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The structure of the O-specific polysaccharide of *Salmonella arizonae* O62

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ABSTRACT

The O-specific polysaccharide was liberated by mild acid hydrolysis of the lipopolysaccharide (LPS) isolated from *S. arizonae* O62 by phenol–water extraction. The branched hexasaccharide repeating-unit of the O-specific chain of the O62 LPS contained L-rhamnose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galacturonic acid in molar ratios of 4:1:1. On the basis of methylation analysis, ^{1}H and ^{13}C NMR spectroscopy, including 2D shift-correlated (COSY) and 1D NOE spectroscopy, the following structure for the repeating unit of the O-specific polysaccharide was established:

$$\begin{array}{c} \alpha\text{-D-Gal}p\text{ANAc} \\ 1 \\ \downarrow \\ 2 \end{array}$$

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow$$

INTRODUCTION

Salmonella arizonae strains form the subgenus III of the "genus" *Salmonella* and are mainly distributed in reptiles but are also encountered in fowls, mammals as well as in man[1,2], in all of which they may produce severe and even fatal infections. Recently, the structures of some of them, e.g., those of serogroups O21 (ref 3), O45 (ref 4), and O61 (ref 5) have been established and have revealed that, in contrast to salmonellae of subgenus I, often feature negatively charged components, such as uronic acids or *N*-acetylneuraminic acid as part of the repeating unit. Here we report on the structural elucidation of another negatively charged O-specific polysaccharide, namely that of *S. arizonae* O62.

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RESULTS AND DISCUSSION

The lipopolysaccharide (LPS) of *S. arizonae* O62 was isolated from dry bacterial cells by extraction[6] with hot phenol–water. Degradation of the LPS with dilute acetic acid afforded the O-specific polysaccharide (PS), which was isolated by gel-permeation chromatography on Sephadex G-50.

Acid hydrolysis of the PS revealed the presence of 2-amino-2-deoxy-D-glucose and L-rhamnose, identified by GLC as the alditol acetates and as peracetylated (*S*)-2-octyl (for rhamnose) or (*S*)-2-butyl (for glucosamine) glycosides. The ^{13}C NMR spectrum of the PS displayed the signals for six anomeric carbons at 96–104 ppm, two carbons bearing nitrogen at 50.8 and 57.3 ppm, one hydroxymethyl group (C-6 of GlcN) at 62.8 ppm, four methyl groups of the rhamnose residues at 17.9–18.1 ppm, one carboxyl group at 174.9 ppm, 22 other sugar carbons in the region 68–83 ppm, and two *N*-acetyl groups (Me at 23.3 and 23.8 ppm, CO at 175.4 and 175.8 ppm). These data showed that the PS had a hexasaccharide repeating-unit containing four residues of rhamnose, one GlcNAc residue, and one residue of a *N*-acetylated aminouronic acid, which was identified as 2-acetamido-2-deoxygalacturonic acid (see later).

In the low-field region of the ^1H NMR spectrum of the PS (4.6–5.1 ppm), signals for six anomeric protons were present; these belonged to the four rhamnose residues (broadened singlets at 4.80, 4.90, 4.95, and 5.10 ppm), plus one α-linked (δ 5.06 ppm, $J_{1,2}$ 3.5 Hz) and one β-linked sugar residue (δ 4.63 ppm, $J_{1,2}$ 8 Hz). Additionally, a singlet at 4.75 ppm was present which most likely arose from H-5 of the GalAN residue. The spectrum contained also the signals for the four Me groups of the rhamnose residues (doublets at 1.19, 1.22, 1.22, and 1.29 ppm, $J_{5,6}$ 6.5 Hz), several other sugar protons in the region 3.2–4.3 ppm, and two NAc groups (Me at 1.98 and 2.00 ppm).

The ^1H NMR spectrum of the PS was completely assigned (Table I) by using 2D shift-correlated spectroscopy (COSY), relayed COSY ([COSYRCT (Fig. 1) and COSYRCT2]), and 1D NOE spectroscopy (Fig. 2, Table II) with selective pre-irradiation of each anomeric proton. The absence of the H-6 protons and the coupling constants $J_{1,2}$ 3.5, $J_{2,3}$ 11, $J_{3,4}$ 3, and $J_{4,5} < 1$ Hz indicated that the sixth component of the PS is a derivative of galacturonic acid and is α-linked. The GlcNAc residue was characterised by the coupling constant $J_{1,2}$ 8 Hz as β-linked. The absence of NOEs on H-3,5 of the four rhamnose residues after pre-irradiation of H-1 of these sugar units indicated that they are all α-linked.

With the ^1H NMR spectrum assigned, the ^{13}C NMR spectrum of the PS could be interpreted (Table III) with the aid of heteronuclear ^{13}C, ^1H shift-correlated spectroscopy (XHCORRD, Fig. 3). The chemical shift 50.8 ppm of the signal for C-2 of the uronic acid residue proved it to be 2-acetamido-2-deoxygalacturonic acid.

The low-field positions of the signals for C-3 of GlcNAc (unit **A**) at 82.9 ppm, C-2 and C-3 of one of the rhamnose residues (unit **B**) at 76.0 and 79.7 ppm, C-2 of

TABLE I

^{1}H NMR data (δ in ppm, $^3J_{H,H}$ in Hz)

H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{6a,6b}$	$J_{5,6b}$
→ 3)-β-D-Glc*p*NAc-(1 → (unit **A**)						
4.63	3.66	3.53	3.27	3.35	3.59	3.85
8	9.5	9.5	9	7	12	2.5
H-1	H-2	H-3	H-4	H-5	H-6	
$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$		
→ 3)-α-L-Rha*p*-(1 → (unit **B**)						
	2 ↑					
4.90	4.29	3.85	3.50	3.65	1.22	
<2	3.5	10	10	6.5		
→ 2)-α-L-Rha*p*-(1 → (unit **C**)						
5.10	4.00	3.88	3.38	3.76	1.29	
<2	3	10	10	6.5		
→ 3)-α-L-Rha*p*-(1 → (unit **D**)						
4.80	4.05	3.76	3.50	3.65	1.22	
<2	3	10	10	6.5		
→ 2)-α-L-Rha*p*-(1 → (unit **E**)						
4.95	3.73	3.82	3.40	3.94	1.19	
<2	3	10	10	6.5		
α-D-Gal*p*NAc-(1 → (unit **F**)						
5.06	4.10	3.94	4.29	4.75		
3.5	11	3	<1			

two other rhamnose residues (units **C** and **E**) 79.0 and 81.0, respectively, and C-3 of the fourth rhamnose residue (unit **D**) at 79.0 ppm, as compared with the corresponding positions in the spectra of the unsubstituted monosaccharides[7], showed that the PS was branched with the 2,3-disubstituted unit **B** as the branching point. Furthermore, units **A** and **D** are 3-substituted and units **C** and **E** are 2-substituted. The lateral location of GalNAc could then be deduced from these data and was also confirmed by the close chemical-shift values of this PS-associated residue and those of the corresponding unsubstituted sugar[8].

This substitution pattern was additionally confirmed by methylation analysis[9] of the PS, which produced 3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 4-*O*-methylrhamnose, and 2-deoxy-4,6-di-*O*-methyl-2-*N*-(methylamino)glucose, identified by GLC as alditol acetates. As in the sugar analysis, the corresponding derivative of 2-amino-2-deoxygalacturonic acid was not detected.

The interresidue NOEs observed in the PS (Fig. 2, Table II) were in accordance with the modes of substitution of the monosaccharide residues. In each experiment, together with the strong NOE on the transglycosidic proton, weaker NOEs were observed on the neighbouring protons due to the spin diffusion that is typical

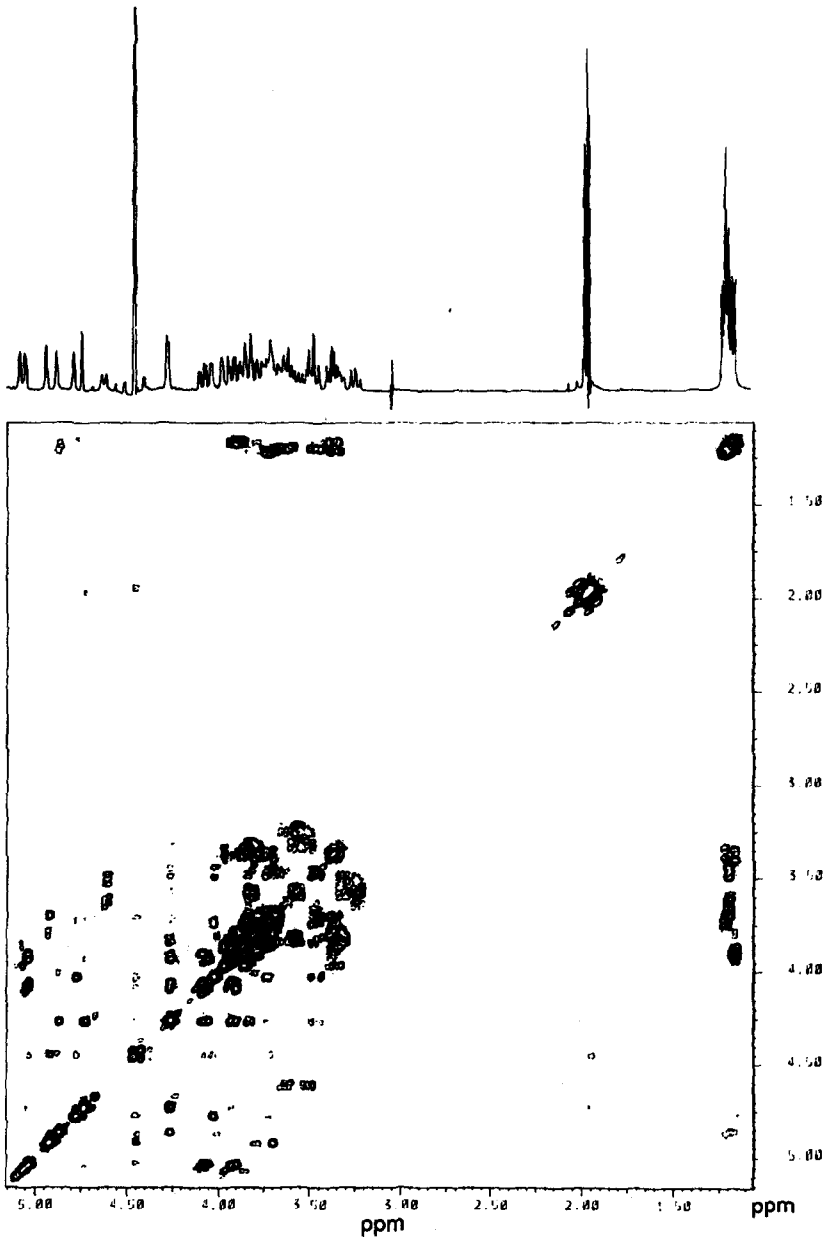


Fig. 1. Two-dimensional one-step relayed COSY spectrum (COSYRCT). One dimensional ^{1}H NMR spectrum is displayed along the horizontal (F1) axis.

for polymers. In the (1 → 2)-linked disaccharide fragments B-C, D-E, and F-B the spatial proximity of the anomeric protons of the two sugar constituents was reflected in the appearance of NOE contacts H-1–H-1′. The NOE data allowed

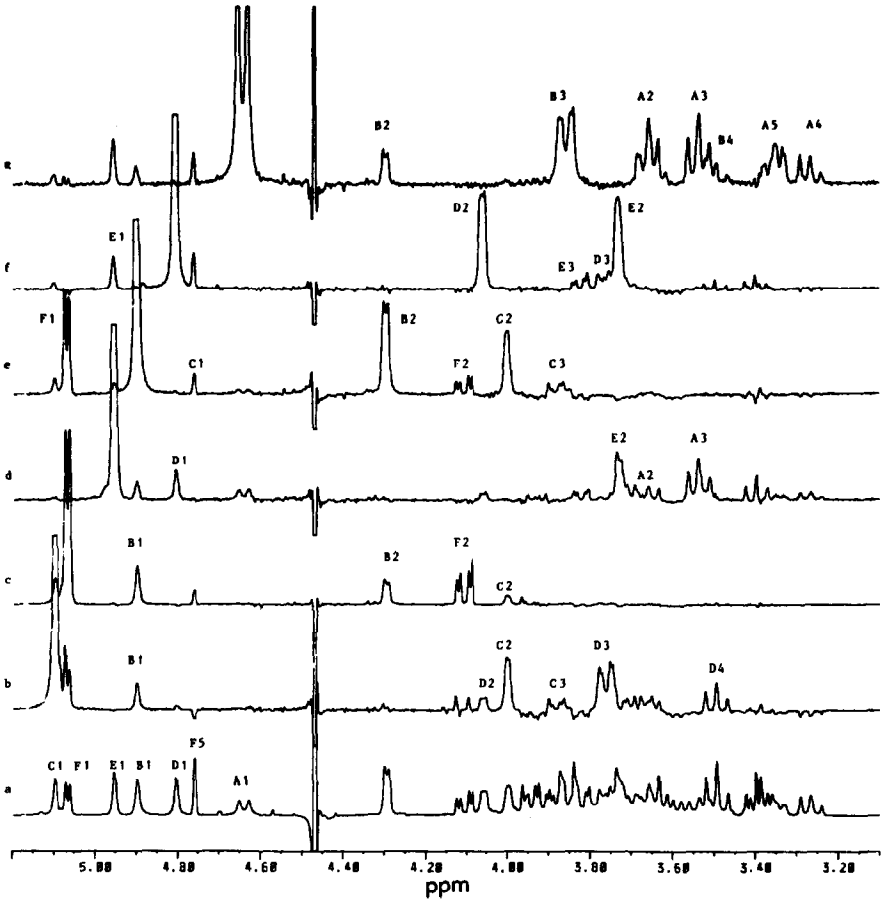
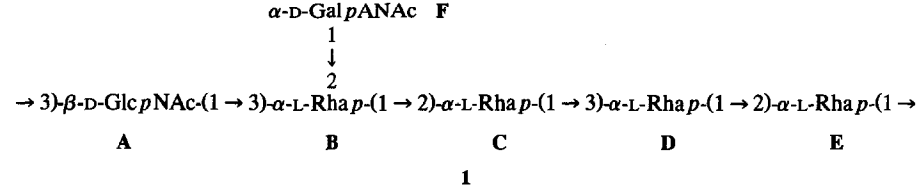


Fig. 2. Part of the 360-MHz ^{1}H-NMR spectra of the O-specific polysaccharide. (a) One-dimensional resolution-enhanced ^{1}H-NMR spectrum. (b–g) One-dimensional NOE spectra with pre-irradiation of H-1 of (b) Rha **C**, (c) GalANAc **F**, (d) Rha **E**, (e) Rha **B**, (f) Rha **D**, and (g) GlcNAc **A**.

also the unambiguous determination of the sequence of the sugar units as shown in structure **1**.



1

Knowing its site of attachment and the configuration of the glycosidic linkage, the absolute configuration of GalANAc was determined by using the known regularities observed in the glycosylation effects in ^{13}C NMR spectra[10]. The chemical shift 96.2 ppm for C-1 of this sugar unit corresponded to a relatively

TABLE II

NOE data with pre-irradiation of anomeric protons

Pre-irradiated unit	NOE observed on [a]																			
	A				B				C			D				E			F	
	H-2	H-3	H-4	H-5	H-1	H-2	H-3	H-4	H-1	H-2	H-3	H-1	H-2	H-3	H-4	H-1	H-2	H-3	H-1	H-2
A	++	++	+	++		+	++	+												
B						++			+	++	+								++	+
C					+					++	+		+	++	+					
D													++	+		+	++	+		
E	+	++										+					++			
F					++	++				+									++	

[a] ++, Strong NOE; +, medium or weak NOE.

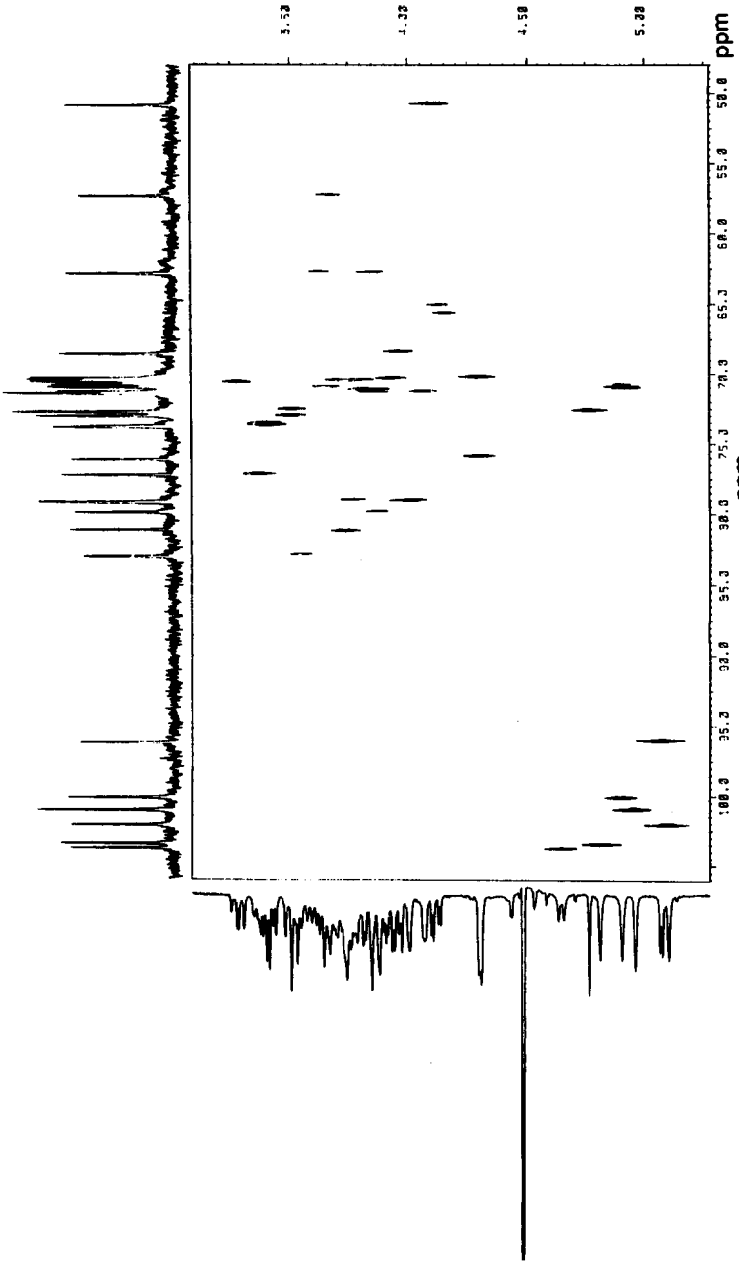


Fig. 3. Two-dimensional $^{13}C/^{1}H$ shift-correlated spectrum (HECTOR). The corresponding parts of the one-dimensional ^{1}H and ^{13}C spectra are displayed along vertical (F1) and horizontal (F2) axes, respectively.

TABLE III

^{13}C NMR chemical shifts (δ in ppm)

Sugar unit	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Glc*p*NAc-(1→ **(A)**	103.8	57.3	82.9	70.6	77.1	62.8
→3)-α-L-Rha*p*-(1→ **(B)**	100.1	76.0	79.7	72.9 [a]	70.8	17.9 [b]
2 ↑						
→2)-α-L-Rha*p*-(1→ **(C)**	102.1	79.0	71.3	73.7	70.4	18.0 [b]
→3)-α-L-Rha*p*-(1→ **(D)**	103.4	71.3	79.0	72.6 [a]	70.4	18.0 [b]
→2)-α-L-Rha*p*-(1→ **(E)**	101.0	81.0	71.2	73.7	70.3	18.1 [b]
α-D-Gal*p*ANAc-(1→ **(F)**	96.2	50.8	68.5	70.2	72.6	174.9

[a,b] Assignments may be interchanged.

small (about +4 ppm) effect being characteristic for different absolute configurations of units **F** and **B** (a much larger effect of 7–8 ppm would be expected in case of the same absolute configuration of these two units[10]). Since unit **B** has L configuration, GalANAc must have the D configuration.

The structural elucidation of the O-antigen of *S. arizonae* O62 containing 2-acetamido-2-deoxy-D-galacturonic acid showed again that acidic O-specific polysaccharide chains are rather common for *S. arizonae*, unlike the findings for other *Salmonella* species studied so far[11]. Thus, D-glucuronic acid, *N*-acetylneuraminic acid, and 7-acetamido-3,5,7,9-tetradeoxy-5-[(*R*)-3-hydroxybutanamido]-D-*glycero*-L-*galacto*-nonulosonic acid were found as acidic components of the O-antigens of *S. arizonae* O45 (ref 4), O21 (ref 3), and O61 (ref 5), respectively.

This polysaccharide has a significant structural similarity with those of *Shigella flexneri* O-antigens[12,13], in possessing a tetrasaccharide sequence (units **A–D**) which differs from the main chain of *Shigella flexneri* O-antigens only by the attachment of glucosamine to C-3 of L-rhamnose instead of C-2 as in the case of *Shigella flexneri*.

EXPERIMENTAL

General methods.—NMR spectra were recorded with a Bruker AM-360 spectrometer for solutions in D_2O at 50°C using acetone as an internal standard (δ_H 2.23 ppm, δ_C 31.45 ppm) by using standard Bruker software for carrying out 1D NOE and 2D shift-correlated spectroscopy. GLC was carried out on a Varian model 3700 gas chromatograph with a flame-ionisation detector with a fused-silica capillary column (25 m × 0.32 mm i.d.) coated with SE 54 stationary phase and using a temperature gradient from 140 to 250°C at 3°C/min; carrier gas H_2, 0.1 mPa. GLC–MS was performed with a Hewlett–Packard model 5985 instrument at 70 eV under the same chromatographic conditions. Gel-permeation chromatogra-

phy was performed on a column (45 × 2.4 cm) of Sephadex G-50 in pyridine–acetate buffer, pH 4.5, with monitoring by a Knauer differential refractometer.

Bacterial strain, growth, and isolation of lipopolysaccharide and O-specific polysaccharide.—S. arizonae O62:Z_4,Z_{32}: The organisms were cultivated under aerobic conditions at 37°C in a fermentor in a complex medium[14] at pH 7.2. After reaching the stationary phase, the bacteria were killed by adding phenol to an end concentration of 1% and were then sedimented by centrifugation. The bacterial sediment was subsequently washed with acetone and diethyl ether, and subjected to the hot phenol–water procedure[6] at 65–68°C. The aqueous phase was purified by repeated ultracentrifugations (105 000 × *g*, 4 h, 3 times)[6].

The lipopolysaccharide (180 mg) was heated with 2% AcOH (10 mL) for 3 h at 100°C, the lipid precipitate was removed by centrifugation, the water-soluble fraction was separated on Sephadex G-50 to give the O-specific polysaccharide (40 mg).

Sugar analysis.—The PS (1 mg) was hydrolysed with 2 M CF_3CO_2H (120°C, 3 h), the hydrolysate was evaporated, the sugars were converted conventionally into alditol acetates, and were analysed by GLC.

Two portions of the PS (2 mg each) were treated with (*S*)-2-butanol (0.2 mL) or (*S*)-2-octanol (0.2 mL) and AcCl (0.025 mL) in sealed ampoules (3 h, 100°C), evaporated, acetylated with Ac_2O in pyridine (0.3 mL of each, 16 h, 20°C), evaporated, and analysed by GLC in comparison with the authentic samples of the corresponding sugar glycosides[15].

Methylation analysis.—An excess of dry powdered NaOH was added under stirring to the solution of the PS (5 mg) in dry Me_2SO (0.4 mL), and then MeI (0.5 mL) was added and the mixture was stirred at room temperature for 30 min. Methyl iodide was removed by a stream of N_2, and then water (2 mL) was added and the methylated PS was absorbed on a Sep-Pak (Waters) cartridge, which was washed first with water in order to remove Me_2SO and salts, then the PS was eluted with MeOH, hydrolysed with 2 M CF_3CO_2H (1.5 h, 100°C), reduced with $NaBD_4$, acetylated, and analysed by GLC–MS.

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