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RESEARCH

Location of the *O*-methyl groups in the O polysaccharide of *Pseudomonas syringae* pv. phaseolicola

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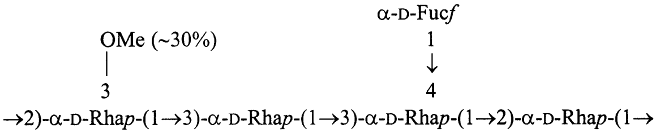
Abstract

The O-methylation pattern of the O polysaccharide (OPS) of the lipopolysaccharide of *Pseudomonas syringae* pv. phaseolicola GSPB 1552 was revealed by methylation (CD_3I) analysis, Smith degradation, and NMR spectroscopy. Together with the major O repeats consisting of D-rhamnopyranose (D-Rha*p*) and D-fucofuranose (D-Fuc*f*), there are minor repeats (~ 30%) containing 3-*O*-methyl-D-rhamnose (D-acofriose), which is 2-substituted in the interior repeats and occupies the terminal non-reducing end of the OPS. It was suggested that the methylated O repeats are linked to each other nearby the non-reducing end of the OPS and that the 'biological' O repeat of the OPS has the following structure:



$$\rightarrow 2)\text{-}\overset{\substack{\text{OMe }(\sim 30\%)\\ | \\ 3}}{\alpha\text{-D-Rha}p}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow 3)\text{-}\overset{\substack{\alpha\text{-D-Fuc}f\\ 1\\ \downarrow\\ 4}}{\alpha\text{-D-Rha}p}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Rha}p\text{-}(1\rightarrow$$

Keywords: Lipopolysaccharides; O polysaccharides; O-Methylation; Phytopathogens; *Pseudomonas syringae*

1. Introduction

On the basis of distinctive pathogenicity to one or more host plants, phytopathogenic *Pseudomonas syringae* strains have been classified into more than 50 infraspecific taxa, so called pathovars.[1] Recently, a total of 48 pathovars of *P. syringae* and eight related species have been studied by DNA–DNA hybridization and ribotyping, and the existence of nine discrete genomic species has been delineated.[2] In particular, *P. syringae* pv. phaseolicola fell in genomospecies 2, together with *P. syringae* pvs. ulmi, mori, lachrymans, sesami, tabaci, morsprunorum, glycinea, ciccaronei, eriobotriae, mellea, aesculi, hibisci, myricae, photiniae, and dendropanacis, as well as nomenspecies *Pseudomonas savastanoi*, *Pseudomonas ficuserectae*, *Pseudomonas meliae*, and *Pseudomonas amygdali*.

Serological studies of these bacteria using lipopolysaccharide (LPS)-specific polyclonal antisera[3–5] and monoclonal antibodies[6–8] have demonstrated a high degree of antigenic het

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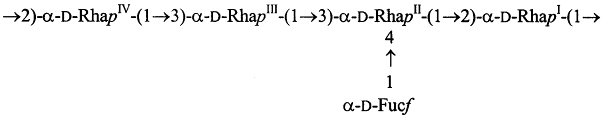
erogeneity, which is defined by structural diversity of the O polysaccharide (OPS) chain of the LPS.[7,8] The serotype and OPS chemotype have been correlated to the host specificity and both suggested to be a phenotypic character of high taxonomic impact.[8]

Chemical studies have shown that *P. syringae* strains produce LPSs with linear or branched OPSs having a backbone consisting of L-, D-, or both L- and D-rhamnose residues and different lateral sugar substituents (Refs. 8–11 and refs. cited therein). Strains with a D-rhamnan backbone having the tetrasaccharide O repeat (Structure **1**, chemotype 1A) have been classified in serogroup O1.[8] Branched OPSs of serogroup O1 include lateral α-D-Rha*p*, β-D-Glc*p*NAc or α-D-Fuc*f* residues attached to the chemotype 1A backbone (chemotypes 1B, 1C, and 1D, respectively).[8,9] Chemotype 1D (O repeat structure **2**[12]) and the corresponding serotype O1[(1-2)a↓,(1-2)d,1d] are typical of all smooth strains of *P. syringae* pv. phaseolicola studied.[8,12]

$$\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{\mathrm{IV}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p^{\mathrm{III}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p^{\mathrm{II}}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{\mathrm{I}}\text{-}(1\rightarrow \qquad \mathbf{1}$$



$$\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{\mathrm{IV}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p^{\mathrm{III}}\text{-}(1\rightarrow 3)\text{-}\underset{\substack{4\\ \uparrow\\ 1\\ \alpha\text{-D-Fuc}f}}{\alpha\text{-D-Rha}p^{\mathrm{II}}}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{\mathrm{I}}\text{-}(1\rightarrow \qquad \mathbf{2}$$

A characteristic feature of D-rhamnan-based OPSs of *P. syringae* is O-methylation (Ref. 13 and G.M. Zdorovenko, unpublished data), but the location of *O*-methyl groups remained unknown so far. In the present work, we elucidated the O-methylation pattern of the chemotype 1D OPS from the LPS of *P. syringae* pv. phaseolicola GSPB 1552.

2. Results and discussion

A loosely bound LPS was isolated by extraction with Tris–ETDA buffer[7,8] and degraded with dilute acetic acid to give a high-molecular mass OPS. Sugar analysis of the OPS, including determination of the absolute configuration, demonstrated the presence of D-rhamnose, D-fucose, and 3-*O*-methyl-D-rhamnose (D-acofriose, D-Rha3Me) in the ratios 4:0.8:0.3.

In addition to the derivatives of rhamnose and fucose expected from structure **2** (see above), methylation analysis of the OPS using CD_3I showed the presence of terminal and 2-substituted acofriose residues. In GLC of partially methylated alditol acetates, the peak for the derivative of 3-*O*-methyl-2,4-di-*O*-trideuteromethylramnose (from terminal acofriose) coincided with that of 2,3,4-tri-*O*-trideuteromethylramnose (from terminal rhamnose). As judged by the relative intensities of the ion peaks at *m*/*z* 164 and 167 for the C-1–C-3 fragment and at *m*/*z* 178 and 181 for the C-3–C-6 fragment, the content of terminal acofriose was twice as high as that of terminal rhamnose. The latter may either result from incomplete O-methylation of the terminal monosaccharide of the O chain or from cleavage to a small extent of rhamnosidic linkages during mild acid degradation of the LPS; it cannot be also excluded that it represented a component of the LPS core.[13,14] The peaks for the derivatives of 3,4-di-*O*-trideuteromethylramnose (from 2-substituted rhamnose) and 3-*O*-methyl-4-*O*-trideuteromethylramnose (from 2-substituted acofriose) were partially superimposed (retention times 10.864 and 10.910 min, respectively). The former was predominant but the ratio of the two derivatives could not be reliably determined.

Each of the ^1H and ^{13}C NMR (Fig. 1) spectra of the OPS contained the major series of signals, which resembled the spectra of the OPS of *P. syringae* pv. phaseolicola 120a studied earlier.[12] The assignment of the major signals using 2D COSY, TOCSY and H-detected $^1H,^{13}C$ HMQC experiments (Tables 1 and 2) and the NOESY correlation pattern confirmed that the major, non-O-methylated O repeat of the OPS of *P. syringae* pv. phaseolicola GSPB 1552 has structure **2**.

The NMR spectra also contained signals for *O*-methyl groups and a minor series of sugar signals with the intensities of about one third of those of the major signals. The $^1H,^{13}C$ HMQC spectrum showed minor cross-peaks for two *O*-methyl groups at δ_H/δ_C 3.49/58.3 and 3.45/57.5 in the ratio ~3:1. A NOESY experiment revealed a correlation of the OMe signal at δ 3.49 to a signal for H-3 of α-

Rha3Me at δ 3.66, which was assigned using 2D COSY and TOCSY experiments. Furthermore, the signal for H-1 of an α-Rha residue at δ 5.07 gave cross-peaks with the signals for H-2 of α-Rha3Me at δ 4.32 and the OMe group at δ 3.49. For its turn, the signal for H-1 of α-Rha3Me at δ 5.20 showed a correlation to the signal for H-3 of another α-Rha residue at δ 3.91. These data demonstrated an α-Rha*p*-(1 → 2)-α-Rha*p*3Me-(1 → 3)-α-Rha*p* trisaccharide fragment.

Methylation of Rha3Me at O-3 and glycosylation at O-2 were further confirmed by downfield displacements in the ^{13}C NMR spectrum of the signals for C-2 and C-3 of Rha3Me to δ 74.7 and 80.9, respectively, as compared with their position at δ 72.1 and 71.3 in nonsubstituted α-Rha*p*.[15] The position of the signal for C-1 of Rha3Me at δ 102.0 and of the Rha residue linked to Rha3Me at δ 101.4 demonstrated that both these residues are 2-substituted (compare δ 103–104 for C-1

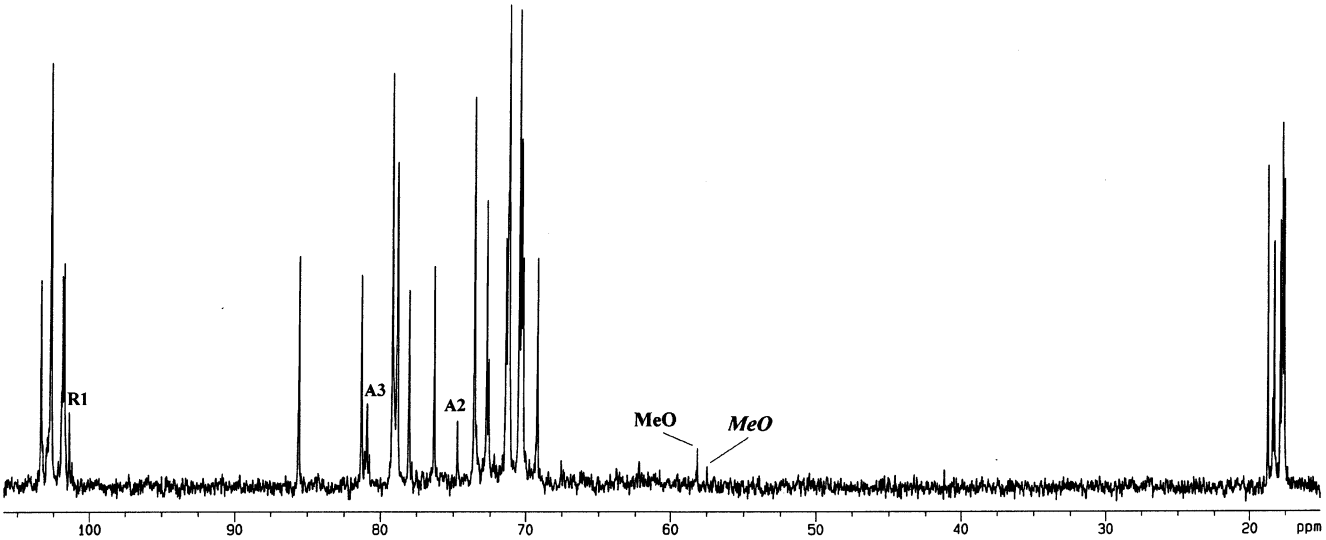


Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide. Denoted are signals from the minor O-methylated O repeat **3**. Arabic numerals refer to carbons in Rhap^{I} (R) and Rha$p$3Me^{IV} (A, acofriose); OMe and *OMe* indicate the *O*-methyl group in the interior and terminal O repeat, respectively.

Table 1
500-MHz ^{1}H NMR data (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	OMe
*Polysaccharide (O repeat **2**)* [a]							
α-D-Fuc*f*-(1 →	5.28	4.03	3.95	3.51	3.86	1.20	
→ 2)-α-D-Rhap^{I}-(1 →	5.10	4.09	3.90	3.49	3.73	1.29	
	(5.07)						
→ 3,4)-α-D-Rhap^{II}-(1 →	4.97	4.16	3.98	3.70	3.83	1.36	
→ 3)-α-D-Rhap^{III}-(1 →	4.99	4.08	3.91	3.59	3.92	1.30	
→ 2)-α-D-Rhap^{IV}-(1 →	5.18	4.07	3.94	3.51	3.80	1.31	
	(5.19)	(4.32)	(3.66)	(3.54)		(3.49)	
*Oligosaccharide **5***							
α-D-Rha$p$3Me^{IV}-(1 →	5.09	4.33	3.58	3.49	3.86	1.29	3.45
→ 3)-α-D-Rhap^{III}-(1 →	5.03	4.15	3.93	3.58	3.91	1.31	
→ 3)-α-D-Rhap^{II}-(1 →	4.96	4.10	3.90	3.59	3.89	1.29	
→ 2)-Gro	3.73	3.80	3.67 [b]				

[a] When different, data for the interior O-methylated O repeat **3** with Rha$p$3Me^{IV} are given in parentheses.
[b] H-3a; H-3b at δ 3.77.

Table 2
125-MHz ^{13}C NMR data (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	OMe
*Polysaccharide (O repeat **2**)* [a]							
α-D-Fuc*f*-(1 →	102.7	78.1	76.3	85.6	70.5	18.8	
→ 2)-α-D-Rhap^{I}-(1 →	101.8	79.2	71.2	73.6	70.3	17.9 [b]	
	(101.4)						
→ 3,4)-α-D-Rhap^{II}-(1 →	102.7	71.3	78.8	81.3	69.2	18.4	
→ 3)-α-D-Rhap^{III}-(1 →	103.4	71.4	78.8	72.7	70.5	17.7 [b]	
→ 2)-α-D-Rhap^{IV}-(1 →	101.9	79.2	71.2	73.6	70.2	17.8 [b]	
	(102.0)	(74.7)	(80.9)	(72.6)			(58.2)
*Oligosaccharide **5***							
α-D-Rha*p*3Me$^{\mathrm{IV}}$-(1 →	103.7	67.3	80.8	72.3	70.5	18.0	57.5
→ 3)-α-D-Rhap^{III}-(1 →	103.6	71.3	79.7	72.7	70.7	18.0	
→ 3)-α-D-Rhap^{II}-(1 →	100.6	71.5	79.7	72.7	70.4	18.0	
→ 2)-Gro	62.7	79.5	61.6				

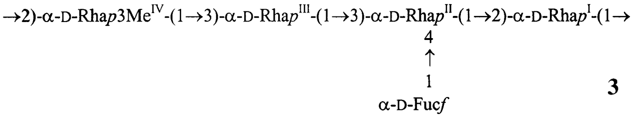
[a] When different, data for the interior O-methylated O repeat **3** with Rha*p*3Me$^{\mathrm{IV}}$ are given in parentheses.

[b] Assignment could be interchanged.

of α-Rha*p* nonsubstituted at position 2[10–12]). The NMR chemical shifts for H-1 and C-1 of Rha3Me and for all atoms of the two neighboring Rha residues were similar or identical to those in the correspondingly substituted rhamnose residues in the major series. Therefore, the minor rhamnose residues could be correlated to the major residues in **2** as follows:

$$\rightarrow 2)\text{-}\alpha\text{-Rha}p^{\mathrm{I}}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-Rha}p3\mathrm{Me}^{\mathrm{IV}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-Rha}p^{\mathrm{III}}\text{-}(1\rightarrow$$

These data suggested that, in addition to the major O repeats **2**, the OPS contained minor O repeats **3**, which differ from **2** in O-methylation of Rha$^{\mathrm{IV}}$ at position 3. The second minor series in the ^{1}H and ^{13}C NMR spectra that included the smaller OMe signal at δ_H 3.45 and δ_C 57.5 was not assigned owing to a low intensity of sugar signals, but could be suggested to belong to a terminal Rha3Me residue at the non-reducing end of the OPS (see below).



$$\rightarrow 2)\text{-}\alpha\text{-D-Rha}p3\mathrm{Me}^{\mathrm{IV}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Rha}p^{\mathrm{III}}\text{-}(1\rightarrow 3)\text{-}\underset{\substack{4\\ \uparrow\\ 1\\ \alpha\text{-D-Fuc}f}}{\alpha\text{-D-Rha}p^{\mathrm{II}}}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Rha}p^{\mathrm{I}}\text{-}(1\rightarrow$$

3

To confirm the position of the *O*-methyl groups, the OPS was subjected to Smith degradation. The products of the mild acid hydrolysis of the degraded polysaccharide were borohydride reduced, fractionated by GPC on TSK HW-40 (Fig. 2), and studied by methylation analysis, ^{1}H and ^{13}C NMR spectroscopy. The data of the major product (Fraction 4) were identical to published data of disaccharide-glycerol **4**.[16] This oligosaccharide was evidently derived from the non-O-methylated O repeat **2**. Methylation (CD$_3$I) analysis of an oligosaccharide eluted earlier (Fraction 3) revealed terminal acofriose and 3-substituted rhamnose. The ^{1}H and ^{13}C NMR spectra of this product contained signals for three rhamnose residues and one *O*-

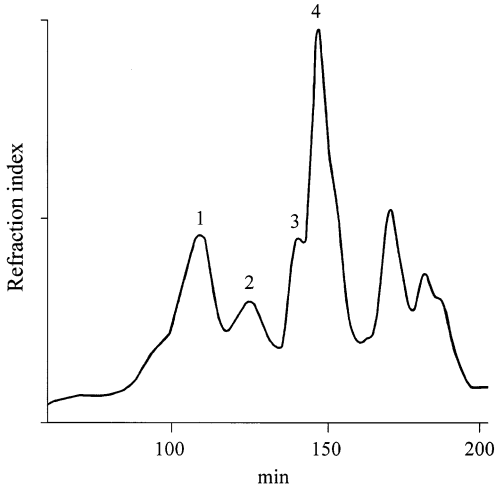


Fig. 2. TSK HW-40 gel chromatography elution profile of Smith degradation products from the O-specific polysaccharide. Fractions 1 and 2 correspond to oligomers of the oxidized O repeats **3**, Fractions 3 and 4 to oligosaccharides **5** and **4**, respectively.

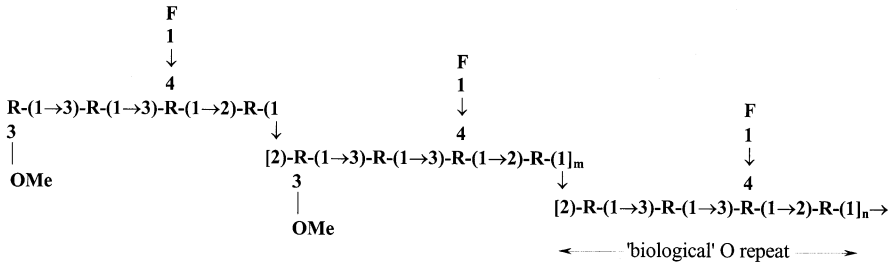


Fig. 3. Proposed structure of the O-specific polysaccharide of *P. syringae* pv. phaseolicola GSPB 1552. O-Methylation in both terminal and interior O repeats may be incomplete. R and F stand for α-D-rhamnopyranose and α-D-fucofuranose, respectively; $m \leq 3$, $m + n \geq 10$.

methyl group (Tables 1 and 2). Full assignment of the spectra using COSY, TOCSY, and ^{1}H,^{13}C HMQC experiments, as well as linkage and sequence analyses using a 2D rotating-frame NOE spectroscopy (ROESY) experiment, demonstrated that this oligosaccharide has structure **5**, which is in agreement with the structure of the O-methylated O repeat **3**. The ^{1}H and ^{13}C NMR chemical shifts δ_H 3.45 and δ_C 57.5 for the *O*-methyl group of terminal acofriose in **5** were identical to those for the minor *O*-methyl group in the OPS (see above), which thus terminates the OPS at the non-reducing end.

α-D-Rha*p*$^{\text{III}}$-(1 → 3)-α-D-Rha*p*$^{\text{II}}$-(1 → 2)-Gro **4**

α-D-Rha*p*3Me$^{\text{IV}}$-(1 → 3)-α-D-Rha*p*$^{\text{III}}$-(1 → 3)-
α-D-Rha*p*$^{\text{II}}$-(1 → 2)-Gro **5**

Higher oligosaccharides (Fractions 2 and 1) included terminal acofriose, 2-substituted acofriose, and 3-substituted rhamnose. Their ^{1}H NMR spectra contained signals for two *O*-methyl groups: one from terminal acofriose at δ 3.45 and the other from 2-substituted acofriose at δ 3.52 in the ratio ~1:1.3 and ~1:3 in Fractions 2 and 1, respectively. In addition to the signals for the anomeric protons of Rha$^{\text{II}}$–Rha$^{\text{IV}}$, the ^{1}H NMR spectra of these products contained a signal at δ 5.25, which could be assigned to H-1 of the oxidized 2-substituted Rha$^{\text{I}}$. The ratio of intensities of this signal and that for H-1 of terminal acofriose at δ 5.09 in both oligosaccharides was consistent with the ratio of the signals for the major and minor *O*-methyl groups given above.

These findings suggested that the higher-molecular mass products from Fractions 1 and 2 resulted from incomplete hydrolysis of the (1 → 2) linkage between the oxidized 2-substituted rhamnose residue (Rha$^{\text{I}}$) and the non-oxidized acofriose residue (Rha3Me$^{\text{IV}}$), whereas the linkage of the oxidized Rha$^{\text{I}}$ in non-O-methylated O repeats cleaved smoothly. Since the oligomers included up to four, i.e., all or almost all acofriose residues present in the OPS, the O-methylated O repeats linked to each other. These data and termination of the OPS with acofriose enabled a further suggestion that the O-methylated O repeats form a sequence starting from the non-reducing end of the OPS; they also defined the structure of the ‘biological’ O repeat as shown in Fig. 3.

O-Methylation of the terminal non-reducing sugar residue occurs in some O-antigens having a homopolymer main chain,[17–19] like, for instance, in the OPS of *P. syringae* pv. atrofaciens IMV 7836, which is a linear D-rhamnan having the structure **1**[8] and is terminated with 2-*O*-methyl-α-D-rhamnopyranose (authors’ unpublished data). It is believed that introduction of the *O*-methyl group to the non-reducing end plays a role of a signal for cessation of the O-antigen chain elongation. The OPS of *P. syringae* pv. phaseolicola GSPB 1552 studied in this work is distinguished by 3-O-methylation of not only terminal but also part of 2-substituted rhamnose residues. O-Methylation in the O polysaccharides of *P. syringae* may be considered as another way of diversification of cell surface molecular structures. The biological role of this phenomenon, in particular, in the bacterium-plant specific interaction, remains to be elucidated.

3. Experimental

Growth of bacterium, isolation of LPS and OPS.—*P. syringae* pv. phaseolicola GSPB

1552 was grown on Potato dextrose agar (Difco Laboratories, USA) at 22 °C for 24 h. LPS was isolated as described[7] and degraded by hydrolysis with aq 2% AcOH for 1.5 h at 100 °C. The OPS was isolated by gel-permeation chromatography on a column (70 × 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate pH 4.5 as eluent and monitoring with a Knauer differential refractometer.

Sugar and methylation analysis.—Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), monosaccharides were identified by GLC–MS as the alditol acetates[20] on a Hewlett–Packard 5890 chromatograph (USA) equipped with a DB-5 capillary column and a NERMAG R10-10L mass spectrometer (France) using a temperature gradient of 160 °C (3 min) to 250 °C at 3 °C/min. The absolute configurations were determined by GLC of acetylated glycosides with (−)-2-octanol,[21] using D-acofriose from the lipopolysaccharide of *Campylobacter fetus* serotype B[19] as the authentic sample. Methylation was carried out with CD_3I in Me_2SO in the presence of solid NaOH,[22] partially methylated monosaccharides were released, converted into the alditol acetates, and analyzed by GLC–MS as above.

Smith degradation.—The OPS (30 mg) was oxidized with 0.1 M $NaIO_4$ in the dark for 48 h at 20 °C, after adding an excess of ethylene glycol, reduction with $NaBH_4$ and desalting on a column (80 × 1.6 cm) of TSK HW-40 (S) in water, the product was hydrolyzed with aq 2% AcOH for 2 h at 100 °C, reduced with $NaBH_4$, desalted by treatment with a KU-2 cation-exchange resin (H^+-form) followed by evaporation and repeated adding–evaporation of methanol. Fractionation by GPC on TSK HW-40 (S) in aq 1% AcOH gave oligosaccharide-glycerols **4** and **5**, together with higher-molecular mass products (Fig. 2).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying from D_2O. NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 60 °C for the OPS and 30 °C for the oligosaccharides. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45). Bruker software XWINNMR 2.1 was used to acquire and process the NMR data. A mixing time of 100 ms was used in TOCSY and NOESY experiments.

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