Carbohydrate Research, 148 (1986) 199–207
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

^{13}C-N.M.R.-SPECTROSCOPIC INVESTIGATION OF AGAROSE OLIGOMERS

CYRILLE ROCHAS*, MARC LAHAYE, WILFRED YAPHE,
Department of Microbiology and Immunology, McGill University, 3775 University Street, Montréal, P.Q. H3A 2B4 (Canada)

AND MINH TAN PHAN VIET
Département de Chimie, Université de Montréal, Montréal, P.Q. H3C 3V1 (Canada)

(Received July 5th, 1985; accepted for publication, November 4th, 1985)

ABSTRACT

A complete, unambiguous assignment of all of the ^{13}C-n.m.r.-spectral signals of agarose oligomers produced by enzymic hydrolysis has been achieved. The ^{1}J ^{13}C-H coupling constants are reported, and the chemical shifts and coupling constants of both the agarose polymer and oligomers are compared.

INTRODUCTION

Agarose[1], a gel-forming polysaccharide extracted from the cell wall of certain red algae, is composed of a repeating unit called neoagarobiose, namely, *O*-3,6-anhydro-α-L-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→3) (see Fig. 1). Agarose represents an extreme in structure[2], because different types of substituent groups can be found on the repeating unit, thereby dramatically influencing the physical properties of the polymer[3,4]. ^{13}C-N.m.r. spectroscopy has been shown to be a rapid method for structural investigation of agarose[5], and assignments of the signals of carbon atoms were made from comparison of chemical shifts observed in the spectra of model compounds, and by applying the classical, n.m.r.-spectral rules of substitution[6–9]. However, such model compounds as methyl 3,6-anhydro-α-D-galactopyranoside and methyl β-D-galactopyranoside do not exactly represent the unit sugars in the repeating unit of the polymer, and we have recently shown that errors in assignments could be avoided by taking oligomers of a polysaccharide as model compounds[10,11].

*To whom correspondence should be addressed. Present address: CERMAV-CNRS, B.P. 68, F-38402 Saint Martin d'Hères, France.

0008-6215/86/$ 03.50

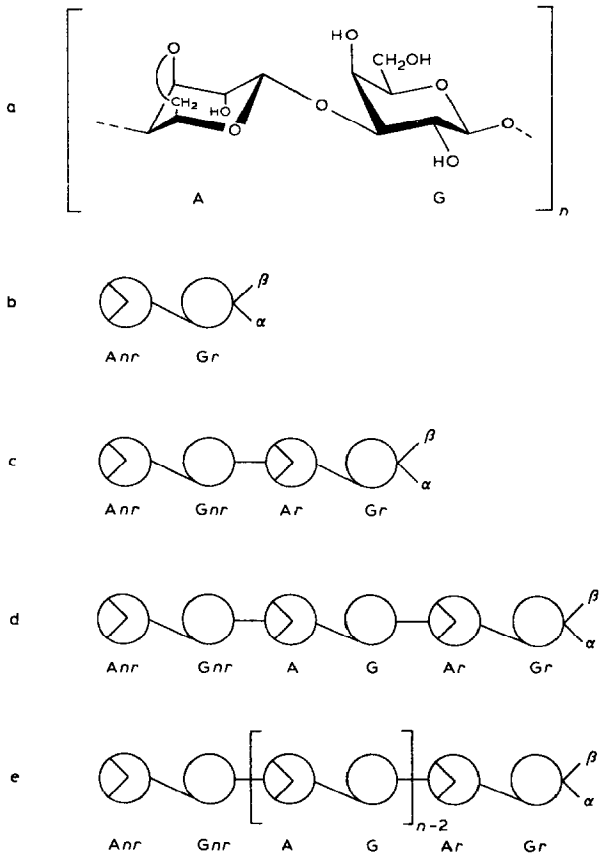

Fig. 1. Repeating disaccharide of agarose (a), and simplified structures of agarose oligomers of d.p. 1 (b), d.p. 2 (c), d.p. 3 (d), and d.p. *n* (e).

MATERIALS AND METHODS

A 0.5% solution of Seakem agarose (lot 291402, Marine Colloid, Rockland, Maine, U.S.A.) in distilled water was hydrolyzed by an excess of β-agarase for several days at 42°. (The enzyme had been purified from the cell-free supernatant solution of a culture of *Pseudomonas atlantica*, according to the methods of Duckworth and Yaphe[12] or Morrice *et al.*[13].) The hydrolyzate was separated on a column (100 × 2.4 cm) of Bio-Gel P-2 (Bio-Rad Laboratories) with distilled water as the eluant at a flow rate of 40 mL/h, the elution (see Fig. 2) being monitored by a refractometer (Pharmacia Fine Chemicals), and the oligosaccharide eluates recovered were concentrated *in vacuo* and freeze-dried.

Proton-decoupled ^{13}C-n.m.r. spectra were recorded with a Bruker WH-400 spectrometer at 100.6 MHz. Samples dissolved in D_2O (4–5%, w/v) were analyzed at 30 or 80° under the following conditions: sweep width, 10 kHz; pulse width, 60°

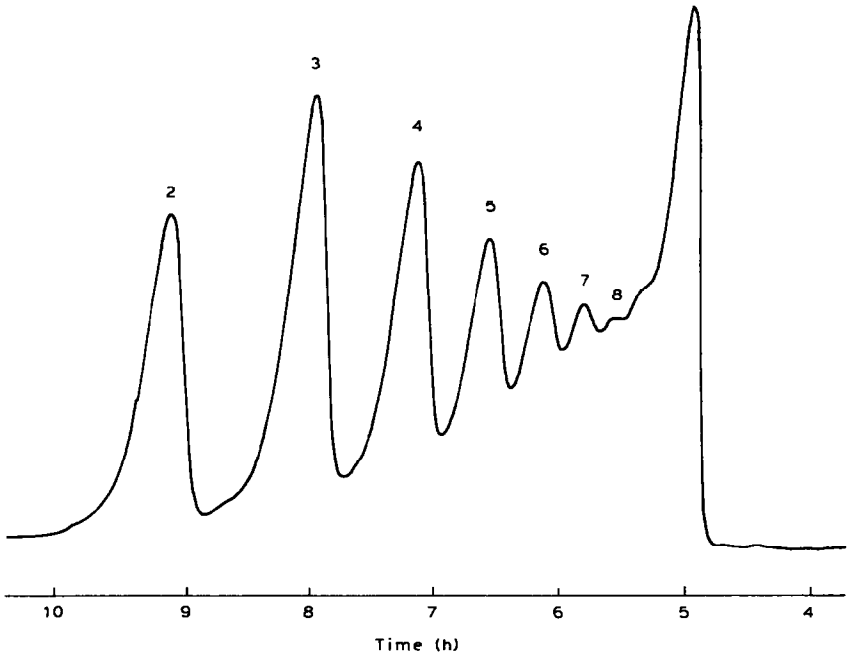

Fig. 2. Chromatogram of agarose oligomers separated by Bio-Gel P-2 (see text for conditions).

(10–15 μs); acquisition time, 0.8 s on 16 k data points; 350 and 1500 transients for oligomers and polymer, respectively. Chemical shifts (p.p.m.) were measured relative to internal dimethyl sulfoxide, and converted to values relative to external Me_4Si (conversion constant, 39.6).

The ^{13}C-^{1}H, polarization transfer, *J*-spectroscopy pulse sequence[14] was used, and 160 transients were collected for each of the 256 values of the evolution period. The spectral widths for F_1 and F_2 were 360 and 5000 Hz, respectively; 8 k data points were collected in F2 (acquisition time: 0.82 s) and the relaxation delay was 1.5 s. A sine-bellfunction was applied in both directions and the data matrix was zero-filled to 8 k × 1 k before the Fourier-transform (F.t.), giving a digital resolution of 0.7 and 1.22 Hz/point in F_1 and F_2, respectively.

The semiselective, proton-flip experiment employed the pulse sequence of Bax[15] and Ruther[16], with a phase cycling to eliminate the long-range couplings; 160 transients were collected for each of the 256 values of the evolution period. The spectral widths for F_1 and F_2 were, respectively, 400 and 4500 Hz; the acquisition time was 0.9 s (8 k points in F2); the relaxation delay was 5 s and the value of *J* selected was 150 Hz. A sine-bellfunction was applied in both dimensions, and the data matrix was zero-filled to 8 k × 2 k before F.t., giving a digital resolution of 391 mHz/point in F_1.

RESULTS AND DISCUSSION

Nomenclature. — In the repeating unit of agarose (see Fig. 1), G represents the 3-*O*-linked β-D-galactopyranose (circle) and A represents the 4-*O*-linked 3,6-anhydro-α-L-galactopyranose (sectioned circle). A*r* and G*r* denote residues at the reducing end (*r*) of the repeating disaccharide, where G*r* is present as the α (G*r*α) or β (G*r*β) anomer. The sugars in the repeating disaccharide at the nonreducing end (*nr*) are represented by A*nr* and G*nr*. The internal repeating units of oligosaccharides having a degree of polymerization (d.p.) equal to, or higher than, 3, and of the polymer, are respectively denoted by A and G. The structures and symbols in this report were previously used for the galactan series[17]. The oligomers (see Fig. 1) having d.p. 1, 2, 3, . . . are currently named neoagarobiose, neoagarotetraose, neoagarohexaose, . . . in the literature.

Assignment of signals in the ^{13}C-n.m.r. spectra of oligomers. — The assignments of signals in the ^{13}C-n.m.r. spectra relied, in part, on their intensity. For quantitative analysis, experimental conditions and data processing should be taken into consideration[18]. For the purpose of signal identification, we found that the conditions for optimum signal/noise (*i.e.*, tip pulse-angle 60°, repetition time $\sim 1T_1$) were sufficient. The spectra from oligomers of d.p. 1, 2, 3, and 4 are depicted in Fig. 3, and those from oligomers of d.p. 5 and 6 are omitted, as they show similar patterns, only with different intensities. A line-broadening function of 5–7 Hz was applied before F.t., in order to equalize the line width of the signals, and the relative intensity could be obtained from the peak height. This line-broadening obscured the small chemical-shift differences, but a resolution-enhancement function (Gaussian multiplication) and zero-filling of the data in order to obtain a digital resolution of better than 0.2 Hz/point allowed the observation of very small chemical-shift differences. The DEPT spectrum confirmed the assignment of the methylene carbon atoms.

Oligomer having d.p. 1: A*nr*-G*r*. — The assignment (see Table I) of the 18 peaks (6 for A*nr*, 6 for G*r*α, and 6 for G*r*β) of the disaccharide of d.p. 1, namely, A*nr*-G*r*, was straightforward. The transformed spectrum, with a 5–7-Hz line-broadening, is similar to one published by Hamer *et al.*[19]. From their relative intensity, the signals corresponding to each unit (G*r*α, G*r*β, and A*nr*) were identified, and the complete assignment could be achieved by using the chemical-shift data of the monomer units α,β-D-galactopyranose[20] and methyl 3,6-anhydro-α-D-galactopyranoside[21], and the usual substituent-effect parameters. The same ratio α/β (40:60) of the G*r* unit was observed for the oligomers, and this ratio is of the same order as those obtained for oligomers of *kappa*-[10] and *iota*-[11]carrageenan. This well resolved, transformed spectrum revealed that the orientation of the anomeric hydroxyl group had a small effect on the shifts of the carbon atoms in the adjacent unit, namely, A*nr*. The signals of C-1 and C-4 were split by 0.04 and 0.025 p.p.m. (4 and 2.5 Hz), while the lines for other carbon atoms were broader. This spectral characteristic allowed the assignment of the unit A adjacent to the

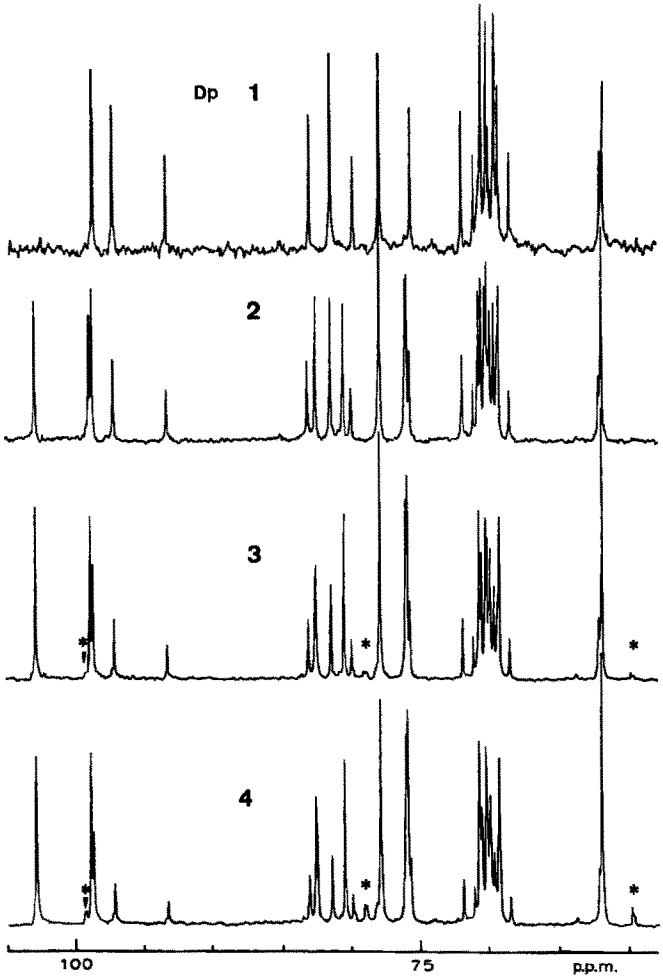

Fig. 3. ^{13}C-N.m.r. spectra of agarose oligomers of d.p. 1 (1), d.p. 2 (2), d.p. 3 (3), and d.p. 4 (4). (*Refers to signals in the neoagarobiose repeating unit methylated on O-2 of the anhydrogalactose; see ref. 6.)

reducing-terminal unit, G*rα,β*. Direct comparison of the spectrum from the oligomer having d.p. 1 with those of model compounds was sufficient to allow precise assignment of the signals in the spectrum; this was possible because the signals of each type of sugar had different intensities, and did not overlap. However, this was not possible for oligomers of higher d.p.

*Oligomer having d.p. 2: A*nr-*G*nr-*A*r-*G*r. — The well resolved spectrum of the oligomer having d.p. 2 contained 32 signals. The signals of the terminal unit G*rα/β* were readily identified, and had practically the same chemical shifts as those observed for d.p. 1; these signals were always present, and their relative intensities

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS[a], AT ROOM TEMPERATURE, OF AGAROSE OLIGOMERS

D.p.	*Unit*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
1	G*rβ*	97.11	71.91	82.86	69.20	75.55	61.76
	G*rα*	93.11	68.38	79.67	69.84	70.99	61.94
	A*nr*(*β*)	98.52	70.04	81.33	70.44	77.55_5	69.45_5
	A*nr*(*α*)	98.56	70.04	81.33	70.46_5	77.55_5	69.45_5
2, 3, or 4							
	G*nr*	102.69	70.58	82.44	69.08(.09)	75.72	61.75
	G	102.69	70.56	82.50	69.08	75.72	61.75_5
	G*rβ*	97.10	71.87	82.91(.92)[a]	69.19(.20)	75.53(.54)	61.75_5
	G*rα*	93.09	68.36	79.75	69.83	70.97	61.94
	A*nr*	98.54(.55)[a]	70.00	81.32	70.42	77.75	69.45
	A	98.73	70.10	80.39	77.69	75.89	69.72
	A*r*(*β*)	98.73	70.10(.12)[a]	80.39	77.69(.70)[a]	75.89(.88)[a]	69.72
	A*r*(*α*)	98.76(.77)	70.10(.13)	80.39	77.69(.72)	75.89(.88)	69.72

[a]The numbers in parentheses are the decimal values for the oligomer of d.p. 2. A (*β*) or A (*α*) means that the sugar is linked to galactose G*rα* or G*rβ*.

became smaller in the spectra of the higher oligomers. The signals of the adjacent unit, A*r*, were identified by the small effects of the anomeric hydroxyl group on the chemical shift of C-1, C-2, and C-4; these signals were separated by 0.04, 0.01, and 0.02 p.p.m. (4.0, 1.4, and 2.2 Hz), respectively, from those of the A*nr* unit, while the signals of C-3, C-5, and C-6 were broader. Due to the presence of the (1→4) linkage, the chemical shifts of C-3, C-4, and C-5 of A*r* were significantly different from those of the unit A*nr*. The substituent effect was −0.9, +7.3, and +1.9 p.p.m. for C-3, C-4, and C-5, respectively. The signals for C-6 of the A*r* and A*nr* units, assigned at 69.46 and 69.72 p.p.m. respectively, were identified from the DEPT spectrum, and by comparison with the assignments for d.p. 1. The signals of the nonreducing-end unit, A*nr*, of d.p. 2 and higher oligomers had practically the same chemical shifts as those of those of d.p. 1 (see Table I). The remaining signals belonged to the G*nr* unit, which was flanked by two A units. As expected from the additional linkage (which did not exist on the G*rβ* unit), the respective signals of C-1 and C-2 had different chemical shifts, while the signals of the other carbon atoms remained unchanged. The complete assignment for d.p. 2 is given in Table I. Carbon atoms 3, 5, and 6 of the A*r* unit are involved in the stiff anhydro-bridge and remained insensitive to the anomeric configuration, *α* or *β*, at the reducing end.

Oligomers having d.p. 3 and higher. — Compared to d.p. 2, these oligomers have additional, internal, repeating units, labelled A and G. These sugars have the same environment and type of glycosidic linkages as A*r* and G*nr*, and therefore the signals of these respective units should have the same chemical shifts. In fact, in the well resolved spectrum, only very small differences in the chemical shifts between the signals of G and G*nr* were observed, and the carbon atoms of A had exactly

TABLE II

^{13}C-N.M.R. CHEMICAL SHIFTS AT 80° OF UNITS A AND G OF THE OLIGOMER OF D.P. 4, AND OF THE POLYMER

Sugar	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
G in oligomer	102.45	70.31	82.24	68.80	75.40	61.48
G in polymer	102.44	70.27	82.24	68.79	75.55	61.43
A in oligomer	98.31	69.93	80.15	77.39	75.68	69.46
A in polymer	98.24	69.92	80.13	77.36	74.67	69.43

(within ±0.01 p.p.m.) the same chemical shifts as those for A*r*. For oligomers of higher d.p., no difference was observed, except for the signals of units A and G, which became increasingly more intense over those of the reducing and non-reducing end-units as the d.p. increased. In fact, the G*nr* and A*r* units of the oligomers of d.p. 2 had chemical shifts very close to those of units A and G of oligomers of higher d.p., and, probably, those of the polymer. Similarity in the chemical shifts of oligomers of d.p. >2 and those of the polymer was shown at a temperature of 80° (see Table II); this comparison cannot be made at lower temperatures, as the polymer is then a gel[22]. The chemical shifts of the A and G units of the polymer and oligomers were identical within experimental error. From these observations, it may be concluded that the different degrees of freedom, and the environment (electronic, hydration, *etc.*), of the A and G units of the oligomer and polymer are identical at high temperatures, although the molecular size and viscosity are different.

^{1}J ^{13}*C-H Coupling constants.* — The ^{1}J ^{13}C-H coupling constants were obtained from the coupled spectra (regular and INEPT) of d.p. 2 and 3, and from the 2D, *J*-resolved spectrum of d.p. 3. These oligomers were chosen because features of the central and terminal units could be readily observed. The coupling values deduced from different spectra are identical within experimental error. The coupling constants of the well separated carbon atoms were measured with higher precision on the coupled spectra. The ^{1}J ^{13}C-H values for the oligomers of d.p. 2 and 3 were identical, and are reported in Table III. Most of the coupling constants observed for the anhydrogalactose were higher than those for the galactose, probably resulting from the ring tension of the anhydro bridge. These values confirmed the carbon chemical-shift assignments and are in agreement with the few reported for the polymer by Nicolaïson *et al.*[8].

CONCLUSION

The ^{13}C-n.m.r. chemical shifts of agarose oligomers reported herein verified the assignments for the polymer, and will help in the location, by ^{13}C-n.m.r. spectroscopy, of substituent groups that can occur in agar polymers. These small oligosaccharides proved to be good models to use for study of the parent polymer,

TABLE III

$^1J_{C\text{-}H}$ VALUES (Hz) OF AGAROSE OLIGOMERS HAVING D.P. 2 AND 3

Unit	$J_{C\text{-}1,H\text{-}1}$	$J_{C\text{-}2,H\text{-}2}$	$J_{C\text{-}3,H\text{-}3}$	$J_{C\text{-}4,H\text{-}4}$	$J_{C\text{-}5,H\text{-}5}$	$J_{C\text{-}6,H\text{-}6}$
G*nr*	160.9	151.1	152.1[a]	147.0	141.6	144.9
G	160.9	153.6	146.1[a]	147.0	141.6	144.9
G*rβ*	162.0	149.9	145.6	148.3	141.3	144.9
G*rα*	168.4	146.9	145.0	152.9	143.3	144.1
A*nr*	165.8	149.6	160.1	157.9	161.5	152.6
A	165.8	150.4	162.0	158.8	162.1	149.3
A*r*(*β*)	165.8	150.4	162.0	158.8	162.1	149.3
A*r*(*α*)	165.7	150.4	162.0	158.8	162.1	149.3

[a]These assignments may have to be reversed.

as previously demonstrated for carrageenan. The chemical-shift values indicated that agarose oligomers and agarose are composed of equivalent repeating units (structure and conformation), with the exception of A*nr* and G*r* (at the non-reducing and reducing end, respectively).

ACKNOWLEDGMENT

This study was supported by a grant from the Natural Sciences and Engineering Research Council, Canada.

REFERENCES

1 C. ARAKI, *Proc. Int. Seaweed Symp., 5th,* Pergamon Press, Oxford, 1966, pp. 3–17.
2 M. DUCKWORTH AND W. YAPHE, *Carbohydr. Res.*, 16 (1971) 189–197.
3 S. ARNOTT, A. FULMER, W. E. SCOTT, I. C. M. DEA, R. MOORHOUSE, AND D. A. REES, *J. Mol. Biol.*, 90 (1974) 269–284.
4 K. B. GUISELEY, *Carbohydr. Res.*, 13 (1970) 247–256.
5 A. I. USOV, *Bot. Mar.*, 27 (1984) 189–202.
6 A. I. USOV, S. V. YAROTSKY, AND A. S. SHASHKOV, *Biopolymers*, 19 (1980) 977–990.
7 A. I. USOV, E. G. IVANOVA, AND A. S. SHASHKOV, *Bot. Mar.*, 26 (1983) 285–294.
8 F. M. NICOLAÏSEN, I. MEYLAND, AND K. SCHAUMBURG, *Acta Chem. Scand., Ser. B*, 34 (1980) 103–107.
9 M. LAHAYE, W. YAPHE, AND C. ROCHAS, *Carbohydr. Res.*, 143 (1985) 240–245.
10 C. ROCHAS, M. RINAUDO, AND M. VINCENDON, *Int. J. Biol. Macromol.*, 5 (1983) 111–115.
11 C. GREER, C. ROCHAS, AND W. YAPHE, *Bot. Mar.*, 28 (1985) 9–14.
12 M. DUCKWORTH AND W. YAPHE, *J. Chromatogr.*, 49 (1970) 482–487.
13 L. M. MORRICE, M. W. MCLEAN, F. B. WILLIAMSON, AND W. F. LONG, *Eur. J. Biochem.*, 135 (1983) 553–558.
14 D. M. THOMAS, M. R. BENDALL, D. T. PEGG, D. M. DODDRELL, AND J. FIELD, *J. Magn. Reson.*, 42 (1981) 298–306.
15 A. BAX, *J. Magn. Reson.*, 52 (1983) 330–334.
16 V. RUTAR, *J. Magn. Reson.*, 56 (1984) 87–100.
17 K. YOUNG, K. C. HONG, M. DUCKWORTH, AND W. YAPHE, *Proc. Int. Seaweed Symp., 7th,* 1971, 469–477.

18 C. H. SOTAK, C. L. DUMOULIN, AND G. C. LEVY, *Top. ^{13}C Spectrosc.*, 4 (1984).
19 G. K. HAMER, S. S. BHATTACHARJEE, AND W. YAPHE, *Carbohydr. Res.*, 54 (1977) c7–c10.
20 P. A. J. GORIN AND M. MAZUREK, *Can. J. Chem.*, 53 (1975) 1212–1223.
21 F. BALZA, N. CYR, G. K. HAMER, A. S. PERLIN, H. J. KOCH, AND R. S. STUART, *Carbohydr. Res.*, 59 (1977) c7–c11.
22 F. M. NICOLAISEN, I. MEYLAND, AND K. SCHAUMBURG, *Acta Chem. Scand., Ser. B*, 34 (1980) 579–583.