Carbohydrate Research, 241 (1993) 309–316
Elsevier Science Publishers B.V., Amsterdam

Note

Structure of the O-specific polysaccharide of *Xanthomonas campestris* NCPPB 45 lipopolysaccharide

Alexander V. Bukharov [a], Igor M. Skvortsov [a], Vladimir V. Ignatov [a], Alexander S. Shashkov [b], Yuriy A. Knirel [b] and Janusz Dabrowski [c]

[a] *Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov (Russian Federation)*
[b] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, Moscow B-334 (Russian Federation)*
[c] *Max Planck Institute for Medical Research, Jahnstrasse 29, Heidelberg 1 (Germany)*

(Received February 7th, 1992; accepted June 8th, 1992)

X. campestris, the type species of *Xanthomonas*, is an aerobic Gram-negative bacterium belonging to the Pseudomonoadaceae; many strains of this species are phytopathogenic. Data on the composition and structure of the surface polysaccharides of *X. campestris* may serve as a chemotaxonomic criterion and allow one to determine its relationship to other bacteria, particularly to *Pseudomonas*. An exopolysaccharide of *X. campestris*, xanthan, has been actively studied[1], whereas data on outer membrane lipopolysaccharides (LPSs) are limited[2,3]. The uncommon sugars D-rhamnose and 3-acetamido-3,6-dideoxy-D-galactose have been identified as components of the strain B-1459 LPS[3].

We report now the structure of the O-specific polysaccharide of LPS from *X. campestris* strain NCPPB 45.

The LPS was extracted by the phenol–water procedure[4] and cleaved with 1% CH_3COOH to give an acidic O-specific polysaccharide (PS) isolated from the water-soluble portion by gel-permeation chromatography on Sephadex G-50 and purified by anion-exchange chromatography on DEAE-Toyopearl 650M. The PS had $[\alpha]_D + 22°$ (*c* 0.4).

Acid hydrolysis of the PS revealed the presence of rhamnose, galactose, and galacturonic acid, which were identified by TLC.

The ^{13}C NMR spectrum of the PS was typical of a regular polymer (Fig. 1). In the region of the anomeric carbon resonances, there were six signals, with nearly equal intensities, pointing to a hexasaccharide repeating unit. The spectrum

Correspondence to: Dr. A.V. Bukharov, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, Russian Federation.

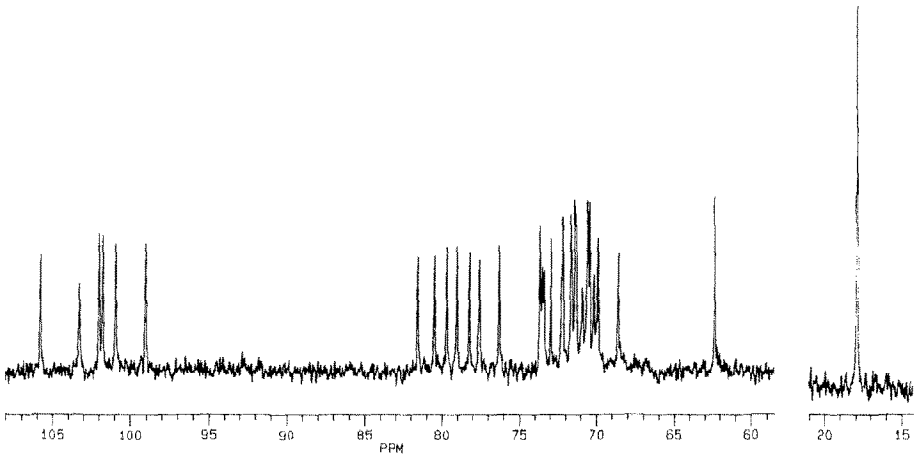

Fig. 1. 90.5-MHz ^{13}C NMR spectrum of the O-specific polysaccharide.

contained also the signals for one hydroxymethyl group (C-6 of Gal) at 62.4 ppm, one carboxyl group (C-6 of GalA) at 174.3 ppm, and four methyl groups (C-6 of Rha) at 17.9 ppm, as well as 24 other signals in the region 68–82 ppm. Therefore, the repeating unit of the PS consisted of four Rha residues, one residue of Gal, and one residue of GalA.

The coupling constants $J_{C\text{-}1,H\text{-}1} < 172$ Hz, determined from the gated-decoupling ^{13}C NMR spectrum of the PS, indicated that all of the sugar residues were pyranoid[5]. The values of 168–172 Hz for five of the anomeric carbons (δ 99–103.5 ppm) showed that the corresponding sugars were α-linked, whereas a smaller value of 161 Hz for the signal at 105.8 ppm proved the sixth monosaccharide to be β-linked[6].

The ^1H NMR spectrum of the PS (Fig. 2) contained eight one-proton signals at low field (4.6–5.35 ppm). Two of them, which overlapped within the 4.64–4.65 ppm range, were doublets with $J_{1,2} \sim 7$ and ~ 3 Hz, the former belonging to a β-linked residue of Gal or GalA. Another doublet with $J_{1,2} \sim 3$ Hz appeared at 5.104 ppm, and the remaining five signals were broadened singlets. The position of one of the singlets (δ 4.816 ppm at 70°C) was strongly temperature-dependent, which is characteristic of H-5 of uronic acids. This proton was coupled to the proton resonating at 4.652 ppm (d, $J_{3,4} \sim 3$ Hz), which was assigned to H-4 of the GalA residue (see below), and, hence, was H-5 of this residue. Four other singlets in the low-field region belonged, therefore, to H-1 of the residues of Rha, and the doublet at 5.104 ppm to an α-linked residue of GalA or Gal.

The ^1H NMR spectrum of the PS was completely assigned (Table I) with the help of sequential, selective spin-decoupling experiments[7], 1D homonuclear Hartmann–Hahn spectroscopy (HOHAHA) performed in the difference mode[8] (Fig. 3), as well as 2D homonuclear shift-correlated spectroscopy (COSY) and relayed coherence transfer spectroscopy (COSYRCT). As a result, it was evident that the

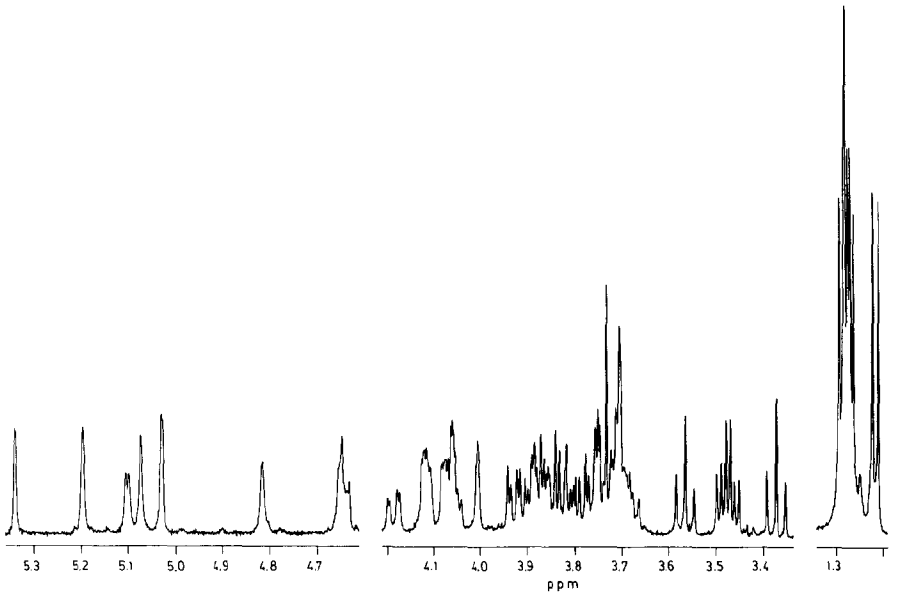

Fig. 2. 500-MHz ^{1}H NMR spectrum of the O-specific polysaccharide.

β-linked residue was Gal (unit **A**) and, hence, the GalA residue (unit **B**) as well as all four Rha residues (units **C**–**F**) were α-linked.

In the 2D NOE spectrum (ROESY) of the PS (Fig. 4), the correlation peaks corresponding to inter-residue coupling between the following protons were observed: H-1 **F**–H-4 **B**; H-1 **A**–H-3 **B**; H-1 **B**–H-2 **C**; H-1 **C**–H-2 **D**; H-1 **D**–H-2 **E**, H-3 **E**, and H-5 **C**; H-1 **E**–H-2 **A** or H-3 **A**. From these data, the sequence of the sugar residues and the substitution patterns of units **B**–**E** became clear (see the structure shown below).

Although two NOEs (on H-2 and H-3) were observed for unit **E**, it was evident that unit **E** was 3-substituted since, in the case of a 1,2-linkage, no NOE on H-3 would be observed. At the same time, an NOE on H-2 is characteristic of an α-(1 → 3) linkage between two Rha*p* residues of the same absolute configuration[9], because spatial proximity of H-1 of the glycosylating residue and H-2 of the glycosylated residue is possible in this case. Similarly, the cross-peak correlating H-1 **D** and H-5 **C** may be explained by the spatial proximity of these two protons in the predominant conformations[10] characteristic of α-(1 → 2)-linked α-Rha*p* residues with the same absolute configuration.

The complete assignment of the ^{13}C NMR spectrum of the PS (Table II) was performed with the aid of 2D inversely correlated heteronuclear ^{1}H–^{13}C spectroscopy. Low-field positions of the signals for carbons carrying glycosyl substituents, relative to those corresponding to the free monosaccharides, confirmed the sites of glycosylation of units **B**–**E** and proved unit **A** to be 3-substituted (for

TABLE I

Data of the ^{1}H NMR spectrum of the O-specific polysaccharide (δ in ppm, J in Hz)

	H-1	H-2	H-3	H-4	H-5	H-6
→ 3)-β-D-Gal*p*-(1 → (unit **A**)						
δ	4.640	3.718	3.710	4.009	~ 3.69 [a]	3.837 [b] 3.742 [c]
J	$J_{1,2}$ 7.0	$J_{2,3}$ 9.5	$J_{3,4}$ ~ 3	$J_{4,5}$ < 2	$J_{5,6a}$ 7.0	$J_{5,6b}$ 4.8
→ 3)-α-D-Gal*p*A-(1 → (unit **B**) 4 ↑						
δ	5.104	4.057	4.190	4.652	4.816	
J	$J_{1,2}$ 3.5	$J_{2,3}$ 10.1	$J_{3,4}$ 2.2	$J_{4,5}$ < 2		
→ 2)-α-L-Rha*p*-(1 → (unit **C**)						
δ	5.077	4.112	3.879	3.480	3.742	1.269
J	$J_{1,2}$ < 2	$J_{2,3}$ 3.1	$J_{3,4}$ 9.6	$J_{4,5}$ 9.6	$J_{5,6}$ 6.1	
→ 2)-α-L-Rha*p*-(1 → (unit **D**)						
δ	5.199	4.079	3.932	3.472	3.807	1.291
J	$J_{1,2}$ < 2	$J_{2,3}$ 3.0	$J_{3,4}$ 9.6	$J_{4,5}$ 9.6	$J_{5,6}$ 6.2	
→ 3)-α-L-Rha*p*-(1 → (unit **E**)						
δ	5.032	4.128	3.893	3.569	3.862	1.280
J	$J_{1,2}$ < 2	$J_{2,3}$ 3.1	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6}$ 6.1	
α-L-Rha*p*(1 → (unit **F**)						
δ	5.341	4.064	3.768	3.375	3.681	1.220
J	$J_{1,2}$ < 2	$J_{2,3}$ 3.1	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6}$ 6.1	

[a] Determined from the ^{13}C–^{1}H heteronuclear shift-correlated spectrum. [b] H-6a, $J_{6a,6b}$ 11.2 Hz. [c] H-6b.

example, the C-3 chemical shifts were 81.6 ppm for unit **A** in the PS and 74.1 ppm for β-Gal*p*[11]).

Analysis of the effects of glycosylation[12,13] in the ^{13}C NMR spectrum confirmed that units **C**, **D**, and **E** had the same absolute configuration and indicated that unit **B** had the same absolute configuration (most probably D) as unit **A**, which was opposite to the configuration of units **C**–**E**. Consequently, units **C**–**E** were identi-

TABLE II

Chemical shifts in the ^{13}C NMR spectrum of the O-specific polysaccharide (δ in ppm)

Sugar unit		C-1	C-2	C-3	C-4	C-5	C-6
→ 3)-β-D-Gal*p*-(1 →	(**A**)	105.8	72.2	81.6	70.0	76.4	62.4
→ 3)-α-D-Gal*p*A-(1 → 4 ↑	(**B**)	99.1	68.6	80.5	78.4	72.2	174.3
→ 2)-α-L-Rha*p*-(1 →	(**C**)	101.0	77.7	70.9	73.4	70.6	17.9
→ 2)-α-L-Rha*p*-(1 →	(**D**)	102.1	79.8	71.4	73.5	70.5	17.9
→ 3)-α-L-Rha*p*-(1 →	(**E**)	103.4	71.4	79.1	73.0	70.5	17.9
α-L-Rha*p*-(1 →	(**F**)	101.9	71.7	71.7	73.4	70.3	17.9

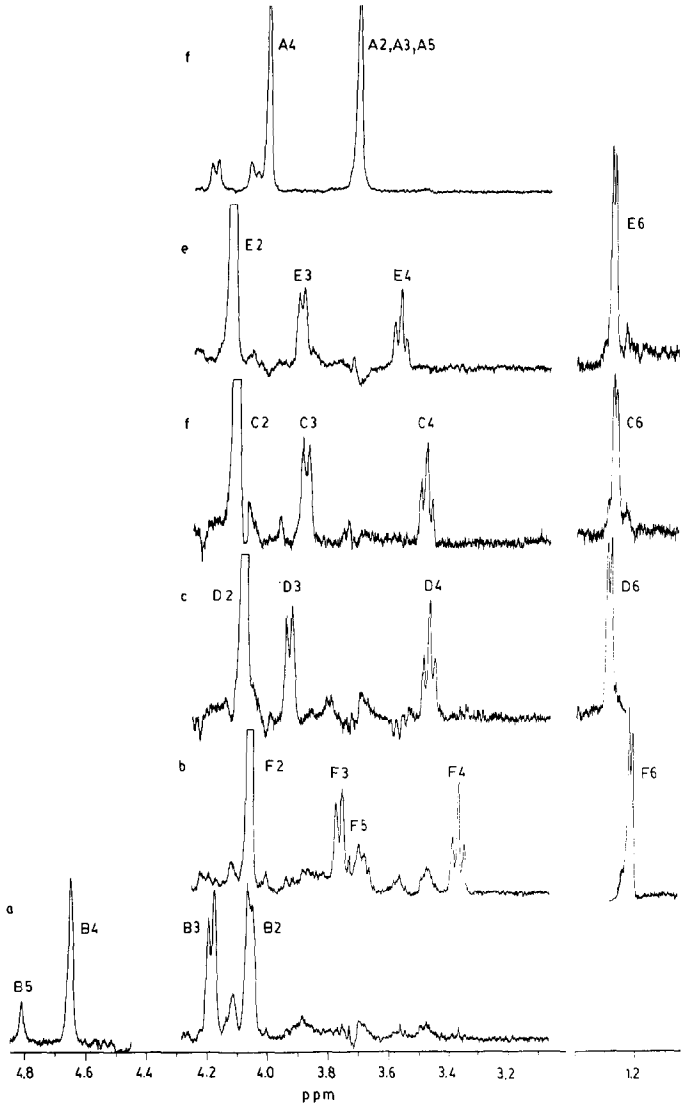

Fig. 3. One-dimensional 500-MHz HOHAHA spectra of six sugar residues of the O-specific polysaccharide, obtained with selective excitation of the anomeric protons of (a) GalA **B**, (b) Rha **F**, (c) Rha **D**, (d) Rha **C**, (e) Rha **E**, and (f) Gal **A**. Arabic numbers refer to the protons in the sugar residues denoted by letters.

fied as residues of L-rhamnose. The configuration of the fourth Rha residue could not be deduced by such an analysis[12], but the closeness of the value $[\alpha]_D$ +7.2° for rhamnose isolated from the hydrolysate of the PS by preparative PC with the reported[14] value +8.9° showed that it too was L-rhamnose. The D configuration of galactose and galacturonic acid was also confirmed by the values of specific optical rotation of the isolated monosaccharides.

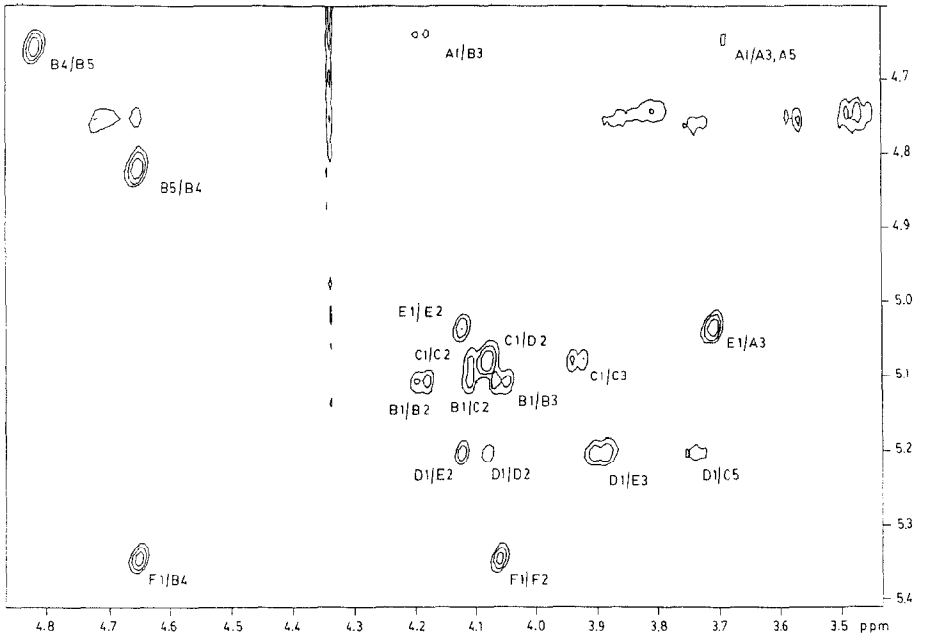

Fig. 4. Part of a 500-MHz ROESY spectrum of the O-specific polysaccharide. Arabic numbers refer to the protons in the sugar residues denoted by letters.

Therefore, the O-specific polysaccharide of *X. campestris* strain NCPPB 45 has the following structure:

$$\begin{array}{l} \qquad\qquad\qquad\quad \alpha\text{-L-Rha}p \quad \mathbf{F} \\ \qquad\qquad\qquad\quad\ \ 1 \\ \qquad\qquad\qquad\quad\ \ \downarrow \\ \qquad\qquad\qquad\quad\ \ 4 \\ \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal}p\mathrm{A}\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow \\ \mathbf{A} \qquad\qquad \mathbf{B} \qquad\qquad \mathbf{C} \qquad\qquad \mathbf{D} \qquad\qquad \mathbf{E} \\ \qquad\qquad\qquad\qquad\quad\ \ \mathbf{1} \end{array}$$

The elucidation of this rather complex structure of a hexasaccharide repeating unit shows the efficiency of the combination of 1D and 2D NMR methods, complemented by the analysis of the effects of glycosylation on ^{13}C chemical shifts.

EXPERIMENTAL

General methods.—Solutions were freeze-dried or evaporated in vacuo at < 40°C.Optical rotations were measured with a Jasco DIP 360 polarimeter at 30°C for solutions in water. The ^{13}C NMR spectrum was recorded with a Bruker AM-360 instrument in D_2O at 70°C. ^1H NMR spectra were recorded with a Bruker AM-500 instrument in D_2O at 70°C. Acetone was used as the internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). The 1D TOCSY-HOHAHA, 2D COSY,

and 2D ROESY spectra were obtained as previously described[15]. The 2D proton-detected $^{1}H-^{13}C$ heteronuclear multiple-quantum correlation (HMQC) was performed as described[16].

TLC on DC-Alufolien Cellulose (Merck) plates was performed with pyridine–EtOAc–acetic acid–water (5:5:3:1); sugars were detected with an anisidine phthalate reagent. Preparative PC was carried out on FN-11 paper (Filtrak, FRG) with the same system and detection with alkaline silver nitrate. Gel-permeation chromatography used a column (55 × 1.8 cm) of Sepharose CL-4B or a column (65 × 1.8 cm) of Sephadex G-50 in pyridine–acetic acid buffer (pH 4.3). Anion-exchange chromatography was performed on a column (20 × 2.3 cm) of DEAE-Toyopearl 650M (Toyo Soda) with a gradient of NaCl from 0 to 1 M in 0.01 M Tris–HCl buffer (pH 7.2). Elution profiles were recorded with a Contiflo sugar analyzer (Hungary) or refractometer LKB 2142.

Growth of bacterium and isolation of LPS and O-specific polysaccharide.—The culture of *X. campestris* strain NCPPB 45 was obtained from the All-Union Collection of Microorganisms (Moscow). The culture was continuously grown in a 10-L ANKUM-2M fermenter for 1 week at 30°C in a medium containing peptone (10 g), NaCl (5 g), and D-glucose (10 g) in 1 L of water, to give 60 L of culture fluid with cells. The cells were separated by centrifugation and dried with acetone; the yield was 89 g. The cells (50 g) were extracted with phenol–water[4] and the LPS was purified by gel-permeation chromatography on Sepharose CL-4B; the yield of LPS was 1.2% of the dry weight of the cells.

The LPS (242 mg) was hydrolysed with aq 1% acetic acid (40 mL, 100°C, 3 h); a lipid precipitate was removed by centrifugation at 105 000*g*. The supernatant solution was subjected to gel-permeation chromatography on Sephadex G-50, to give the PS (122 mg) which was further purified by anion-exchange chromatography on DEAE-Toyopearl 650M and desalted on Sephadex G-50; the yield of the PS was 23.5%.

Acid hydrolysis.—The PS (50 mg) was hydrolysed with 4 M CF_3COOH for 4 h at 100°C. The hydrolysate was concentrated and a portion analysed by TLC. The remainder was separated by preparative PC to give: L-rhamnose, $[\alpha]_D$ + 7.2° (*c* 1.2), lit.[14] +8.9° (H_2O); D-galactose, $[\alpha]_D$ +42.6° (*c* 0.5), lit.[14] +80.2° (H_2O); and D-galacturonic acid, $[\alpha]_D$ +15.8° (*c* 0.3), lit.[14] +50.9° (H_2O).

ACKNOWLEDGMENT

J.D. was supported by the Fritz Thyssen Stiftung.

REFERENCES

1 J.F. Kennedy and I.J. Bradshaw, *Mol. Appl. Tradit. Biotechnol.*, (1984) 320–371.
2 W.A. Volk, *J. Bacteriol.*, 91 (1966) 39–42.
3 J. Hickman and G. Ashwell, *J. Biol. Chem.*, 241 (1966) 1424–1428.

4 O. Westphal and K. Jann, *Methods Carbohydr. Chem.,* 5 (1965) 83–89.
5 N. Cyr and A.S. Perlin, *Can. J. Chem.,* 57 (1979) 2504–2511.
6 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2,* (1974) 293–297.
7 N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, N.K. Kochetkov, and G.B. Pier, *J. Biol. Chem.,* 263 (1988) 11291–11295.
8 A. Bax and D.G. Davis, *J. Magn. Reson.,* 65 (1985) 355–360.
9 G.M. Lipkind, A.S. Shashkov, S.S. Mamyan, and N.K. Kochetkov, *Carbohydr. Res.,* 181 (1989) 1–12.
10 S.S. Mamyan, G.M. Lipkind, A.S. Shashkov, N.E. Bairamova, A.V. Nikolaev, and N.K. Kochetkov, *Bioorg. Khim.,* 14 (1988) 205–215.
11 G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, and N.K. Kochetkov, *Carbohydr. Res.,* 175 (1988) 59–75.
12 A.S. Shashkov, G.M. Lipkind, Y.A. Knirel, and N.K. Kochetkov, *Magn. Reson. Chem.,* 26 (1988) 735–747.
13 N.K. Kochetkov, A.S. Shashkov, G.M. Lipkind, and Y.A. Knirel, *Sov. Sci. Rev., Sect. B,* 13, Part 2 (1989) 1–73.
14 J. Stanek, M. Černy, J. Kocourek, and J. Pácák, *The Monosaccharides*, Publishing House of the Czechoslovak Academy of Sciences, Prague, 1963, pp 89, 434, and 704.
15 A.S. Shashkov, E.V. Vinogradov, Y.A. Knirel, N.E. Nifant'ev, N.K. Kochetkov, J. Dabrowski, E.V. Kholodkova, and E.S. Stanislawski, *Carbohydr. Res.,* 241 (1993) 177–188.
16 A. Bax, R.H. Griffey, and B.L. Howkins, *J. Magn. Reson.,* 55 (1983) 301–315.