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Carbohydrate Research 264 (1994) 73–81

CARBOHYDRATE RESEARCH

Structure of the putative O10 antigen from *Acinetobacter baumannii*

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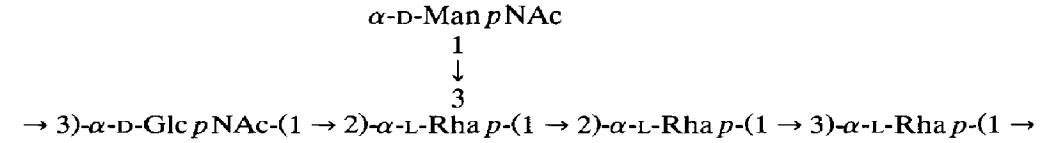
Received 14 March 1994; accepted 26 April 1994

Abstract

A polysaccharide containing L-rhamnose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-mannose was obtained from an aqueous phenol extract of isolated cell walls from the reference strain for *Acinetobacter baumannii* serogroup O10. By means of NMR studies and chemical degradations, the repeating unit of the polymer (the putative O10 antigen) was identified as a branched pentasaccharide of the structure shown.



Keywords: Acinetobacter baumannii; Lipopolysaccharide; O Antigen

1. Introduction

Acinetobacter baumannii is a Gram-negative bacterium associated with nosocomial infections [1,2]. To assist in epidemiological monitoring, a scheme for typing clinical isolates by their heat-stable antigens [assumed to correspond to lipopolysaccharides (LPSs)] has been devised [3,4]. The existence of at least 27 distinct serogroups suggests that differentiation of the strains is based on the presence and structural variability of O-specific side chains in S-type LPSs, even though early studies of *Acinetobacter* species indicated that they produced R-type

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SSDI 0008-6215(94)00179-J

LPSs [5]. Further evidence that some strains may elaborate a polymeric O antigen is the detection of a typical ladder pattern on fractionation of the LPS by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) [6–9]. The isolation of a regular polymer from an 'LPS' extract of a clinical isolate of *A. baumannii* is consistent with this result, although the molecular origin of the polymer was not proved [10]. Encouraged by this preliminary finding, we are undertaking a systematic study of the LPSs produced by the 27 reference strains [3,4]. The first 6 LPSs examined all contain polymeric fractions. Here we report evidence for the structure of the putative O10 antigen.

2. Results and discussion

Growth of the reference strain for *A. baumannii* serogroup O10 in an aerated fermenter gave problems: the culture frothed excessively, and most of the cells were removed from the medium by flotation and collected in the overflow aspirator (yield, 140 g wet cells from a 20-L batch). This material was used for the preparation and analysis of LPS, via mechanical disintegration of the cells and extraction of the defatted cell walls with hot aqueous phenol. Because of our inability to control or specify the growth parameters, and consequent doubts about the physiological state of the cells and the validity of the product, LPS was also isolated from whole cells grown in small-scale shake-flask culture. The ^{1}H NMR spectra of the polymeric fractions from both LPS preparations were identical, which allowed us to proceed with structural studies using the more plentiful product from the fermenter-grown cells.

The yield of LPS from defatted cell walls (2.0 g) was 560 mg. SDS-PAGE analysis of the product showed a clear ladder pattern, indicative of S-type LPS. After mild acid hydrolysis of the LPS, followed by chromatography on Sephadex G-50, the polymeric fraction was isolated in 18% yield. The monosaccharide components of the polymer were identified as L-rhamnose, 2-amino-2-deoxy-D-glucose, and 2-amino-2-deoxy-D-mannose. A regular structure based on a pentasaccharide repeating-unit of Rha (3), GlcNAc (1), and ManNAc (1) residues was apparent from the NMR spectra. The ^{1}H NMR spectrum contained signals for anomeric protons at δ 5.17, 5.05, 5.00, and 4.94 (each unresolved) and one at δ 5.03 ($J_{1,2} \sim 4$ Hz), attributable to α-Glc*p*NAc, as well as methyl singlets at δ 2.07 and 2.06, and methyl doublets at δ 1.33, 1.30, and 1.27 (each with $J_{5,6} \sim 6$ Hz). The ^{13}C NMR spectrum (Fig. 1) contained 33 signals (one of double intensity), including signals for anomeric carbons at δ 101.60, 101.48, 99.77, 96.47, and 96.38, signals for the two acetyl groups at δ 175.29, 174.72, 22.89, and 22.77, for C-2 of the acetamido sugars at δ 54.02 and 53.52, for unsubstituted hydroxymethyl carbons at δ 61.13 and 60.95, and for three methyl carbons from Rha residues at δ 17.74, 17.54, and 17.31. Reliable values of $^1J_{CH}$ (diagnostic for anomeric configuration [11]) could not be obtained by gated decoupling because of background noise and incomplete resolution of signals, but the δ values for the anomeric protons

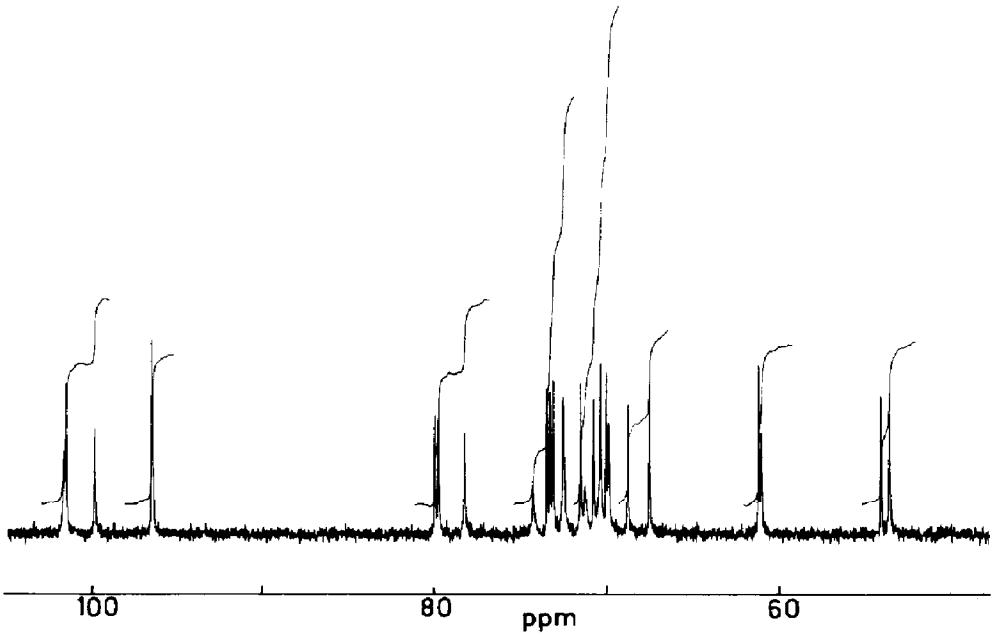
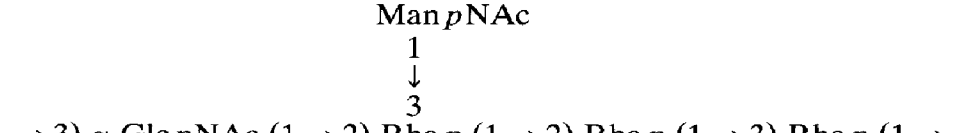


Fig. 1. ^{13}C NMR spectrum of the O10 polymer. The spectrum for the sample in D_2O was recorded at 100 MHz and 40°C with acetone (δ 31.07) as the internal reference. In addition to the signals shown, the spectrum contained acetyl signals at δ 175.29, 174.72, 22.89, and 22.77, and methyl signals (for Rha) at δ 17.74, 17.54, and 17.31.

favour the α configuration even for sugars with the *manno* configuration. This tentative inference was confirmed by more detailed interpretation of the NMR data (see later).

Methylation analysis, monitored by GLC–MS of the methylated alditol acetates, showed that the polymer was branched, and confirmed that all sugars occurred as pyranosyl residues. The products detected were derived from unsubstituted and 3-substituted HexN residues, together with those from 2-substituted, 3-substituted, and 2,3-disubstituted Rha residues. Periodate oxidation of the native polymer led to the loss of ManN (hence present as the non-reducing terminal residue) and the 2-substituted Rha residue. Methylation analysis of the oligomeric product (SD) obtained after mild acid hydrolysis of the oxidised and reduced polymer (Smith degradation) gave the derivatives of unsubstituted Rha, 2-substituted Rha, and 3-substituted GlcN. These data are only consistent with the partial structures **1** and **2** for the repeating unit in the O10 polymer



1

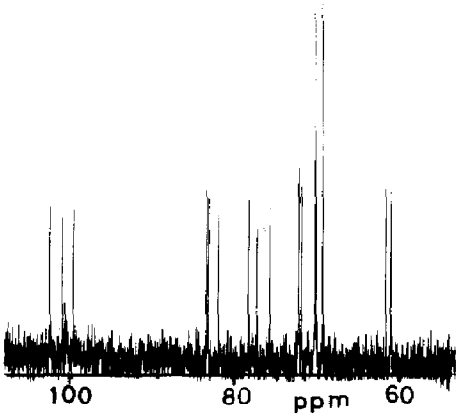


Fig. 2. ^{13}C NMR spectrum of the O10 deamination product (DA). The spectrum for the sample in D_2O was recorded at 150 MHz. In addition to the signals shown, the spectrum contained methyl signals (for Rha) at δ 17.14, 17.12, and 17.04.

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{Man}p\text{NAc} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 3 \\ \rightarrow 3)\text{-}\alpha\text{-Glc}p\text{NAc-}(1 \rightarrow 2)\text{-Rha}p\text{-}(1 \rightarrow 3)\text{-Rha}p\text{-}(1 \rightarrow 2)\text{-Rha}p\text{-}(1 \rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 2 \end{array}$$

In order to distinguish between these alternative structures, the polymer was subjected to *N*-deacetylation, deamination, and reduction to give a tetrasaccharide-anhydroalditol (DA), the partial structure of which should be **3** (derived from **1**) or **4** (derived from **2**). The ^1H NMR spectrum of DA contained three anomeric signals (each 1 H) at δ 5.17 ($J_{1,2}$ 1.6 Hz), 4.98 ($J_{1,2}$ 1.7 Hz), and 4.86 ($J_{1,2}$ 1.9 Hz), and methyl doublets at δ 1.30, 1.29, and 1.26 (each with $J_{5,6} \sim 6.3$ Hz). The ^{13}C NMR spectrum (Fig. 2) showed 22 discrete signals (two of double intensity), including those for anomeric carbons at δ 102.79, 101.27, and 99.91, unsubstituted hydroxymethyl carbons at δ 61.98 and 61.38, and methyl carbons at δ 17.14, 17.12, and 17.04. Insufficient material was available to establish completely the correlations between ^1H and ^{13}C chemical shift data, but the ^1H NMR spectrum was fully assigned with the aid of COSY, relayed COSY, and TOCSY experiments (Table 1). The relatively high chemical shifts for H-3 and H-5 showed that all Rha residues had the α configuration [12], and this was confirmed by the absence of intra-residue NOEs for these protons when the respective anomeric protons were irradiated. The inter-residue contacts (Table 2) identified RhaI as the 2-substituted residue, RhaII as the unsubstituted residue, RhaIII as the 3-substituted residue, and the overall sequence as that shown in structure **3**. Accordingly, the complete structure **5** can be assigned to the deamination product DA. This tetrasaccharide-anhydroalditol has, in fact, been isolated previously as a degradation product from other bacterial O antigens: *Shigella flexneri* variant Y [13], and a strain of *Burkholderia* (*Pseudomonas*) *solanacearum* [14]. The NMR data for

Table 1
NMR data [a] for the tetrasaccharide-anhydroalditol (DA) obtained by *N*-deacetylation, deamination, and reduction of the O10 polymer

Atom		Residue			
		RhaI	RhaII	RhaIII	2,5-Anhydromannitol
1	H	5.17	4.98	4.86	~ 3.71
	H′				~ 3.75
	C	101.27	102.79	99.91	61.98 or 61.38
2	H	4.06	4.07	3.99	~ 4.07
3	H	3.93	3.79	3.80	4.09 [b]
4	H	3.48	3.46	3.59	4.18
5	H	3.78	3.72	3.83	3.96
6	H	1.29	1.26	1.30	3.70
	H′				3.76

[a] Spectra were obtained at 70°C and 400 MHz (^{1}H), or 25°C and 150 MHz (^{13}C). Values for chemical shifts are given relative to acetone (δ_H 2.22; δ_C 31.07).
[b] Inferred from the NOE contact (Table 2).

anomeric signals given in Table 1 are close to those reported previously [13,14], and the overall data for the protons of individual residues in DA are comparable with other values in the extensive literature on corresponding residues of Rha [e.g., 15–17] and 2,5-anhydromannitol [18,19]

Rha*p*-(1 → 2)-Rha*p*-(1 → 3)-Rha*p*-(1 → 3)-2,5-Anhydromannitol

3

Rha*p*-(1 → 3)-Rha*p*-(1 → 2)-Rha*p*-(1 → 3)-2,5-Anhydromannitol

4

α-L-Rha*p*-(1 → 2)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-2,5-Anhydro-D-mannitol

5

To finalise the structure of the repeating unit in the intact O10 polymer, the NMR spectra were analysed further. Not all resonances could be identified (Table 3) because of signal overlap (^{1}H) and the absence of some connectivities (^{1}H–^{13}C). However, the signals for the anomeric protons of the acetamido sugar residues (*c* and *d*) were readily recognised from the ^{1}H–^{13}C and COSY spectra, and differentiated by the value of $J_{1,2}$ (~ 4 Hz for α-GlcNAc, unresolved for ManNAc) and the

Table 2
NOE contacts and assignments for the tetrasaccharide-anhydroalditol DA

Anomeric H irradiated	Intra-residue NOE	Inter-residue NOE
RhaI (δ 5.17)	H-2	RhaIII H-3
RhaII (δ 4.98)	H-2	RhaI II-2
RhaIII (δ 4.86)	H-2	2,5-Anhydromannitol H-3

Table 3
NMR data [a] for the O10 polymer

Atom		Rha (*a*)	Rha (*b*)	GlcNAc (*c*)	ManNAc (*d*)	Rha (*e*)
1	H	5.17	5.05	5.03	5.00	4.94
	C	101.48	99.77	96.47 [b]	96.38 [b]	101.60
2	H	4.05	4.29	4.06	4.36	3.87
	C	79.72	72.47	54.02	53.52	71.44
3	H	3.92	3.95	3.82	4.08	~ 3.78
	C	70.69	73.22	79.94	70.28	78.19
4	H	3.45	3.68	3.66	3.65	3.56
	C	73.04	nd	68.71	67.43	72.47
5	H	~ 3.74	~ 3.78	nd	nd	4.01
	C	nd	nd	nd	nd	69.81
6	H	1.30	1.33	nd	nd	1.27
	C	17.74 [c]	17.54 [c]	60.95 [d]	61.13 [d]	17.31 [c]

[a] Residues (*a*–*e*) are identified in structure **6**. Spectra were obtained at 70°C and 400 MHz (^{1}H), or 40°C and 100 MHz (^{13}C). Values for chemical shifts are given relative to acetone (δ_H 2.22; δ_C 31.07); nd, not determined.
[b–d] Sets of signals for which assignments may be interchanged.

low-field shift (δ 4.36) for H-2 of ManNAc). The α configuration for the Man*p*NAc residue (*d*) was inferred from the chemical shifts for H-3 and C-3 [12]. Virtually complete assignments of proton resonances for the three Rha residues were achieved with the aid of COSY and relayed COSY spectra. The 2-substituted residue (*a*) was readily identified from the good agreement between its proton resonances and those of the corresponding residue (RhaI) in the deamination product (Table 1), and from the downfield location of the signal for C-2. Likewise, the proton resonances for the 3-substituted residue (*e*) were in accord with those reported [20] for such a residue in an identical chemical environment. The partial sequence $b \rightarrow a \rightarrow e$ was established by inter-residue NOE contacts, supporting previous inferences. Thus, the repeating unit of the O10 polymer has the structure **6**.

$$\begin{array}{c} \alpha\text{-D-Man}p\text{NAc} \quad d \\ 1 \\ \downarrow \\ 3 \\ \rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1 \rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow \\ c \qquad\qquad b \qquad\qquad a \qquad\qquad e \end{array}$$

6

Polymers based largely or entirely on Rha and GlcNAc residues are relatively common among bacterial O antigens, and a tetrasaccharide backbone identical to that found in the putative O10 polymer of *A. baumannii* occurs (unsubstituted) in several strains of *Burkholderia solanacearum* [14,21] and in *Serratia marcescens* O22 [20], while glycosylated derivatives have also been found in *Serratia marcescens* O10 [22] and other strains of *Burkholderia solanacearum* [21]. Similar polymers

occur in *Shigella flexneri* [15], *Escherichia coli* O1 [23], *Escherichia coli* O2 [24], and yet more strains of *Burkholderia solanacearum* [21,25] *inter alia*.

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—For preparative purposes, the reference strain for serogroup O10 of *A. baumannii* was grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 12 h at 30°C with aeration at 10 L min^{-1} and stirring at 500 rpm. Despite the use of antifoam [poly(ethylene glycol) P-2000], part of the culture (~ 1 L) foamed out of the fermenter, carrying by flotation the bulk of the cells (140 g wet weight). The cells were disintegrated, and the cell walls were purified and used for the isolation of LPS as in previous studies [10]. LPS (560 mg) was recovered from the upper phase after extraction of the defatted cell walls with hot aqueous phenol. LPS was also obtained from whole cells (2.7 g dry weight) grown in shake-flask culture (total volume 3.6 L) for 24 h at 30°C in the same medium. The crude LPS was purified by repeated ultracentrifugation (yield, 160 mg). Polymeric fractions were obtained from both preparations after mild acid hydrolysis (aq 1% AcOH, 100°C, 2.5 h), followed by chromatography of the water-soluble products on Sephadex G-50 in pyridine–AcOH buffer (pH 5.4), monitoring the eluate for total carbohydrate (phenol–H_2SO_4 method).

General methods.—NMR spectra for samples (intact polymer, Smith-degradation product SD, deamination product DA) in D_2O were mostly recorded with a Jeol JNM-GX270 or a Bruker WH-400 spectrometer. A Varian DXR600S spectrometer was also used to obtain the ^{13}C spectrum, the ^{1}H–^{13}C correlation spectrum (HMQC pulse sequence), and 2D ^{1}H spectra (COSY-45, phase-sensitive TOCSY) for the product DA. The ^{1}H NMR data reported were obtained at 70°C with acetone (δ 2.22) as reference, while ^{13}C NMR data were obtained at 40°C with acetone (δ 31.07) as reference (intact polymer) or at 25°C (product DA). The equipment used for GLC, GLC–MS, and HPLC, and the methods used for PC and electrophoresis (paper and SDS-PAGE) were those listed previously [10].

Determination of sugar composition.—Hydrolysis conditions used were 2 M HCl at 105°C for 2 h (for neutral sugars), and 6.1 M HCl at 105°C for 4 h (for amino sugars). Rhamnose was identified by PC, HPLC (Bio-Rad HPX-87P and Dionex CarboPac PA100), and GLC of the alditol acetate. It was shown to be the L isomer by (a) GLC (BP1) of the (+)-but-2-yl glycoside acetates, (b) the CD spectrum of the alditol acetate [26], and (c) reaction with L-rhamnose isomerase (EC 5.3.1.14) [27], monitored by HPLC (Dionex). 2-Amino-2-deoxyglucose and 2-amino-2-deoxymannose were identified by PC, high-voltage paper electrophoresis, and HPLC (Dionex), and by GLC of their alditol acetates and of the products obtained by deamination [28] followed by reduction and acetylation (acetates of 2,5-anhydromannitol and glucitol, respectively). The O10 polymer contained equimolar amounts of each hexosamine, and both were identified as the D isomer by a positive reaction with hexokinase and ATP [29]. The conversion of the hexosamines into their 6-phosphates was monitored by HPLC (Dionex).

Degradative methods.—Oxidation of the polymer (~ 20 mg) with 50 mM $NaIO_4$ was carried out for 5 days at 4°C. After the addition of ethylene glycol, reduction ($NaBH_4$), acidification (AcOH), dialysis, and freeze-drying, the product was treated with 1 M trifluoroacetic acid for 16 h at room temperature. The oligomeric degradation product was isolated by HPLC (TSKgel G-Oligo-PW). Methylation analysis of the intact polymer and of the Smith-degradation product followed standard procedures [30–32]. The conditions for *N*-deacetylation, deamination, and reduction of the $NaBH_4$-treated O10 polymer were those used in related studies [10], and the oligosaccharide-anhydroalditol (DA) was isolated by HPLC (TSKgel G-Oligo-PW).

Acknowledgments

We thank Mrs. H.M. Aucken (Central Public Health Laboratory, London) for the reference culture of *A. baumannii* O10, and Dr. W.D. Fessner (University of Freiburg) for a gift of L-rhamnose isomerase. We also thank the S.E.R.C. for a grant to purchase the Dionex HPLC system and for an allocation on the high-field NMR service at the University of Warwick, and the University of Hull for a Scholarship (S.R.H.). We are also grateful to the following: Dr. O. Howarth and staff at Warwick, Dr. J.A. Parkinson (University of Edinburgh), and our colleagues Dr. D.F. Ewing and Mrs. B. Worthington for NMR spectra; Ms. B. Banfield (National Chiroptical Spectroscopy Centre, University of London) for the CD measurement on rhamnitol acetate; Mr. A.D. Roberts for GLC–MS; Miss. L. Galbraith for technical assistance.

References

[1] H. Seifert, R. Baginski, A. Schulze, and G. Pulverer, *Zentralbl. Bakteriol.*, 279 (1993) 544–552.
[2] Y. Gräser, I. Klare, E. Halle, R. Gantenberg, P. Buchholz, H.D. Jacobi, W. Presber, and G. Schönian, *J. Clin. Microbiol.*, 31 (1993) 2417–2420.
[3] W.H. Traub, *J. Clin. Microbiol.*, 27 (1989) 2713–2716.
[4] W.H. Traub, *Zentralbl. Bakteriol.*, 175 (1991) 487–494.
[5] P. Borneleit, B. Blechschmidt, R. Blasig, P. Franke, P. Günther, and H.-P. Kleber, *Curr. Microbiol.*, 19 (1989) 77–81.
[6] P. Borneleit, H. Binder, and H.-P. Kleber, *Acta Biotechnol.*, 10 (1990) 117–123.
[7] A.W. Smith and K.E. Alpar, *J. Med. Microbiol.*, 34 (1991) 83–88.
[8] M.J. Loeffelholz, F. Rana, M.C. Modrazakowski, and J. Blazyk, *Biochemistry*, 26 (1987) 6644–6648.
[9] W.H. Traub and D. Bauer, *Zentralbl. Bakteriol.*, 279 (1993) 244–258.
[10] S.R. Haseley, L. Galbraith, and S.G. Wilkinson, *Carbohydr. Res.*, 258 (1994) 199–206.
[11] K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
[12] P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[13] L. Kenne, B. Lindberg, K. Petersson, and E. Romanowska, *Carbohydr. Res.*, 56 (1977) 363–370.
[14] Y. Akiyama, S. Eda, K. Kato, and H. Tanaka, *Carbohydr. Res.*, 133 (1984) 289–296.
[15] P.-E. Jansson, L. Kenne, and T. Wehler, *Carbohydr. Res.*, 166 (1987) 271–282.
[16] A.V. Bukharov, I.M. Skvortsov, V.V. Ignatov, A.S. Shashkov, Y.A. Knirel, and J. Dabrowski, *Carbohydr. Res.*, 241 (1993) 309–316.

[17] N.K. Kochetkov, G.M. Lipkind, A.S. Shashkov, and N.E. Nifant'ev, *Carbohydr. Res.*, 221 (1991) 145–168.
[18] J.L. DiFabio, M.B. Perry, and J.-R. Brisson, *Biochem. Cell Biol.*, 66 (1988) 107–115.
[19] J.L. DiFabio, J.-R. Brisson, and M.B. Perry, *Biochem. Cell Biol.*, 67 (1989) 78–85.
[20] D. Oxley and S.G. Wilkinson, *Carbohydr. Res.*, 203 (1990) 247–251.
[21] N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, N.E. Nifant'ev, N.K. Kochetkov, L.D. Varbanets, N.V. Moskalenko, O.S. Brovarskaya, V.A. Muras, and J.M. Young, *Carbohydr. Res.*, 250 (1993) 275–287.
[22] D. Oxley and S.G. Wilkinson, *Carbohydr. Res.*, 187 (1989) 303–311.
[23] B. Jann, A.S. Shashkov, D.S. Gupta, S.M. Panasenko, and K. Jann, *Carbohydr. Polym.*, 18 (1992) 51–57.
[24] P.-E. Jansson, H. Lennholm, B. Lindberg, U. Lindquist, and S.B. Svenson, *Carbohydr. Res.*, 161 (1987) 273–279.
[25] A.S. Shashkov, N.A. Kocharova, Y.A. Knirel, L.D. Varbanets, N.M. Moskalenko, and A.V. Zdokhliy, *Bio-org. Khim.*, 19 (1993) 1089–1093.
[26] G.M. Bebault, J.M. Berry, Y.M. Choy, G.G.S. Dutton, N. Funnell, L.D. Hayward, and A.M. Stephen, *Can. J. Chem.*, 51 (1973) 324–326.
[27] G.F. Domagk and R. Zech, *Methods Enzymol*, 9 (1966) 579–582.
[28] D. Horton and K.D. Philips, *Carbohydr. Res.*, 30 (1973) 367–374.
[29] D.H. Brown, in H.U. Bergmeyer, *Methods of Enzymatic Analysis*, 2nd ed., Vol. 3, Academic, London, 1974, pp 1251–1256.
[30] B. Lindberg and J. Lönngren, *Methods Enzymol.*, 50C (1978) 3–33.
[31] L.R. Phillips and B.A. Fraser, *Carbohydr. Res.*, 90 (1981) 149–152.
[32] A.J. Mort, S. Parker, and M.-S. Kuo, *Anal. Biochem.*, 133 (1983) 380–384.