H	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	β-Ara4N-	β-Ara4N-
4q	37, 44	β-GalAPu	H	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	β-Ara4N-	β-Ara4N-
4r	37, 44	β-GalASp	H	α-Hep-(1→2)-α-DDHep-	β-GlcNAc-(1→4)-α-GlcN-	β-Ara4N-	β-Ara4N-

ac, (*R*)-3-hydroxytetradecanoic acid.

an Aqua C_{18} column (Phenomenex, 1×25 cm) in water.

O-*Deacylation of LPS*.—LPS (50 mg) was dissolved in anhyd hydrazine (2 mL) and kept for 1 h at 50 °C. The cooled mixture was poured into stirred acetone (200 mL) and the precipitated material was collected by centrifugation and fractionated on a Sephadex G50 superfine gel (Pharmacia) column (2.5×80 cm) using 0.05 M pyridinium acetate buffer (pH 4.6) as the eluant. The eluate was monitored by refractive-index detection and the collected fractions were lyophilized.

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