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Highly Phosphorylated Core Oligosaccaride Structures from Cold-Adapted *Psychromonas arctica*

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Abstract: Many cold habitats contain plenty of microorganisms that represent the most abundant cold-adapted life forms on earth. These organisms have developed a wide range of adaptations that involve the cell wall of the microorganism. In particular, bacteria enhance the synthesis of unsaturated fatty acids of membrane lipids to maintain the membrane fluidity, but very little is known about the adaptational changes in the structure of the lipopolysaccharides (LPSs), the main constituent of the outer leaflet of the outer membrane of Gram-negative bacteria. The aim of this study was to investigate the chemical structure of these LPSs for insight into the temperature-adaptation mechanism. For this objective, the cold-adapted *Psychromonas arctica* bacterium, which lives in the arctic seawater near Spitzbergen (Svalbard islands, Arctic) was cultivated at 4 °C. The lipooligosaccharides (LOSs) were isolated and analysed by means of chemical analysis and electrospray ionisation high-resolution Fourier transform mass spectrometry. The LOS was then degraded either by mild hydrazinolysis (*O*-deacylation) or with hot 4 M KOH (*N*-deacylation). Both products were investigated in detail by using ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. The core consists of a mixture of species that differ because of the presence of nonstoichiometric D-fructose and/or D-galacturonic acid units.

Keywords: lipooligosaccharides · microorganisms · NMR spectroscopy · *Psychromonas arctica* · structure elucidation

Introduction

Cold-adapted bacteria are microorganisms that thrive at low temperatures in permanently cold environments (0–10 °C). These habitats abound on earth, especially when one considers that these include not only the polar and alpine regions but also a large proportion of seawaters and seasonally and artificially cold environments.[1,2]

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Cold-adapted microorganisms, also called psychrophiles, can be divided in two types, eury-psychrophiles and steno-psychrophiles.[2,3] The terminology refers to the tolerance of a given microorganism to temperature fluctuations. The steno-psychrophiles can grow in a narrow temperature range restricted to low values, whereas the eury-psychrophiles, despite preferring to grow under freezing conditions, tolerate temperature fluctuation and can extend their growth range towards the mesophilic one.

Cold-adapted microorganisms have been forced to develop a wide range of adaptations because the low temperature exponentially affects the rate of biochemical reactions,[4,5] and water viscosity around 0 °C is almost twice as high relative to that at 37 °C. At low temperatures, all cellular processes are affected: transcription and translation rates and the transport of nutrients and waste products are strongly decreased because of the decrease in membrane fluidity.[5] To counteract these detrimental effects from lowering the temperature, many adaptive responses have been described in psychrophilic microorganisms.[1,5–8]

To maintain membrane fluidity, the cold-adapted microorganisms enhance the synthesis of unsaturated fatty acids, also changing the fatty acid chain length[5,6,8] and the phos-

phorylation quality and quantity.[9,10] The outermembrane of the Gram-negative bacteria forms a barrier for the cell and it is composed of phospholipids, outer membrane proteins (OMPs), and lipopolysaccharides (LPSs). The LPSs are complex amphiphilic macromolecules embedded in the outer leaflet of the external membrane, of which they are the major constituents. Smooth-form LPSs (S-LPSs) consist of three covalently linked regions: the glycolipid lipid A (also known as the endotoxin for human pathogens), the oligosaccharide region (core region), and the O-specific polysaccharide (O-chain, O-antigen). Rough-form LPSs (R-LPSs), also named lipooligosaccharides (LOSs), lack the polysaccharidic portion.[11,12]

Despite their important role as a component of the Gram-negative outermembrane, very little is known about the LPS structure of cold-adapted microorganisms.[9,13–15] This lack of information greatly limits our understanding of the role played by LPSs in the adaptation of psychrophilic bacteria to the harsh cold lifestyle.

Herein, we report the structural characterisation of the carbohydrate backbone of a LOS from *Psychromonas arctica*, a Gram-negative bacterium isolated in the arctic seawater near Spitzbergen (Svalbard islands, Arctic).[16] This biofilm-forming microorganism is classified as an eury-psychrophile, which can actively duplicate in the 0–25 °C temperature range.

The LOSs were degraded either by mild hydrazinolysis (*O*-deacylation) or with hot 4 M KOH (*N*-deacylation). Both products were investigated by ^{1}H and ^{13}C NMR spectroscopy, electrospray ionisation high-resolution Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS), and chemical analysis. The chemical structural variations induced by the growth of *P. arctica* at 20 °C are also reported.

Results

Isolation and purification of LOS: *Psychromonas arctica* was grown at 4 °C as reported in the Experimental section. Dried bacteria were first extracted by using the phenol/$CHCl_3$/light petroleum ether method of Galanos and Lüderitz[17] and the phenol/water method of Westphal and Jann.[18] Only the first method was successful in LPS recovery and the yield was 1.5 % of dried cells. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) showed, after silver nitrate gel staining, the presence of two fast migrating species that are typical of LOSs.

Compositional analysis: Sugar and lipid analyses were obtained by methanolysis of the LOS followed by extraction with methanol/hexane to recovery the fatty acids as methyl esters. The hexane layer was analysed by GC–mass spectrometry, thus revealing the presence of 3-hydroxytetradecanoic acids, dodecanoic acids, and cyclopropanoid tetradecanoic acids, the latter having already been found in *Pseudoalteromonas haloplanktis* TAC125.[9] The methyl glycosides recovered in the methanol layer were then acetylated and injected into the GC—mass spectrometer. This analysis revealed the presence of galacturonic acid (GalA), glucosamine (GlcN), and glucose (Glc), whereas neither heptose nor 3-deoxy*manno*oct-2-ulosonic acid (Kdo) were recovered. To check if these residues were not revealed because they were phosphorylated, hydrolysis with 48 % aqueous HF was performed on the LOS fraction to remove the phosphate groups and the sugar analysis was repeated. The presence of L,D-heptose and Kdo in this analysis suggested their phosphorylation in the LOS structure. Methylation analysis indicated the presence of terminal galacturonic acid, terminal glucose, 4-linked glucose, 2,4-linked heptose, 6-linked glucosamine, and 5-linked Kdo units.

The absolute configuration determination revealed a D configuration for glucosamine, galacturonic acid, and glucose. L-*Glycero*-D-*manno*-heptose was identified, as alditol acetate, by comparison with an authentic sample.

Mass spectrometric analysis: The LOS fraction was analysed in the negative-ion mode by using electrospray ionisation Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS), which revealed the presence of seven molecular species termed M1–M7 (Figure 1). Taking into account the glycosyl analysis, all the signals were identified as reported in Table 1.

The most abundant molecular species **M1** is an hexacyl pentaphosphorylated LOS with the core oligosaccharide containing one Kdo, one heptose, one hexose, and two uronic acid units. One less uronic acid was present in **M3**. All the other glycoforms differed from these two species only by the acyl substitutions and/or phosphates content. In source fragmentation by capillary skimmer dissociation (CSD), the Y and B fragments were generated (see Domon

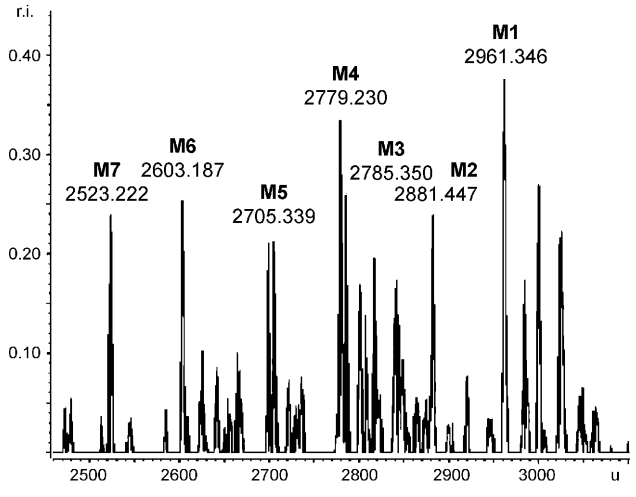


Figure 1. Charge-deconvoluted ESI FT-ICR mass spectrum of the LOS fraction isolated from the *P. arctica* bacterium. The spectrum was acquired in the negative-ion mode. The mass numbers given refer to monoisotopic peak of the neutral molecular species. The peaks not stated clearly belong to the sodium and potassium adducts. r.i. = relative intensity.

Table 1. Composition of the main species present in the ESI FT-ICR mass spectrum of the LOS fraction isolated from *P. arctic*a.

Glycoform	Observed mass [u]	Calcd mass [u]	Composition
M1	2961.346	2961.358	$HexHexA^2HepKdoHexN^2P^5[C14:0(3OH)]^4(C14:1)(C12:0)$
M2	2881.447	2881.392	$HexHexA^2HepKdoHexN^2P^4[C14:0(3OH)]^4(C14:1)(C12:0)$
M3	2785.350	2785.326	$HexHexAHepKdoHexN^2P^5[C14:0(3OH)]^4(C14:1)(C12:0)$
M4	2779.230	2779.188	$HexHexA^2HepKdoHexN^2P^5[C14:0(3OH)]^4(C14:1)$
M5	2705.339	2705.360	$HexHexAHepKdoHexN^2P^4[C14:0(3OH)]^4(C14:1)(C12:0)$
M6	2603.187	2603.156	$HexHexAHepKdoHexN^2P^5[C14:0(3OH)]^4(C14:1)$
M7	2523.222	2523.190	$HexHexAHepKdoHexN^2P^4[C14:0(3OH)]^4(C14:1)$

and Costello[19] for the fragment nomenclature) from the rupture of the labile lipid A/Kdo linkage,[20] thus confirming the presence of a bis- and monophosphorylated hexa- and penta-acylated lipid A and the presence of at least two core oligosaccharide structures. The core fragments could be identified by an additional fragment ion because of the decarboxylation of Kdo ($\Delta m = -44$ u).[20] The fragment at 1166.16 u was in agreement with the composition of one hexose, two uronic acid, one heptose, one Kdo, and three phosphate groups, whereas the other fragment (990.12 u) was consistent with the lack of one uronic acid.

We prepared *O*-deacylated LOS (LOS-OH) by mild hydrazinolyis and the sample was analysed by using negative-ion ESI FT-ICR MS, thus showing by CSD (Figure 2) the corresponding *O*-deacylated lipid A with 952.48 and 872.50 u, in agreement with structures consisting of two glucosamine, two amide-linked hydroxytetradecanoic acid, one and two phosphate units, respectively, and the two usual core oligosaccharide fragments (1166.16 and 990.12 u) that differ from each other in one unit of uronic acid (HexA).

Treatment of LOS-OH with KOH followed by purification with gel-permeation chromatography revealed two molecular species of 1410.72 and 1572.78 u, which differ from each other by one hexose unit (mass spectra not shown). The first species could be attributed the following composition $HexHexAHepKdoGlcN^2P^4$, according to the calculated mass of 1410.22 u. From these results, it is not possible to exclude that these compositions can correspond to several molecular species that differ at the phosphate positions. However, the lack of a signal that corresponds to a molecular species containing two uronic acid residues suggested that one uronic acid is linked to the carbohydrate backbone by a phosphodiester linkage, cleaved during the strong alkaline treatment.

NMR spectroscopic analysis of a fully deacylated LOS fraction: The complete structure of the fully deacylated LOS was obtained by one- and two-dimensional ^1H, ^{31}P, and ^{13}C NMR spectroscopic analysis. Chemical shifts were assigned utilizing DQF-COSY, TOCSY, ROESY, $^1H,^{13}C$ DEPT-HSQC, $^1H,^{13}C$ HMBC, and $^1H,^{31}P$ HSQC experiments. All the residues, except for the fructose (Fru), were present as pyranose rings, as indicated by ^1H and ^{13}C NMR chemical shifts and methylation analysis.

The NMR spectroscopic data confirmed the existence of a mixture of two main oligosaccharides that differ in the length of the sugar backbone. The ^1H NMR spectra of the fully deacylated fraction (Figure 3) together with the $^1H,^{13}C$ DEPT-HSQC experiment revealed the presence of six main anomeric signals. In particular, the signals for anomeric protons at $\delta = 5.69$, 5.37, 5.21, 4.94, 4.51, and 4.47 ppm, assigned to residues **A**–**F**, respectively, were identified by their correlation with the signals for carbon atoms at $\delta = 92.4$, 99.2, 102.4, 100.1, 104.3 and 104.5 ppm, respectively (Table 2). In contrast, the on-resonance ^{13}C NMR spectrum (Figure 4) indicated the presence of two additional signals for anomeric carbon atoms at $\delta = 100.8$ and 104.9 ppm, assigned to residues **G** and **H**, respectively (Table 2). Residue **G** was recognised as Kdo because in the ^1H NMR spectrum the typical signals for the H-3 methylene protons at $\delta = 2.01$ and 2.23 ppm (H-3ax and H-3eq, respectively) were both connected in the $^1H,^{13}C$ DEPT-HSQC experiment to a signal for the carbon atom at $\delta = 35.4$ ppm.[21] The difference between the proton chemical shifts of H-3ax and H-3eq suggested an α configuration for this residue.[22] A long-range

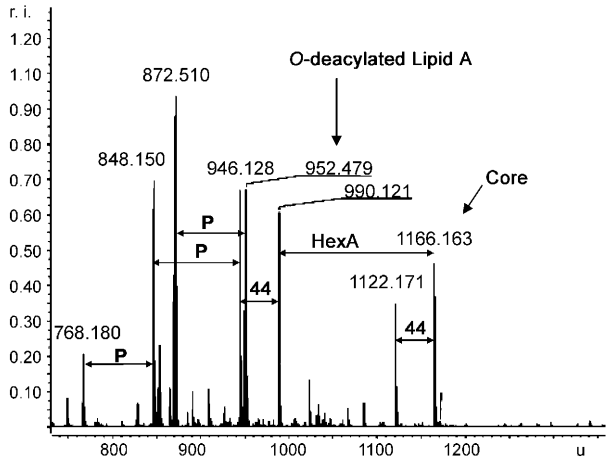


Figure 2. Charge-deconvoluted CSD/ESI FT-ICR mass spectrum of the LOS-OH fraction isolated from *P. arctic*a. The spectrum was acquired in the negative-ion mode.

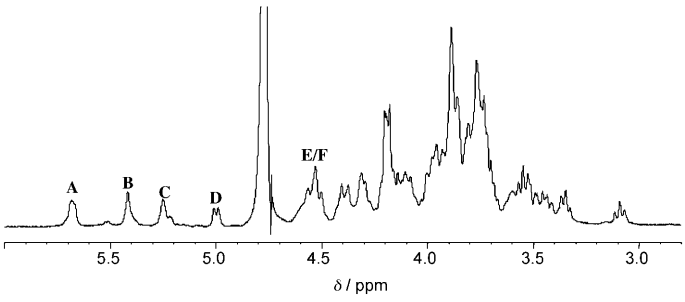


Figure 3. ^1H NMR anomeric and carbinolic region of the fully deacylated fraction isolated from *P. arctica* at 298 K. The spectrum was recorded in D_2O at 500 MHz. The letters refer to the residues described in Table 2.

Table 2. ^{1}H–^{13}C NMR assignments of **OS1** and **OS2**.[a]

Residue	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 C6	H7 C7	H8 C8
α-6-GlcN*p1P*	5.69	3.38	3.89	3.82	4.14	4.28–4.26		
A	92.4	55.4	70.6	72.8	73.9	70.2		
α-2,4-Hep*p3P*	5.37	4.13	4.47	4.14	4.04	n.d.	3.80–3.76	
B	99.2	80.8	73.7	78.2	70.3		63.6	
α-*t*-GalA*p3P*	5.21	3.95	4.36	4.48	4.51	–		
C	102.4	69.1	75.5	71.1	71.1	176.6		
β-6-GlcN*4P*	4.94	3.03	3.83	3.76	3.72	3.51–3.53		
D	100.1	56.8	73.1	75.6	75.4	63.8		
β-4-Glc*p*	4.51	3.29	3.47	3.45	3.56	4.03–3.69		
E	104.3	74.3	76.5	71.2	76.1	62.1		
β-*t*-Glc*p*	4.47	3.29	3.47	3.44	3.56	4.03–3.69		
F	104.5	74.3	76.5	70.9	76.1	62.1		
α-5-Kdo*4P*	–	–	2.01–2.23	4.56	4.26	3.83	3.84	3.93–3.70
G		100.8	35.4	71.1	73.4	73.2	70.2	64.9
β-*t*-Fru*f*	3.72–3.68	104.9	4.14	4.08	3.86	3.65–3.71		
H	61.6		79.0	75.6	82.3	63.7		

[a] All the chemical shifts are referred to acetone as the internal standard ($\delta = 2.225$ and 31.45 ppm for ^{1}H and ^{13}C, respectively); the spectra were recorded at 298 K.

proton–carbon correlation among the signals for the methylene protons and the carbon atom at $\delta = 100.8$ ppm allowed us to assign this latter signal to the anomeric carbon atom of Kdo. In addition, the glycosylation of Kdo at O-5 was suggested from the methylation analysis and confirmed by the chemical shift of the signal of C-5 at $\delta = 73.4$ ppm with respect to the value of $\delta = 67.4$ ppm for an unsubstituted Kdo residue.[21]

As the sugar analysis revealed only the presence of Kdo as a keto sugar, an additional experiment was performed. A mild acid hydrolysis was carried out on the LOS fraction and the obtained free monosaccharides were derivatised as alditols acetates. The GC-MS chromatogram revealed the presence of glucitol and mannitol, thus indicating the occurrence of fructose in the LOS sugar backbone. Thus, residue **H** was assigned as a β-fructose because the chemical shift for the anomeric carbon atom occurred at $\delta = 104.9$ ppm.[23] This value was deduced on the basis of a long-range correlation with methylene protons at $\delta = 3.72$ and 3.68 ppm in a ^{1}H,^{13}C HMBC experiment. In addition, a furanose ring for this residue was suggested by the signal for C-5 at $\delta = 82.3$ ppm in the ^{13}C NMR spectrum.[23]

Both spin systems **A** and **D** were attributed to 2-deoxy-2-aminohexose, on the basis of the correlation of the signals for H-2 at $\delta = 3.38$ and 3.03 ppm with nitrogen-bearing carbon atoms at $\delta = 55.4$ and 56.8 ppm, respectively, in a ^{1}H,^{13}C DEPT-HSQC spectrum. The signal of the anomeric proton of residue **A** at $\delta = 5.69$ ppm was assigned to the proximal GlcN1*P* residue of the lipid A skeleton because of the typical coupling constants with ^{31}P ($^{3}J_{H,P} = 6.0$ Hz), whereas the α configuration of residue **A** was deduced from the chemical shift of the anomeric carbon atom at $\delta = 92.4$ ppm and from $^{3}J_{H1,H2} = 3.1$ Hz. Finally, the substitution at C-6 of residue **A** was inferred from the chemical shift of C-6 due to the glycosylation effect at $\delta = 70.2$ ppm.

Residue **D** was recognised to be the lipid A distal β-glucosamine unit because of $^{3}J_{H1,H2} = 8.4$ Hz and its proton spin system. The ROESY experiment revealed that residues **D** and **A** were (1→6) connected. Residues **B** and **C** were revealed to be α configured from both their low $^{3}J_{H1,H2}$ values. Residue **B** was recognised as α-heptose from its internal spin-system connectivities, which were revealed from the COSY, TOCSY, and ROESY experiments, in which the latter experiment showed for the anomeric proton a NOE intraresidue interaction with only H-2. Moreover, residue **B** was 2,4-linked, as suggested by methylation analysis and confirmed by the downfield chemical shift of the signals for C-2 and C-4 at $\delta = 80.8$ and 78.2 ppm, respectively. Residue **C** was identified as a terminal α-galacturonic acid species because of the correlation in the TOCSY experiment of the anomeric proton with only four densities, which is typical of a *galacto* configuration, and the long-range connectivity between the signals for H-5 at $\delta = 4.51$ ppm and the carbon atom at $\delta = 176.6$ ppm. Finally, the signals for the anomeric units of **E** (H-1/C-1: $\delta = 4.51/104.3$ ppm) and **F** (H-1/C-1: $\delta = 4.47/104.5$ ppm) were assigned to two β-glucose residues on the basis of the proton multiplicities obtained by the DQF-COSY and TOCSY experiments.

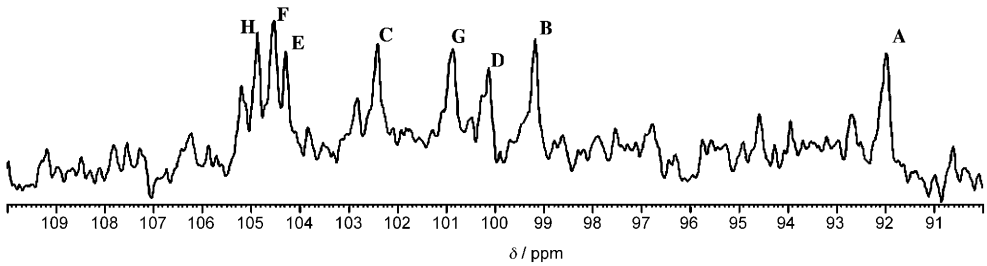


Figure 4. ^{13}C NMR anomeric region of the fully deacylated fraction fraction from *P. arctica* at 298 K. The spectrum was recorded in D_2O at 100 MHz.

The sequence and the attachment points of the residues were deduced from HMBC experiments that indicated the following correlations: H-1 of **C** with C-2 of **B**; H-1 of **B** with C-5 of Kdo; H-1 of **D** with C-6 of **A**; H-1 of both **E** and **F** with C-4 of **B**. These latter correlations, together with the close similarity of the chemical shifts of **E** and **F**, suggest a nonstoichiometric substitution on the glucose residue by a terminal nonreducing fructose unit. Moreover, as no signals for the carbon atoms were shifted downfield for both **E** and **F**, the position of the linkage of fructose was assigned to O-4 on the basis of methylation analysis. These data definitely revealed two oligosaccharides structures, which we named **OS1** and **OS2**.

Interresidue NOE interactions confirmed the sequence of the residues (Figure 5 and arrows in Scheme 1), in particular dipolar couplings were observed between H-1 of **C** and H-2 of **B**, H-1 of **B** and H-5 of **G**, H-1 of **D** and both H-6 and H-4 of **A**. Residue **G** did not show any diagnostic NOE interactions or heteronuclear multiple-bond correlations. Because the chemical shift of C-6 in **D** was in agreement with reported data, the Kdo moeity was placed at C-6 of **D**. The methylation analysis strongly supports this statement. Finally, the NOE interations between H-1 of **B** and both H-5 and H-7 of **G** confirmed the attachment point of **B** to be O-5 of Kdo; in addition, the NOE interaction between H-5 of **B** and H-3ax of **G** suggests a D configuration for Kdo.[24]

The locations of the phosphate groups were deduced by a $^{1}H,^{31}P$ HSQC experiment that showed correlations of the signals for the ^{31}P nuclei at $\delta = 0.98$ and 1.57 ppm with H-1 of **A** and H-4 of **D**, respectively, thus confirming that both glucosamine residues of lipid A were phosphorylated. The phosphorylation at O-4 of the Kdo unit and at O-3 of the residues **B** and **C** was inferred from the correlations among the signals for the ^{31}P nuclei at $\delta = 1.13$, 3.22, and 1.38 ppm and the protons at $\delta = 4.56$, 4.47, and 4.36 ppm, respectively.

All these data revealed the structures of **OS1** and **OS2** as shown in Scheme 1.

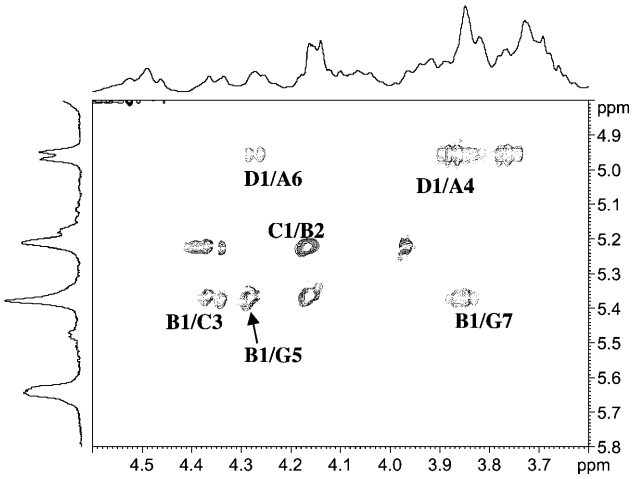
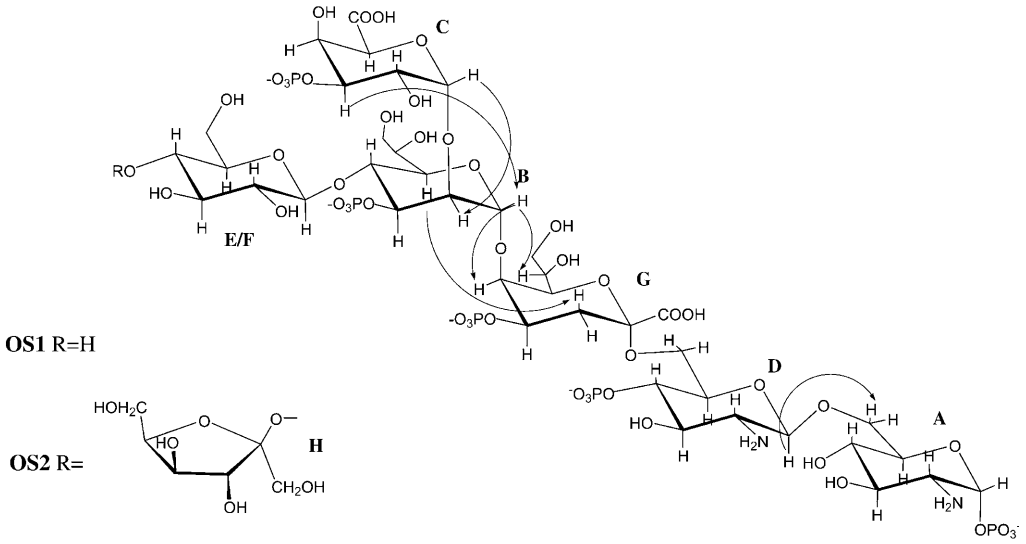


Figure 5. Carbinolic region of the ROESY experiment of the fully deacylated fraction fraction from *P. arctica* at 298 K. The spectrum was recorded in D_2O at 500 MHz. The letters refer to the residues as described in Table 2.

NMR spectroscopic analysis of LOS-OH: To establish the structure of the oligosaccharides including labile groups lost during the harsh alkaline treatment, the LOS-OH was analysed by NMR spectroscopic analysis. Because the presence of fatty acids on the sugar backbone induces the formation of micelles, the best resolution was obtained by dissolving the sample in a solution of SDS in D_2O (1 mg mL^{-1}). Two-dimensional NMR spectroscopic experiments (COSY, TOCSY, ROESY, DEPT-HSQC, HMBC, and $^{1}H,^{31}P$-HSQC) were performed.

The NMR spectroscopic analysis revealed the occurrence of the same carbohydrate backbone as in the samples of the **OS** compounds, except for the presence of an additional spin system, namely, residue **I**, with resonance of H-1 at $\delta =$ 5.49 ppm (Table 3). All the proton resonances were assigned on the basis of COSY, TOCSY, and ROESY experiments.



Scheme 1. The structures of **OS1** and **OS2**. The arrows indicate the interresidue NOE interactions.

Table 3. ^{1}H–^{13}C NMR assignments of LOS-OH.[a]

Residue	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 C6	H7 C7	H8 C8
α-6-GlcN*1P*	5.23	3.78	3.67	3.43	3.89	4.05–4.07		
A	92.5	53.9	71.2	69.5	71.5	68.1		
α-2,4-Hep*p3P*	5.18	4.28	4.48	3.96	3.68	n.d.	n.d.	
B	97.5	78.1	72.8	77.1	73.7			
α-*t*-GalA*p3P*	5.13	3.81	4.22	4.39	n.d.	n.d.		
C	100.9	69.6	72.8	70.0				
β-6-GlcN*4P*	4.55	3.67	3.59	3.46	3.66	3.70		
D	101.1	54.8	73.1	75.5	74.2	61.1		
β-*t*-Glc*p*	4.35	3.18	3.38	3.30	3.21	3.56–3.78		
E	104.3	73.4	75.0	69.2	75.1	59.9		
α-*t*-GalA*p1P*	5.49	3.68	3.79	4.18	4.33	n.d.		
I	95.7	71.3	69.6	70.7	72.7			
α-5-Kdo*4P*	n.d.	n.d.	1.95–2.15	4.31	4.11	3.69	n.d.	n.d.
G			35.4	67.6	71.5	71.3		

[a] All the chemical shifts are referred to acetone as the internal standard ($\delta = 2.225$ and 31.45 ppm for ^{1}H and ^{13}C, respectively); the spectra were recorded at 298 K; n.d. = not determined.

This proton resonance was identified to come from uronic acid, as already suggested by ESI FT-ICR mass-spectrometric analysis. In particular, the values of $^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ were diagnostic of a *galacto* configuration, and all of the chemical shifts for the carbon atoms were in agreement with an unsubstituted residue. Moreover, the $^{3}J_{H1,H2}$ value, the signals for H-1 and C-1 (i.e., $\delta = 5.49$ and 95.7 ppm), and the intraresidual NOE interactions suggested an anomeric α configuration.

The ^{31}P NMR spectrum showed three signals, of which two resonated at $\delta = 3.46$ and 2.10 ppm, typical of monophosphate ester groups, whereas the third signal at $\delta = -2.24$ ppm indicated a phosphate diester group. The position of the phosphate groups was deduced by a ^{1}H,^{31}P HSQC experiment that showed a correlation of the ^{31}P signal at $\delta = 3.46$ ppm with proton signals at $\delta = 5.23$ and 3.78 ppm, attributed to H-1 and H-2 of residue **A**, respectively. The correlation between the ^{31}P signals at $\delta = 2.1$ ppm and the proton signal at $\delta = 4.22$, attributed to H-3 of **C**, confirmed a phosphate group at O-3 of the galacturonic acid residue. The ^{31}P signal at $\delta = -2.24$ ppm correlated to proton resonances at $\delta = 5.49$ and 4.48 ppm, attributed to H-1 of **I** and H-3 of **B**, respectively, thus indicating that the heptose unit was substituted at O-3 by a galacturonic acid unit through a phosphodiester linkage. None of the correlations of phosphate groups of both lipid A distal glucosamine and Kdo were evident in this experiment.

These data allowed the identification of a third oligosaccharide backbone in the LOS fraction from *P. arctica*, the presence of which was not revealed in the ESI FT-ICR mass spectrum of the LOS fraction. However, in the LOS-OH mass spectrum (Figure 6) a further species of low intensity at 2200.729 u that corresponds to the composition $Hex^{2}HexA^{2}HepKdoGlcN^{2}P^{4}[C14:0(3OH)]^{2}$ appeared besides the two main abundant molecular species. To further confirm the presence of an oligosaccharide backbone containing both fructose and the α-galacturonyl phosphate moiety, we performed a negative-ion (linear mode) MALDI TOF mass spectrum of the LOS fraction (data not shown). This spectrum comprised, in addition to the species already found in the ESI FT-ICR mass spectrum (Table 1), a further low-abundant molecular species of 3123.5 u that corresponds to the composition $Hex^{2}HexA^{2}HepKdoHexN^{2}P^{5}$ [C14:0-(3OH)]^{4}(C14:1)(C12:0).

In conclusion, the complete structure of the sugar backbone of the LOS isolated from *P. arctica* is depicted in Figure 7.

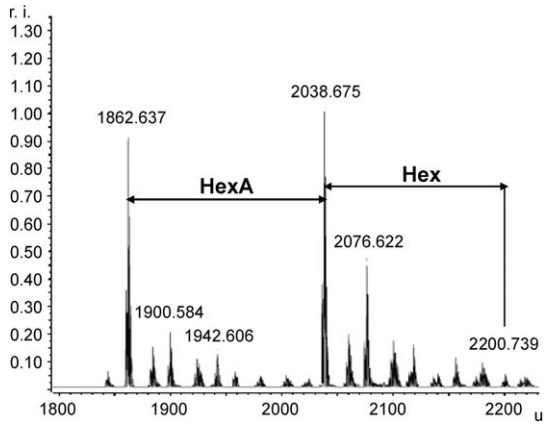


Figure 6. Charge-deconvoluted ESI FT-ICR mass spectrum of the LOS-OH fraction isolated from *P. arctica* bacterium. The spectrum was acquired in negative-ion mode.

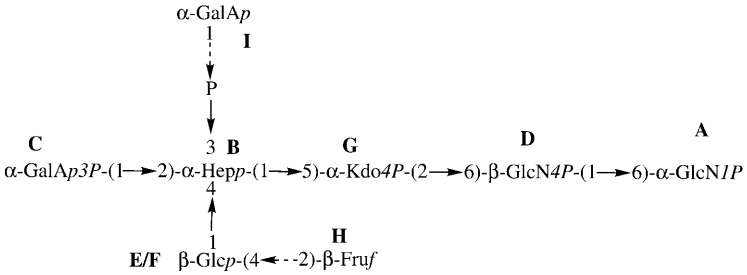


Figure 7. The structure of the phosphorylated carbohydrate backbone of the LOS fraction isolated from *P. arctica* bacterium. The dotted lines indicate nonstoichiometric bonds.

Structural differences induced in the LOS by a growth temperature of 20 °C: To establish the chemical structural variations induced by different growth temperatures in the LOSs, the cells of *P. arctica* were grown at 10 and 20 °C. Because no differences were found in lipid and glycosyl analyses for both the samples, only the structural variations between 4 and 20 °C are discussed. The glycosyl analysis performed on

the LOS sample from *P. arctica* grown at 20 °C showed the same qualitative composition of the LOS from the bacterium grown at 4 °C; however, interestingly, integration of the peaks showed an increase in the amount of glucose, thus suggesting the presence of at least an additional sugar unit in the oligosaccharide structure.

The analysis fatty acids by using GC-MS showed no significant compositional differences between the LOS sample at 4 and 20 °C, even if a higher content of the dodecanoic acid was observed at 20 °C, thus suggesting the presence of an additional hepta-acyl lipid A species.

The ESI FT-ICR mass spectrum of the LOS sample obtained at 20 °C (Figure 8, Table 4) confirmed the above results. In this mass spectrum, most of the signals were found at *m/z* values higher than the corresponding species found in the LOS obtained at 4 °C. In particular, the most aboundant species **N3** at 3125.253 u suggested the presence of an hexa-acyl triphosphorylated LOS with the composition $Hex^3HexA^2HepKdoHexN^2P^3[C14:0(3OH)]^4(C14:1)(C12:0)$, according to the calculated mass of 3125.529 u. Moreover, one less hexose unit was present in **N4**, which corresponded to an hepta-acyl triphosphorylated LOS, in agreement with the analysis of the fatty acids by using GC-MS. Finally, the species **N2** at 3307.413 u suggested the presence of a LOS containing both an additional hexose and a dodecanoic acid.

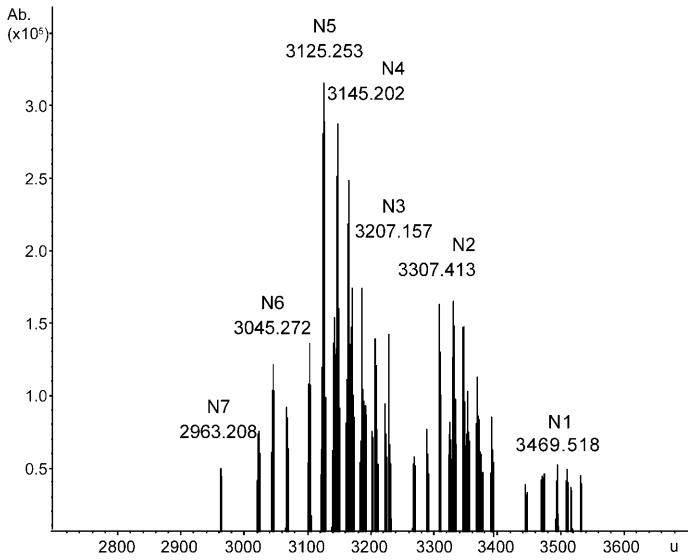


Figure 8. Charge-deconvoluted ESI FT-ICR mass spectrum of the LOS fraction isolated from *P. arctica* bacterium grown at 20 °C. The spectrum was acquired in the negative-ion mode. The mass numbers given refer to the monoisotopic peak of the neutral molecular species. The peaks not stated clearly belong to the sodium and potassium adducts.

Table 4. Composition of the main species present in the ESI FT-ICR mass spectrum of the LOS fraction isolated from *P. arctica* grown at 20 °C.

Glycoform	Observed mass [u]	Calcd mass [u]	Composition
N1	3469.518	3469.752	$Hex^4HexA^2HepKdoHexN^2P^3[C14:0(3OH)]^4(C14:1)(C12:0)^2$
N2	3307.413	3307.699	$Hex^3HexA^2HepKdoHexN^2P^3[C14:0(3OH)]^4(C14:1)\ (C12:0)^2$
N3	3207.157	3207.615	$Hex^4HexA^2HepKdoHexN^2P^2[C14:0(3OH)]^4(C14:1)\ (C12:0)$
N4	3145.202	3145.647	$Hex^2HexA^2HepKdoHexN^2P^3[C14:0(3OH)]^4(C14:1)(C12:0)^2$
N5	3125.253	3125.529	$Hex^3HexA^2HepKdoHexN^2P^3[C14:0(3OH)]^4(C14:1)(C12:0)$
N6	3045.272	3045.563	$Hex^3HexA^2HepKdoHexN^2P^2[C14:0(3OH)]^4(C14:1)(C12:0)$
N7	2963.208	2963.477	$Hex^2HexA^2HepKdoHexN^2P^3[C14:0(3OH)]^4(C14:1)(C12:0)$

Discussion

It is well known that cold-adapted microorganisms are able to control the fluidity of the cell membrane at low temperatures by generally including a preponderance of unsaturated short acyl chains in their lipids.[25] On the contrary, very little information is available on the structural adaptations of the outermembrane of Gram-negative bacteria, which is an asymmetric bilayer in which LPSs are essential components of the outer leaflet, to low temperatures. It is reasonable that the structure of the LPSs also needs to be altered to allow bacterial survival at freezing temperatures.

Up until now, a unique LPS structure was reported for a cold-adapted bacterium, the Antarctic Gram-negative *P. haloplanktis* TAC125.[13–15] The outer membrane of the psychrophilic bacterium contains a LOS made up of the typical phosphorylated disaccharide skeleton of lipid A [*P*4-β-D-GlcN-α-D-(1→6)-GlcN1*P*], which was glycosylated by a phosphorylated Kdo unit and then substituted in turn by an heptose residue. This latter sugar was substituted at the O-4 position by a residue of galactose, which in turn was non-stoichiometrically glycosylated by 2-acetamido-2-deoxymannose.

Herein, we report the complete structure of the sugar backbone of the LPS fraction from *P. arctica,* a marine bacterium isolated from a seawater sample collected near Spitzbergen (Svalbard islands, Arctic). The molecules were extracted by using phenol/$CHCl_3$/petroleum ether and they constituted a LOS fraction. Complete structural determination was achieved by NMR spectroscopic, ESI mass-spectrometric, and chemical analysis. *P. arctica* LOS shares with *P. haloplanktis* TAC125 the lack of the O-chain, which seems to be a characteristic feature of LPSs obtained from cold-adapted microorganisms. Moreover, both LOS structures are highly phosphorylated, when the number of phosphate groups is considered with respect to the number of monosaccharides that constitute the LOS. Another interesting feature of *P. arctica* LOS in common with *P. haloplanktis* TAC125 is the occurrence of a cyclopropanoid fatty acid C14:0Δ in the lipid A.[9] This structural observation may be related to the cold adaptation of the two strains because the cyclopropanoid fatty acids structurally resemble unsaturated fatty acids, the concentration of which in membrane lipids is often increased during growth at low temperatures.[26–28]

An intriguing difference between the two psychrophilic LOS structures is the presence of a terminal β-fructofuranose

residue in the LOS from *P. arctica*. The occurrence of such a monosaccharide as a terminal residue is quite rare in the LOS structure and, to the best of our knowledge, it has been found only in the core oligosaccharide of a LPS from *Vibrio cholerae* strains.[29,30]

In conclusion, we have reported the structural features of a LOS isolated from *P. Arctica*, which evidenced a strong similarity to that obtained from *P. haloplanktis* TAC125, thus suggesting a common evolutionary bacterial adaptation to the extreme environment. The characterisation of more LPS structures from other cold-adapted microorganisms is mandatory to obtain experimental data that prove that the elucidated structures are indeed related to adaptative processes essential for maintaining, for example, membrane fluidity and transmembrane permeability.

As for the results obtained for the LOS from *P. arctica* grown at 20 °C, a decrease in the phosphate content and an increase in the sugar chain length were observed. Moreover, molecular species containing an additional dodecanoic acid unit were found. These data, which seem to be different from those of the LOS from *P. haloplanktis* TAC125,[9] are difficult to explain. However, as the information about the effects induced in cold-adapted bacteria by higher growth temperatures are still poor, it will be particularly interesting to analyse the structure of other outermembrane components, such as phospholipids and proteins.

Experimental Section

Growth of bacteria and isolation of LPS: *Psychromonas arctica* was grown at 4, 10, and 20 °C under aerobic conditions in a modified Luria–Bertani medium,[31] in which sodium chloride was replaced by a mixture of marine salts (10 $g L^{-1}$). The large-scale biomass production was carried out in shaken flasks (total volume = 2.5 L) containing liquid broth (500 mL). After 72 h of incubation at 4 °C, the cells were recovered by centrifugation at 4 °C for 15 min at $3836 \times g$ and lyophilised. Bacterial dried cells (2 g) were extracted with phenol/$CHCl_3$/light petroleum ether (2:5:8, v/v) as described.[17] The yields of the LPSs were 2.9, 1.4, and 2.1 % of dried cells grown at 4, 10, and 20 °C, respectively.

PAGE was performed by using the system of Laemmli and Favre[32] with SDS as the detergent. The separation gel contained acrylamide (15 %), SDS (0.1 %), and 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris—HCl; pH 8.8, 375 mM) and the stacking gel contained acrylamide (4 %), SDS (0.07 %), and Tris–HCl (pH 6.8, 125 mM). LPS samples were prepared at a concentration of 0.05 % in the sample buffer of SDS (2 %), Tris–HCl (pH 6.8, 60 mM), and glycerol (25 %). Bromophenol blue (0.003 % in the sample buffer) was used as the tracking dye. All the concentrations are expressed as a percentage of mass/vol. The electrode buffer was composed of SDS (1 $g L^{-1}$), glycine (14.4 $g L^{-1}$), and Tris (3.0 $g L^{-1}$). Electrophoresis was performed at a constant voltage of 150 V. The gels were fixed in an aqueous solution of ethanol (40 %) and acetic acid (5 %). LPS bands were visualised by silver staining as described previously.[33]

Chemical analysis: Acetylated methyl glycosides were obtained from each crude LPS (0.5 mg). The LPS was dephosphorylated with 48 % aqueous HF at 4 °C for 16 h then freeze-dried. Methanolysis was performed in MeOH/HCl (0.5 mL, 0.5 M) at 85 °C for 45 min, and the sample was extracted twice with hexane. The methanol layer was dried and the residue acetylated. The hexane layer was analysed by using GC-MS.

The linkage positions of the monosaccharides were determined by methylation analysis. Briefly, the product obtained from alkaline hydrolysis of the LPS (2 mg) was dephosphorylated with 48 % aqueous HF at 4 °C for 48 h. The sample was reduced with $NaBH_4$ and then *N*-acetylated with Ac_2O in anhydrous methanol. Methylation was performed with CH_3I in dimethyl sulfoxide (DMSO) and NaOH (2.5 h). The product was then reduced at the carboxyl group with $NaBD_4$. A mild acid hydrolysis was performed with 0.1 M trifluoroacetic acid (TFA) at 100 °C for 30 min to hydrolyse the Kdo glycosidic linkage. This residue was then reduced at the anomeric position with $NaBD_4$. After the usual workup, a second stronger hydrolysis was performed with 2 M TFA at 120 °C for 2 h. After reduction with $NaBD_4$, the sample was acetylated and finally analysed by using GC-MS.

The fructose unit for each LPS sample was revealed by preparation of the corresponding alditol acetates. Briefly, the LPS sample (0.5 mg) was mildly hydrolysed with AcOH 1 % at 100 °C for 4 h. The product was reduced with $NaBD_4$ and, after the usual workup, was treated with MeOH/HCl 1 M at 80 °C for 16 h. An extraction with hexane was performed and afterwards the methanol layer was dried and acetylated using Ac_2O and pyridine at 100 °C for 30 min. The presence of glucitol and mannitol was detected by using GC-MS.

The absolute configuration of the sugars was determined by subjecting the acetylated (*S*)-2-octyl glycosides to GC.[34]

The sugar derivatives were analysed on a Agilent Technologies gas chromatograph 6850 A equipped with a mass-selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex; 30 m × 0.25 mm i.d.; flow rate: 1 $mL min^{-1}$; carrier gas: He). Acetylated methyl glycosides were analysed accordingly with the following temperature program: 150 °C for 3 min, 150→240 °C at 3 °C min^{-1}. The temperature program for partially methylated alditol acetates: 90 °C for 1 min, 90→140 °C at 25 °C min^{-1}, 140→200 °C at 5 °C min^{-1}, 200→280 °C at 10 °C min^{-1}, 280 °C for 10 min. The temperature program for alditol acetates: 150 °C for 5 min, 150→310 °C at 3 °C min^{-1}. The temperature program for the analysis of acetylated octyl glycosides: 150 °C for 5 min, 150→240 °C at 6 °C min^{-1}, 240 °C for 5 min. The temperature program for fatty acid methyl esters: 140 °C for 3 min, 140→280 °C at 10 °C min^{-1}, 280 °C for 20 min.

Deacylation of the LPS: The LPS (20 mg) was dried over phosphorus anhydride under vacuum and then incubated with hydrazine (0.9 mL) at 37 °C for 1.5 h. Cold acetone was added to precipitate the *O*-deacylated LPS. The pellet was recovered after centrifugation at 4 °C and $8000 \times g$ for 30 min, washed three times with acetone, dissolved in water, and lyophilised (12 mg).

The *O*-deacylated LPS was dissolved in 4 M KOH (0.8 mL) and incubated at 120 °C for 16 h. The reaction mixture was neutralised with 4 M HCl until pH 6 and then extracted three times with CH_2Cl_2. The water phase was recovered and desalted on a column (1.5 × 800 mm) of Sephadex G-10 (17 $mL h^{-1}$; fraction volume: 1.7 mL) eluted with water. The eluted oligosaccharide mixture was then lyophilised (7 mg).

NMR spectroscopy: For the structural assignments, 1D and 2D ^1H and ^{13}C NMR spectra were recorded at 298 K with a Varian Inova 500 spectrometer. The chemical shifts were measured in D_2O using acetone at $\delta = 2.225$ and 31.45 ppm as an internal standard for the proton and carbon atoms, respectively. All 2D homo- and heteronuclear experiments (DQF-COSY, TOCSY, ROESY, HSQC-DEPT, and HMBC) were performed using standard pulse sequences available in the Varian software. The $^1H,^{31}P$ HSQC experiments were recorded on a Bruker DRX-400 spectrometer with aqueous 85 % phosphoric acid as an external reference ($\delta = 0.00$ ppm).

Mass spectrometric analysis: High-resolution ESI FT-ICR MS was performed in the negative mode using an ApexII mass analyser (Bruker Daltonics, Billerica, MA) equipped with a 7T actively shielded magnet and an Apollo ESI source. The mass spectra were acquired by using standard experimental sequences as provided by the manufacturer. The samples were dissolved at a concentration of 10 $ng mL^{-1}$ in 2-propanol/water/triethylamine (30:30:0.01, v/v/v) and sprayed at a flow rate of 2 $mL min^{-1}$. The capillary entrance voltage was set to 3.8 kV and the dry gas temperature to 150 °C. The spectra, which showed several charge states for each component, were charge deconvoluted, and the mass numbers given refer to the monoisotopic molecular masses. Capillary skimmer dissocia-

tion (CSD) was induced by increasing the capillary exit voltage from 100 to 350 V.

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