Carbohydrate Research, 203 (1990) 219–227
Elsevier Science Publishers B.V., Amsterdam

The structure of the biological repeating unit of the O-antigen of *Hafnia alvei* O39

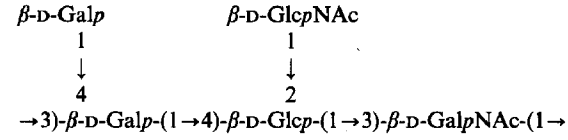
Ewa Katzenellenbogen*, Elzbieta Romanowska,
Department of Immunochemistry, L. Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 12 Czerska, 53–114 Wroclaw (Poland)

Nina A. Kocharova, Alexander S. Shashkov, Grigory M. Lipkind, Yuriy A. Knirel, and Nikolay K. Kochetkov
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R., Moscow (U.S.S.R.)

(Received August 4th, 1989; accepted for publication, November 11th, 1989)

ABSTRACT

On mild acid-catalysed degradation of the lipopolysaccharide from *Hafnia alvei* O39 followed by gel filtration of Sephadex G-50, the O-specific polysaccharide and three oligosaccharides were obtained, which represent the core substituted with 0–2 O-antigen repeating-units. On the basis of sugar and methylation analyses, ^{13}C-n.m.r. data, solvolysis of the polysaccharide with anhydrous hydrogen fluoride, and computer-assisted ^{13}C-n.m.r. analysis of the Smith-degraded polysaccharide, it was concluded that the biological repeating unit of the O39 antigen was



INTRODUCTION

The name *Hafnia alvei* was introduced by Moller in 1954 for the group of enterobacterial micro-organisms previously included in the genus Enterobacter[1]. According to the serological classification of Sakazaki[2], *Hafnia alvei* strains are divided into 68 O- and 34 H-antigenic groups. Numerous serological cross-reactions exist between *Hafnia* and such genera as *E. coli*, *Enterobacter cloaceae*, *Citrobacter freundii*, and *Shigella*.

There has been controversy regarding the pathogenicity and epidemiology of this group of bacteria. Many incidents of nosocomial infections with *Hafnia* have been reported, but generally these bacteria have been found in stool specimens of healthy people[1].

We have reported preliminary chemical characteristics of *Hafnia alvei* lipopoly-

* Author for correspondence.

0008-6215/90/$ 03.50

saccharides isolated from 33 different strains[3], but no immunochemical studies have been described.

The aim of this study was to elucidate the structure of the O-antigen isolated from one of the representatives of this bacterial genus, namely, the strain *Hafnia alvei* O39.

RESULTS AND DISCUSSION

The lipopolysaccharide (LPS) of *Hafnia alvei* O39 was isolated from dry bacteria in a yield of 2.8%. Hydrolysis of the LPS, followed by fractionation of the products on Sephadex G-50, gave one high-molecular-weight fraction corresponding to the O-specific polysaccharide (PS-I), and three other fractions representing the core (OS-III) and the core substituted with shorter (OS-IIB) and longer (OS-IIA) O-specific chains. Kdo (3-deoxy-D-*manno*-2-octulosonic acid) was eluted from the column as the last fraction.

PS-I had $[\alpha]_{578}^{25}$ +20° (water), and hydrolysis with 4M HCl for 18 h at 100° or 0.5M HCl for 18 h at 100° gave galactose, glucose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose in the molar ratios 2.1:0.8:1.0:1.1 (Table I). Hydrolysis of PS-I with 0.5M HCl (18 h, 100°) followed by deamination, borohydride reduction, acetylation, and g.l.c. gave the acetylated derivatives of galactitol, glucitol, 2,5-anhydromannitol, and 2,5-anhydrotalitol in the molar ratios 1.8:1.0:0.6:0.7.

Each sugar has the D configuration, as established by the use, as appropriate, of D-glucose oxidase, D-galactose oxidase, and hexokinase (see Experimental).

The ^{13}C-n.m.r. spectrum of PS-I (Fig. 1, Table II) contained the signals for five anomeric carbons (102.9–105.1 p.p.m.), five CH_2OH groups (61.0–62.3 p.p.m.), two carbons bearing nitrogen (52.5 and 57.0 p.p.m.), 18 other sugar carbons (60.0–83.2 p.p.m.), and two *N*-acetyl groups (CH_3 at 23.5 and 24.2 p.p.m., CO at 175.8 and 175.9 p.p.m.). The $J_{C\text{-}1,H\text{-}1}$ values for each of the anomeric carbons determined by gated-decoupling were 165 Hz; hence, each sugar residue was β-pyranosidic[4].

TABLE I

Sugar composition of *Hafnia alvei* O39 specific polysaccharide [native (PS-I) and Smith-degraded (PS-IM)] and core oligosaccharides (OS-IIA, OS-IIB, and OS-III)

Fraction	*Sugar constituent*									
	Gal		*Glc*		*GlcN*		*GalN*		*Hep*	
	%	MR[a]	%	MR	%	MR	%	MR	%	MR
PS-I	30.0	2.1	12.0	0.77	14.0	1.0	16.0	1.1	n.d.[b]	
PS-IM	30.0	1.36	22.0	1.0	-	-	22.0	1.0	n.d.	
OS-IIA	26.0	6.5	18.0	4.6	11.5	3.0	8.5	2.2	9.0	2.0
OS-IIB	25.0	4.5	18.0	3.2	10.5	1.9	5.5	1.0	10.5	1.6
OS-III	16.0	1.98	18.0	2.2	8.0	1.0	1.5	0.18	17.5	1.8

[a] Molar ratio, [b] Not determined.

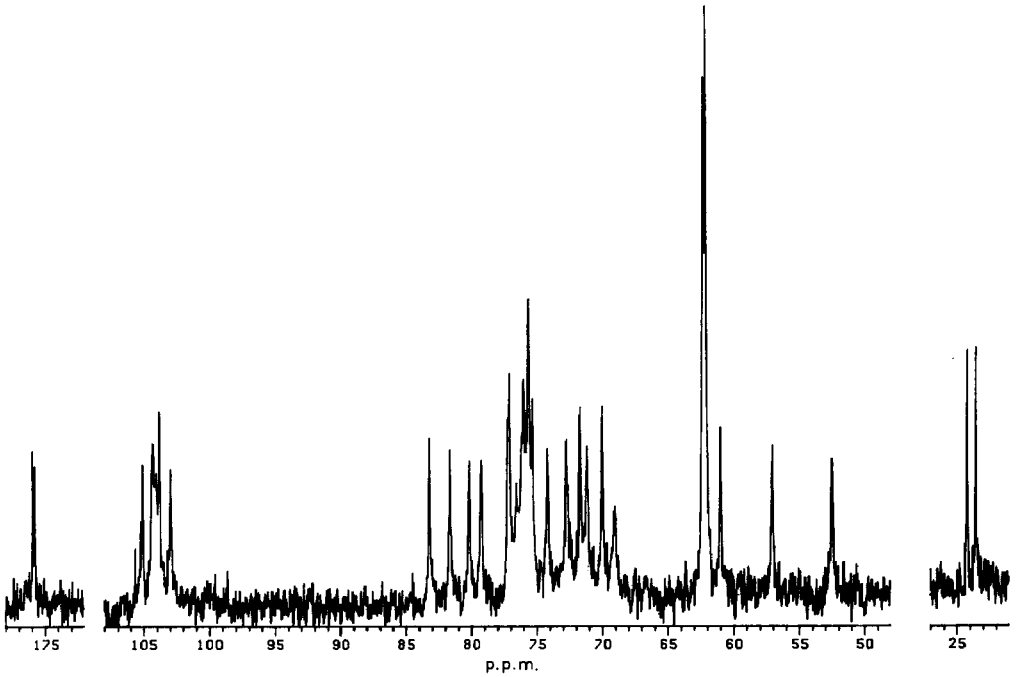


Fig.1. ^{13}C-N.m.r. spectrum of the O-specific polysaccharide (PS-I)

The above data showed PS-I to comprise pentasaccharide repeating-units consisting of β-D-glucose, β-D-galactose, 2-acetamido-2-deoxy-β-D-galactose, and 2-acetamido-2-deoxy-β-D-glucose in the molar ratios 1:2:1:1.

Methylation analysis of PS-I gave 2,3,4,6-tetra-*O*-methylgalactose, 2,6-di-*O*-methylgalactose, 3,6-di-*O*-methylglucose, 2-deoxy-3,4,6-tri-*O*-methyl-2-methylaminoglucose, and 2-deoxy-4,6-di-*O*-methyl-2-methylaminogalactose in the ratios 8:15:6:22:14, which were identified as alditol acetates[5,6]. As judged from the ^{13}C-n.m.r. spectrum, PS-I is regular; thus, the non-stoichiometric ratio of the methylated sugars is due, most likely, to their different stabilities during the acid hydrolysis of methylated PS-I. The methylation data indicated PS-I to be branched and to have two different side-chains terminated with galactose and 2-acetamido-2-deoxyglucose residues, respectively, whereas the glucose residue and the second galactose residue were the branching points.

Smith degradation of PS-I gave a polymeric product (PS-IM). On the basis of sugar analysis (Table I) and ^{13}C-n.m.r. data (Table II), PS-IM comprises trisaccharide repeating-units including, as expected from the methylation analysis data, glucose, galactose, and 2-acetamido-2-deoxygalactose in the molar ratios 1:1:1.

A computer-assisted ^{13}C-n.m.r. analysis[7] of PS-IM indicated that only structure **1** was consistent with the experimental spectrum. This structure was characterized by an S value of 0.7 (see ref. 7), whereas the S values for all of the alternative structures were >1.6 and, hence, were inconsistent with the ^{13}C-n.m.r. spectrum of PS-IM.

TABLE II

^{13}C-N.m.r. data

Sugar unit	*Chemical shifts (p.p.m.[a])*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
PS-I						
→3,4)-β-Gal-(1→	104.0	71.7	83.2	76.6	75.6	62.3
→2,4)-β-Glc-(1→	103.7	80.1	77.1	79.2	75.6	61.0
→3)-β-GalNAc-(1→	104.3	52.5	81.6	69.0	76.0	62.3
β-Gal-(1→	105.1	72.7	74.2	70.0	76.1	62.1
β-GlcNAc-(1→	102.9	57.0	75.3	71.2	77.1	62.1
PS-IM[b]						
→3)-β-Gal-(1→	104.3	71.4	83.1	69.8	76.0	62.3
	(104.0)	(71.0)	(83.2)	(69.7)	(76.3)	(62.2)
→4)-β-Glc-(1→	105.4	73.9	75.5	79.6	76.0	61.3
	(104.9)	(74.4)	(75.6)	(79.9)	(75.9)	(61.3)
→3)-β-GalNAc-(1→	104.3	52.7	81.2	69.2	76.2	62.3
	(104.4)	(52.3)	(82.2)	(69.2)	(76.4)	(62.2)
OS-IIB						
→4)-β-Gal-(1→	104.2	71.5	74.1	78.7	75.8	62.3
→2,4)-β-Glc-(1→	103.8	80.3	77.2	79.3	75.6	61.0
→3)-β-GalNAc-(1→	102.8	52.5	81.7	69.0	76.3	62.3
β-Gal-(1→	105.6	72.8	74.2	69.9	76.4	62.2
β-GlcNAc-(1→	103.0	57.0	75.3	71.3	77.1	62.2
OS-IIA						
Terminal pentasaccharide unit						
→4)-β-Gal-(1→	104.2	71.6	74.3	78.8	75.9	62.4
→2,4)-β-Glc-(1→	103.8	80.3	77.3	79.4	75.7	61.1
→3)-β-GalNAc-(1→	104.4	52.5	81.7	69.1	76.0	62.4
β-Gal-(1→	105.7	72.8	74.3	70.0	76.5	62.2
β-GlcNAc-(1→	103.0	57.1	75.4	71.3	77.1	62.2
Pentasaccharide unit linked to the core						
→3,4)-β-Gal-(1→	104.2	71.8	83.2	76.7	75.7	62.4
→2,4)-β-Glc-(1→	103.8	80.3	77.3	79.4	75.7	61.1
→3)-β-GalNAc-(1→	102.9	52.5	81.7	69.1	76.3	62.4
β-Gal-(1→	105.1	72.6	74.3	70.1	76.2	62.2
β-GlcNAc-(1→	103.0	57.1	75.4	71.2	77.1	62.2

[a] Assignments of signals with chemical shift difference <0.5 p.p.m. could be interchanged. [b] Calculated shifts are given in parentheses.

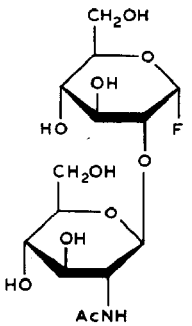
→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→

1

The positions of substitution of the hexose residues in the backbone were confirmed by methylation analysis of PS-IM, which gave 2,3,6-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose.

In order to determine the sites of attachment of the side chains, PS-I was solvolyzed partially with anhydrous HF (1 h, −20°). The main oligosaccharide product was the disaccharide **2** isolated by gel filtration followed by h.p.l.c. on reverse-phase C_{18}. On acid hydrolysis, **2** afforded glucose and 2-amino-2-deoxyglucose in the molar ratio 1:1. Since **2** could not be reduced with sodium borohydride, it was proposed to be a glycosyl fluoride. This conclusion was confirmed by the ^{1}H-n.m.r. spectrum of **2**, which contained signals for two anomeric protons one of which belonged to the 2-acetamido-2-deoxy-β-glucose residue (δ 4.64, d, $J_{1,2}$ 7.9 Hz) and the other to the α-glucosyl fluoride residue (δ 5.79, dd, $J_{1,2}$ 2.2 Hz $J_{H,F}$ 52 Hz). The formation of α-glucosyl fluorides that are stable in water on solvolysis with anhydrous HF has been reported[8,9].

Also formed in the solvolysis were derivatives of a disaccharide (Gal:GalNAc 1:1) that contaminated **2**, a trisaccharide (Glc:GlcNAc:GalNAc 1:1:1), a tetrasaccharide (Glc:Gal:GlcNAc:GalNAc 1:1:1:1), and a pentasaccharide that contained all of the



2

constituents of the repeating unit of PS-I. None of these derivatives could be reduced with sodium borohydride; most probably, they were also glycosyl fluorides.

From the formation of the disaccharide derivative **2**, it followed that, in PS-I, the lateral 2-acetamido-2-deoxyglucose residue was attached to the glucose residue and, hence, the lateral galactose residue was attached to the galactose residue in the backbone. Hence, the repeating unit of PS-I has the structure **3**.

The structure **3** was confirmed by the ^{13}C-n.m.r. spectrum of PS-I (Table II), which was interpreted by comparison with the spectra of PS-IM, galactose, and 2-acetamido-2-deoxyglucose, taking into account the effects of glycosylation[7].

Sugar analyses of OS-IIA and OS-IIB (Table I) showed that, together with core constituents [heptose (2), glucose (2), galactose (2), 2-amino-2-deoxyglucose (1)], they contained glucose, galactose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxygalactose, which are O-antigenic components. In OS-IIA, except for the core oligosaccharide, two repeating units were found (Glc:Gal:GlcN:GalN 2.6:4.5:2.0:2.2); in OS-IIB, one repeating unit was found (Glc:Gal:GlcN:GalN 1.2:2.5:0.9:1.0).

The ^{13}C-n.m.r. spectrum of OS-IIB contained signals for the core (found by comparison with the spectrum of OS-III) and for one O-antigen repeating-unit (Table II). As compared to the spectrum of PS-I, the most significant displacements in the

B-D-Gal*p*
1
↓
4
β-D-Glc*p*NAc
1
↓
2
→3)-*β*-D-Gal*p*-(1→4)-*β*-D-Glc*p*-(1→3)-*β*-D-Gal*p*NAc-(1→

3

spectrum of OS-IIB were observed for the signals of C-3 and C-4 of the galactose residue in the backbone (from 83.2 and 76.6 p.p.m. to 74.1 and 78.7 p.p.m., respectively). Such displacements are characteristic of the effects of glycosylation at position 3 of a galactose residue substituted at position 4 and indicated that, in OS-IIB, this residue is monosubstituted at position 4. Another marked displacement involved the signal of C-1 of the 2-acetamido-2-deoxygalactose residue (from 104.3 p.p.m. in the spectrum of PS-I to 102.8 p.p.m. in the spectrum of OS-IIB). This shift proved that the residue is attached to different sites, namely, to the neighbouring repeating unit in PS-I and to the core in OS-IIB. On the other hand, the chemical shift of the signal of C-2 of the 2-acetamido-2-deoxygalactose residue is the same in both spectra (52.5 p.p.m.) and, hence, in OS-IIB, as in PS-I, this residue is *β*.

The foregoing data show that the O-antigen moiety of OS-IIB, that is, the biological repeating unit of the O-antigen, has the structure **4**.

β-D-Gal*p* *β*-D-Glc*p*NAc
1 1
↓ ↓
4 2
β-D-Gal*p*-(1→4)-*β*-D-Glc*p*-(1→3)-D-Gal*p*NAc

Analysis of the ^{13}C-n.m.r. spectrum (Table II) indicated OS-IIA to consist of the core, to which was attached a block of two repeating units. Thus, the spectrum contained signals for the terminal repeating unit linked to the neighbouring repeating unit and for the penultimate unit linked to the core (Table II).

The structures of OS-IIB and OS-IIA were confirmed by methylation analysis. The appearance of 2,3,6-tri-*O*-methylgalactose and a marked decrease in the content of 2,6-di-*O*-methylgalactose were observed in the mixture of partially methylated sugars derived from OS-IIA, as compared to that from PS-I. The former sugar was also present in the mixture derived from OS-IIB, whereas the latter was almost completely absent from the mixture.

Thus, in *Hafnia alvei* O39, together with the lipopolysaccharide species that contains the core substituted with the polysaccharide chain and the unsubstituted core, there are present species in which the O-antigen part is represented by one or two repeating units. Fraction PS-I constitutes 36% of the total material eluted from the column of Sephadex G-50, and fractions OS-IIA, OS-IIB, and OS-III constitute 10%, 20%, and 13%, respectively.

Comparative analysis of the carbohydrate moieties of all of these species allowed the structure of the biological repeating unit of the O-antigen to be established and to prove that each unit is bound to the neighbouring one and that the first unit is linked to the core via a 2-acetamido-2-deoxy-β-galactose residue.

EXPERIMENTAL

General. — ^{13}C-N.m.r. spectra were recorded with a Bruker AM-300 instrument for solutions in D_2O (internal MeOH, 50.15 p.p.m.) at 30° for oligosaccharides and 60° for polysaccharides. The ^{1}H-n.m.r. spectrum of **2** was obtained with a Bruker WM-250 instrument for a solution in D_2O (internal acetone, 2.24 p.p.m.). Optical rotations were measured on a JASCO DIP 300 polarimeter for solutions in water at 25°.

G.l.c. of sugar alditol acetates and partially methylated alditol acetates was performed on a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector and a glass-capillary column (0.2 mm × 25 m) coated with Ultra-1 and operated with a temperature gradient of 160°→290° at 10°.min^{-1}, or on a Varian 2100 instrument equipped with a flame-ionisation detector and a glass column (0.25 × 200 cm) containing 3% of OV-225 operated at 180° (neutral sugars) or 205° (amino sugars). G.l.c.–m.s. was performed with a Finnigan MAT Ion-Trap mass spectrometer under the same chromatographic conditions as in g.l.c.

Gel chromatography was performed on a column (2 × 100 cm) of Sephadex G-50 in pyridine–acetic acid buffer (pH 5.75) or on a column (1.6 × 80 cm) of Fractogel TSK HW 40 (S) in aqueous 1% acetic acid, and monitored by the phenol–sulfuric acid method[10] or with a Knauer differential refractometer.

H.p.l.c. was performed with a Serva Octadecyl Si 100 column (500 × 7.5 mm) by elution with water or aqueous 5% methanol at 2 mL.min^{-1}, and monitoring of the eluate with a Knauer variable-wavelength detector at 220 nm.

Glucose and galactose were determined after hydrolysis of the polysaccharide with 0.5M HCl for 18 h at 100°, using D-glucose oxidase [11] and D-galactose oxidase[12], respectively. Hexosamines were assayed after hydrolysis of the polysaccharide with 4M HCl for 18 h at 100° by a modified[13] Ludowieg–Benmaman method[14]. Heptose was determined according to Osborn[15]. Neutral and amino sugars were identified simultaneously by g.l.c. after hydrolysis of the polysaccharide with 0.5M HCl for 18 h at 100°, followed by deamination, $NaBH_4$ reduction, and acetylation. Sugars were acetylated according to Sawardeker *et al.*[16], and methylation was carried out according to Stellner *et al.*[5] or Hakomori[17].

Determination of the absolute configuration of the hexose and hexosamine residues.

— The polysaccharide was hydrolysed with 0.5M or 4M HCl for 18 h at 100°. The D configuration of glucose was established using D-glucose oxidase[11] and the D configurations of galactose and 2-amino-2-deoxygalactose were proved by reaction with D-galactose oxidase[12]. The absolute configuration of 2-amino-2-deoxyglucose was determined, as described[18], using hexokinase. The degree of phosphorylation, checked by g.l.c. of enzyme-treated, $NaBH_4$-reduced, and acetylated PS-I hydrolysate was 100%.

Isolation and degradation of lipopolysaccharide. — *Hafnia alvei* O39 strain, obtained from the collection of the Institute of Immunology and Experimental Therapy (Wrocław), was grown in the liquid medium described[19].

The LPS was extracted from the acetone-dried bacteria with phenol–water[20] and purified by filtration on a Sepharose 2B column[21]. The LPS was hydrolysed with aqueous 1% AcOH at 100° for 2.75 h, lipid A was removed by centrifugation, and the supernatant solution was fractionated on a column of Sephadex G-50.

Smith degradation. — A solution of PS-I (45 mg in 4.5 mL of 0.1M $NaIO_4$) was kept for 2 days in the dark, then treated with an excess of ethyleneglycol, reduced with $NaBH_4$, acidified with conc. acetic acid, and desalted by gel chromatography on TSK HW 40. The polymeric product was hydrolysed with aqueous 2% acetic acid (2 h, 100°), the hydrolysate was concentrated, the residue was reduced with $NaBH_4$, and PS-IM was isolated by gel chromatography on TSK HW 40.

Solvolysis with anhydrous hydrogen fluoride. — PS-I (20 mg, dried over P_2O_5 at 80°) was treated with anhydrous HF (1 h, −20°). The mixture was poured into cold heter and was filtered through a stainless-steel filter, the precipitate was washed with ether, and a solution in water was subjected to gel chromatography on TSK HW 40 followed by h.p.l.c., to give di- to penta-saccharide derivatives.

REFERENCES

1 T. Bergan, *Methods Microbiol.*, 14 (1984) 1–41.
2 R. Sakazaki, *Methods Microbiol.*, 14 (1984) 165–186.
3 A. Romanowska, E. Katzenellenbogen, M. Kułakowska, A. Gamian, D. Witkowska, M. Mulczyk, and E. Romanowska, *FEMS Microbiol. Immunol.*, 47 (1988) 151–156.
4 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
5 P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lonngren, *Chem. Commun. Univ. Stockholm*, (1976) 1–75.
6 K. Stellner, H. Saite, and S. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
7 G. M. Lipkind, A. S. Shashkov, Y. A. Knirel, E. V. Vinogradov, and N. K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
8 H. Hardt and D. T. A. Lamport, *Phytochemistry*, 21 (1982) 2301–2303.
9 M.-S. Kuo, A. Dell, and A. J. Mort, *Carbohydr. Res.*, 156 (1986) 173–187.
10 M. Dubois, K. A. Gilles, J. K. Hamilton, P. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
11 H. U. Bergmeyer and E. Bernt, in H. U. Bergmeyer (Ed.), *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, pp. 123–130.
12 H. Roth, S. Segal, and D. Bertoli, *Anal. Biochem.*, 10 (1965) 35–52.
13 E. Romanowska and V. Reinhold, *Eur. J. Biochem.*, 36 (1973) 160–166.
14 J. Ludowieg and J. D. Benmaman, *Anal. Biochem.*, 19 (1967) 80–88.
15 M. J. Osborn, *Proc. Natl. Acad. Sci. U.S.A.*, 50 (1963) 499–506.
16 J. S. Sawardeker, J. H. Sloneker, and A. R. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.
17 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.

18 E. Katzenellenbogen, I. Ekiel, and E. Romanowska, *Carbohydr. Res.*, 179 (1988) 349–357.
19 E. Romanowska and M. Mulczyk, *Eur. J. Biochem.*, 5 (1968) 109–113.
20 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–89.
21 E. Romanowska, *Anal. Biochem.*, 33 (1970) 383–389.