Carbohydrate Research, 187 (1989) 93–101
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE LINKAGE OF 4-*O*-METHYL-L-GALACTOPYRANOSE IN THE AGAR POLYMERS FROM *Gracilaria verrucosa**

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(Received December 31st, 1987; accepted for publication in revised form, April 19th, 1988)

ABSTRACT

4-*O*-Methyl-L-galactopyranose was found in agar preparations from *Gracilaria verrucosa*. Its content varied with the culture conditions and with the tissue age. Highly substituted portions having this residue were obtained by enzymic degradation with β-agarase I. Permethylation analysis and ^{13}C-n.m.r. spectroscopy demonstrated the linkage of this sugar to O-6 of the 3-*O*-linked-D-galactopyranose units. The definition of agar should take into account this occurrence of 4-*O*-methyl-L-galactose side groups.

INTRODUCTION

Agar is a complex mixture of polysaccharides, obtained from red algae, having a basic disaccharide repeat unit, 3-*O*-(3,6-anhydro-α-L-galactopyranosyl)-β-D-galactopyranose, which may be substituted with methyl (at O-6 of D-galactopyranose, O-2 of L-galactopyranose unit) or with sulfate groups (at O-6 of L-galactopyranose unit). 4-*O*-Methyl-L-galactopyranose was first described by Araki *et al.*[1], who isolated it in a small amount from agar of *Gelidium amansii*. Since then, this sugar has been obtained from several polysaccharides of red algae belonging to the *Grateloupiaceae*[2,3] and to the *Gracilariaceae*[4,5] families, and recently from agar of *Gracilaria verrucosa*[6,7].

Because of the very low proportion of 4-*O*-methyl-L-galactopyranose present in the agar that they investigated, Araki *et al.*[1] surmized that it had no structural significance. Craigie *et al.*[5] and Craigie and Wen[8] suggested that this sugar residue may be attached as a side group, because the ratio of L to D units of their species

*Presented at the IXth International Symposium on Glycoconjugates, Lille, July 6–11, 1987.
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0008-6215/89/$ 03.50

lies close to unity, reflecting the fundamental repeating disaccharide structure. They pointed out that the gel strength of agars diminishes markedly with increasing content of 4-*O*-methyl-L-galactopyranose and that its content depends on the tissue age. Hirase[9] also reported side chains of D-galactopyranosyl groups in the agaroid from *Laurencia undulata*.

Although the natural abundance of 4-*O*-methyl-L-galactopyranose in agar from *Gracilaria verrucosa* is low, we were able to enrich certain fractions with this residue. The isolation of highly substituted portions by means of enzymic degradation permitted the elucidation of its branching point.

EXPERIMENTAL

General methods. — *Gracilaria verrucosa* was collected in Wimereux, Northern France, and cultivated as described[10]. Agar polymers were extracted from the main axis, cultivated under growth promoting conditions, by a combined water and ethanol–water extraction procedure[7]. The 6-*O*-methyl- and 4-*O*-methyl-galactopyranose, as well as galactopyranose contents of the polymer were determined by g.l.c. analysis of the alditol acetates, prepared[11] after acid hydrolysis (2M trifluoroacetic acid, 2 h at 100°). *meso*-Inositol was the internal standard (100 μg/mg of fraction). The alditols were separated with a Girdel 300 Chromatograph (Delsi) equipped with a CP-Sil 5CB (Chrompack) capillary column (0.32 mm × 50 m), a flame-ionization detector, an evaporator–injector, and N_2 as the carrier gas. Mass spectra were recorded with a Ribber 10-10 mass spectrometer using an electron energy of 70 eV and an ionization current of 0.2 mA.

Solutions of the different fractions in D_2O (1–4%) were analyzed with a Brucker AM400 spectrometer equipped with a 5-mm dual probe. Proton-decoupled ^{13}C-n.m.r. spectra (100.62 MHz) were recorded at 30° for the monosaccharides and oligomers, and at 80° for the polymers. Chemical shifts (δ) were measured relative to the signal of internal dimethyl sulfoxide (δ 39.6), and converted into values relative to external tetramethylsilane. The distortionless enhancement by polarization-transfer (DEPT) pulse technique was used as described[12].

The carbohydrate content of various fractions was estimated by the phenol–H_2SO_4 reagent. T.l.c. of oligosaccharides was performed on silica gel plates with 2:1:1 butanol–acetic acid–water during 6 h. Oligosaccharides were revealed with an orcinol–H_2SO_4 reagent (orcinol 0.1% in H_2SO_4 20%).

Methylation analysis was carried out according to Paz Parente *et al.*[13]. The spectra were interpreted by use of the data of Fournet *et al.*[14] for the methylated hexosides, and the data of Chizov *et al.*[15] for the derivatives of 3,6-anhydro-galactopyranose.

Enzymic degradation. — Highly substituted fragments of agar polymers were obtained by enzymic degradation with β-agarase I (EC 3.2.1.81) from *Pseudomonas atlantica,* kindly provided by Dr. W. Yaphe. The enzyme was prepared and the

digestion carried out according to Morrice *et al.*[16]. The enzyme-resistant fraction (E.R.F.) was precipitated with ethanol (3 vols.) The supernatant solution was evaporated and the oligosaccharides fractionated on a column (130 × 1.6 cm; distilled water) of Bio-Gel P-2 (Fig. 1).

RESULTS AND DISCUSSION

A 4-*O*-methylhexose was first identified by g.l.c.–m.s. (alditol acetate derivative) as part of the agar extracted from *Gracilaria verrucosa.* This sugar had the same retention time on g.l.c. and the same mass fragmentation pattern as authentic 4-*O*-methyl-L-galactopyranose (obtained by courtesy of Dr. J. S. Craigie, Atlantic Research Laboratory, Halifax, Canada). The ^{13}C-n.m.r. chemical shifts of this sugar and its methyl glycoside derivative were compared with those of α-D-galactopyranose and methyl α-D-galactopyranoside, and with those of 4-*O*-linked α-L-galactopyranose in the desulfated agar precursors[17]. These data were useful in determining the ^{13}C-n.m.r. chemical shifts of 4-*O*-methyl-α-L-galactopyranosyl side groups (Table I, Figure 2a). The L-form of this sugar was assumed by analogy to 4-*O*-methyl-L-galactopyranose found in *G. tikvahiae*[8].

The ^{13}C-n.m.r. spectra (see Table II) established that agarobiose was the main repeat unit in the agar polymer with a low amount of its methylated analog; several unknown resonances probably correspond to 4-*O*-methyl-α-L-galactopyranosyl groups. The 12 signals attributed to agarobiose were in agreement with earlier data[18,19], as well as the resonances for 6-*O*-methylgalactopyranose units.

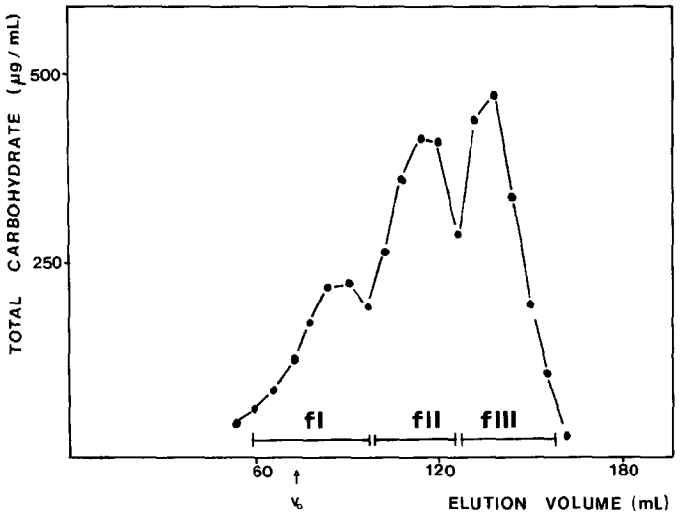


Fig. 1. Fractionation of oligosaccharides obtained by enzymic degradation of agar by β-agarase I, on a Bio-Gel P-2 (column 130 × 1.6 cm), equilibrated in water. Total carbohydrate was estimated with the phenol–sulfuric acid reagent.

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS ASSIGNMENTS OF 4-*O*-METHYL-α-L-GALACTOSE AND REFERENCE COMPOUNDS

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*OMe-1*	*OMe-4*
α-D-Galactopyranose[a]	93.20	69.40	70.20	70.30	71.40	62.20		
4-*O*-Methyl-α-L-galactopyranose	92.91	69.41	70.36	80.53	71.49	61.44		62.08
Methyl α-D-galactopyranoside[a]	100.10	69.20	70.50	70.20	71.60	62.20	56.00	
Methyl 4-*O*-methyl-α-L-galactopyranoside	100.13	69.33	70.74	80.56	71.80	61.55	55.86	62.07
α-L-Galactopyranose in the desulfated agar precursor[b]	100.94	69.37	71.04	79.32	72.20	61.18		

[a]From ref. 23. [b]From ref. 17.

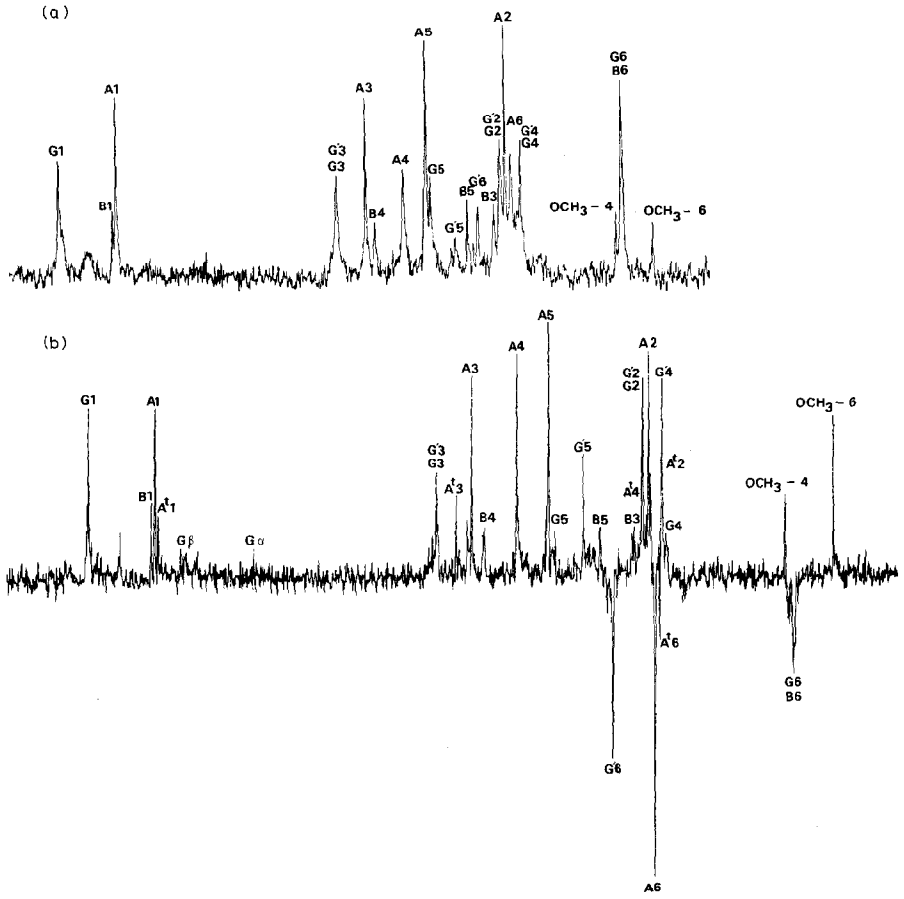


Fig. 2. (a) ^{13}C-N.m.r. spectrum of the polymer recorded at 80°, 28 500 scans, spectral width 22 727 Hz, and acquisition time of 0.360 s. (b) ^{13}C-N.m.r. spectrum of oligosaccharides (DEPT sequence) at 30°, 61 522 scans, 23 809 Hz, and 0.344 s.

In order to obtain highly substituted portions of agar, it was degraded with β-agarase I. The resistant fraction (E.R.F.) represented 14.9% of total agar. The major fractions obtained on Bio-Gel P-2 (Fig. 1) contained neoagarotetraose (FIII, 36.9%) and neoagarohexaose (FII, 24.2%). Both of them contained little amount of the corresponding 6-*O*-methyl-galactopyranose oligosaccharides, as established by t.l.c. FI (13.7%) was enriched in 4-*O*-methylgalactopyranose and had a composition similar to that of E.R.F. (Table III).

E.R.F. contained α-L-galactopyranose 6-sulfate, as shown by ^{13}C-n.m.r. spectroscopy. As the 4-*O*-methyl-L-galactopyranose content of FI was similar to that of E.R.F., the present investigation was continued with this fraction, which presented the advantages that no sulfate was detected and the oligosaccharides

TABLE II

^{13}C-N.M.R. CHEMICAL SHIFTS (δ)[a] OF *Gracilaria verrucosa* AGAR POLYMER

Unit[b]	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*OMe-6*	*OMe-4*
G	102.40	70.27	82.29	68.78	75.37	61.45		
A	98.32	69.91	80.16	77.39	75.67	69.49		
G'				69.01	73.62	71.87	59.10	
B	98.73		70.73	79.46	72.65	61.44		61.84
A^t	98.29	69.74	81.05	70.16	77.50	69.19		

[a]At 80°. [b]*G, A, A*^{t}, *G'*, and *B* refers to D-galactopyranose, 3,6-anhydro-L-galactopyranose, 3,6-anhydro-L-galactopyranose at the nonreducing end, 6-*O*-methyl-D-galactopyranose, and 4-*O*-methyl-L-galactopyranose units, respectively (see Schemes 1 and 2).

TABLE III

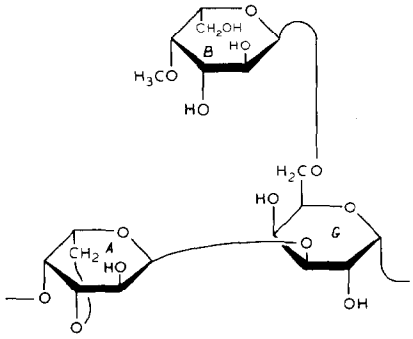
MOLAR RATIOS[a] OF THE POLYMER AND FRACTIONS OBTAINED BY ENZYMIC DEGRADATION

Material	*6-OMe-D-Gal*	*4-OMe-L-Gal*	D-*Gal*	*Percent of total agar*
Agar	0.33	0.12	1	
E.R.F.	1.07	0.36	1	14.9
FI	0.87	0.29	1	13.7
FII	0.05	0.01	1	24.2
FIII	0.02		1	36.9

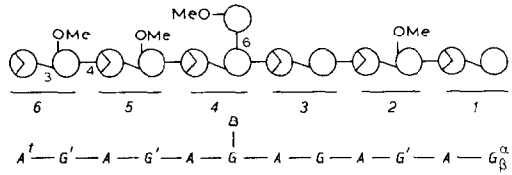
[a]Relative to one residue of galactose.

were water soluble at room temperature. The ^{13}C-n.m.r. spectrum of FI were compared with that for neoagarohexaose[20]. The DEPT sequence (Fig. 2b) permitted assignments of C-6 for units *G, G', A,* and *A*^{t} (see Schemes 1 and 2 for naming of units). The signal at δ 61.43 is broad owing to C-6 of *B* units. It is interesting to note that the position of the OCH_3 resonance of *B* units differs from that of *G'* units. The signals for C-3, C-4, and C-5 were assigned by comparison with data obtained from reference compounds (Table I). The oligosaccharides are terminated at the nonreducing end by a 3,6-anhydro-α-L-galactopyranosyl group (*A*^{t}). By comparison with the neoagarohexaose spectrum, an additional resonance in the anomeric region at δ 98.73 was observed, probably due to C-1 of the *B* unit. An upfield shift (~2 p.p.m.) for the signal of C-1 of an α-L-rhamnosyl group has been reported[21,22], when this sugar is linked to O-6 of a β-D-galactopyranose unit instead of C-3 or C-4; this phenomenon may explain the upfield shift of the signal for C-1 of the *B* unit, δ 98.73, instead of δ 100.94 reported[17] for 4-*O*-linked α-L-galactopyranose units of the desulfated biological precursor and δ 100.13 for methyl 4-*O*-methyl-α-L-galactopyranoside.

The branching point of the *G* residue was established by the permethylation analysis, after comparison of the methyl ethers obtained by permethylation, methanolysis, and acetylation of reduced FI and neoagarohexaitol (Fig. 3). Methyl 2,4,6-tri-*O*-methylgalactopyranoside and 1,2,4,5,6-penta-*O*-methylgalactitol were



Scheme 1. The structure of the branching point of the 4-*O*-methyl-L-galactopyranose unit.



Scheme 2. Schematic representation of the proposed structure for oligosaccharides in FI.

the main methyl ethers obtained from neoagarohexaitol. Several minor compounds were observed, which corresponded to derivatives of 3,6-anhydrogalactopyranose, four of them were identified by g.l.c.–m.s. as methyl 3,6-anhydro-2-*O*-methyl-galactopyranoside, methyl 3,6-anhydro-2,4-di-*O*-methylgalactopyranoside, 3,6-anhydro-2-*O*-methylgalactopyranose dimethylacetal, and 3,6-anhydro-2,4-di-*O*-methylgalactopyranose dimethylacetal.

Permethylation analysis of reduced oligosaccharides (FI) showed the presence of methyl 2,3,4,6-tetra-*O*-methylgalactopyranoside and an equivalent amount of methyl 2,4-di-*O*-methylgalactopyranoside, in addition to the derivatives observed for the hexasaccharide. These two methyl ethers correspond to 4-*O*-methylgalactopyranose and its branching point (C-6 of D-galactopyranose unit *G*), respectively (Scheme 1). The degree of polymerisation of FI (expressed as the number of repeating units), calculated from integration areas, averaged six. The molar ratio of this fraction (Table III) corresponds to this calculation.

The use of β-agarase I from *Pseudomonas atlantica* permitted the isolation of oligosaccharides enriched in 4-*O*-methyl-L-galactopyranosyl side groups. 4-*O*-Methyl-L-galactopyranose was shown to occur as a single branch-unit, attached to position O-6 of the 3-*O*-linked D-galactopyranose residues of the main chain. As the content of this sugar is higher in E.R.F. and FI, it seems to prevent degradation by β-agarase I, probably because of steric hindrance. It seems that substitutions at O-6 of the D-galactopyranose units occurred in blocks averaging six contiguous units containing three 6-*O*-methyl-D-galactopyranose and three D-galactopyranose

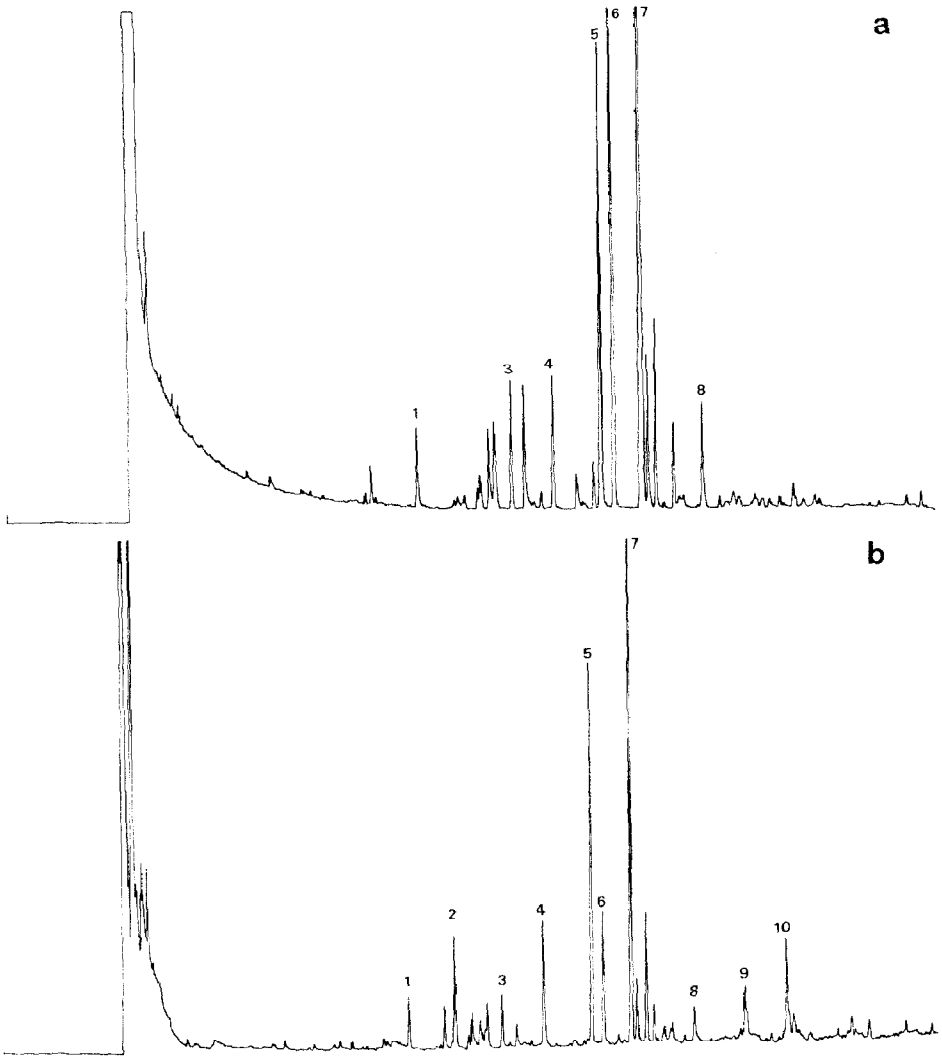


Fig. 3. G.l.c. analysis of the methyl ethers obtained by permethylation, methanolysis, and acetylation of (a) reduced FI, and (b) neoagarohexaitol: (1) Methyl 3,6-anhydro-2,4-di-*O*-methylgalactopyranoside, (2) methyl 2,3,4,6-tetra-*O*-methylgalactopyranoside, (3) methyl 3,6-anhydro-2-*O*-methylgalactopyranoside, (4) 3,6-anhydro-2,4-di-*O*-methylgalactopyranose dimethylacetal, (5) methyl 2,4,6-tri-*O*-methylgalactopyranoside, (6) 1,2,4,5,6-penta-*O*-methylgalactitol, (7) methyl 2,4,6-tri-*O*-methylgalactopyranoside, (8) 3,6-anhydro-2-*O*-methylgalactopyranose dimethylacetal, (9) methyl 2,4-di-*O*-methylgalactopyranoside, and (10) methyl 2,4-di-*O*-methylgalactopyranoside.

residues, one of them bearing the 4-*O*-methyl-L-galactopyranose unit (Scheme 2). As all oligosaccharides are terminated, at their reducing end, by unsubstituted neoagarobiose[16], one residue of D-galactopyranose remains unsubstituted. The specificity of β-agarase I suggests that this unsubstituted repeating unit is adjacent

to be branched residue and that the side groups prevent recognition of the internal neoagarobiose. The use of β-agarase II, which cleaves methylated agarose sequences and produces disaccharides, will be helpful to extend this investigation.

REFERENCES

1 C. ARAKI, K. ARAI, AND S. HIRASE, *Bull. Chem. Soc. Jpn.*, 40 (1967) 959–962.
2 A. J. FARRANT, J. R. NUNN, AND H. PAROLIS, *Carbohydr. Res.*, 19 (1971) 161–168.
3 A. J. ALLSOBROOK, J. R. NUNN, AND H. PAROLIS, *Carbohydr. Res.*, 36 (1974) 139–145.
4 M. DUCKWORTH, K. C. HONG, AND W. YAPHE, *Carbohydr. Res.*, 18 (1971) 1–9.
5 J. S. CRAIGIE, Z. C. WEN, AND J. P. VAN DER MEER, *Bot. Mar.*, 27 (1984) 55–61.
6 C. J. BIRD, R. J. HELLEUR, E. R. HAYES, AND J. MCLACHLAN, *Hydrobiologia*, 151–152 (1987) 207–211.
7 M. ONDARZA, Y. KARAMANOS, D. CHRISTIAEN, AND T. STADLER, *Food Hydrocolloids*, 5–6 (1987) 507–509.
8 J. S. CRAIGIE AND Z. C. WEN, *Can. J. Bot.*, 62 (1984) 1665–1670.
9 S. HIRASE, *Abstr. Int. Seaweed Symp., 11th*, (1983) 93.
10 D. CHRISTIAEN, Y. KARAMANOS, T. STADLER, AND H. MORVAN, *Sci. Eau*, 6 (1987) 121–135.
11 J. H. SLONEKER, *Methods Carbohydr. Chem.*, 6 (1972) 20–24.
12 D. M. DODDRELL, D. T. PEGG, AND M. R. BENDALL, *J. Magn. Reson.*, 48 (1982) 323–327.
13 J. PAZ PARENTE, P. CARDON, Y. LEROY, J. MONTREUIL, B. FOURNET, AND G. RICART, *Carbohydr. Res.*, 141 (1985) 41–47.
14 B. FOURNET, G. STRECKER, Y. LEROY, AND J. MONTREUIL, *Anal. Biochem.*, 116 (1981) 489–502.
15 O. S. CHIZHOV, B. M. ZOLOTAREV, A. I. USOV, M. A. RECHTER, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 16 (1971) 29–38.
16 L. M. MORRICE, M. W. MCLEAN, W. F. LONG, AND F. B. WILLIAMSON, *Eur. J. Biochem.*, 133 (1983) 673–684.
17 M. LAHAYE, W. YAPHE, AND C. ROCHAS, *Carbohydr. Res.*, 143 (1985) 240–245.
18 M. LAHAYE, C. ROCHAS, AND W. YAPHE, *Can. J. Bot.*, 64 (1986) 579–585.
19 A. I. USOV, E. G. IVANOVA, AND A. S. SHASKOV, *Bot. Mar.*, 26 (1983) 285–294.
20 C. ROCHAS, M. LAHAYE, W. YAPHE, AND M. T. P. VIET, *Carbohydr. Res.*, 148 (1986) 199–207.
21 B. A. DMITRIEV, A. V. NIKOLAEV, A. S. SHASHKOV, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 100 (1982) 195–206.
22 L. V. BACKINOWSKY, N. F. BALAN, A. S. SHASHKOV, AND N. K. KOTCHETKOV, *Carbohydr. Res.*, 84 (1980) 225–235.
23 K. BOCK AND C. PEDERSEN, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–65.