*Carbohydrate Research*, 177 (1988) 43–50
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands


# ISOLATION AND STRUCTURE OF A NEW OLIGOSACCHARIDE FORMED AS A BY-PRODUCT DURING THE PREPARATION OF COMMERCIAL LACTULOSE

TÕNIS PEHK, ENDEL LIPPMAA,
*Institute of Physics and Biophysics, Estonian Academy of Sciences, Lenini pui. 10, Tallinn 200001 (U.S.S.R.)*

JAN SCHRAML*, OTOMAR PIHAR, MILAN MINÁRIK,
*Institute of Chemical Process Fundamentals, Czechoslovak Academy of Sciences, Prague 165 02 (Czechoslovakia)*

JÁN HIRSCH,
*Institute of Chemistry, Slovak Academy of Sciences, Bratislava 842 38 (Czechoslovakia)*

HEINRICH KNOBLOCH, AND SAURENDRA NATH DATTA
*Chephasaar, GmbH, Mühlstrasse 50, D-6670 St. Ingebert (F.R.G.)*
(Received October 26th, 1987; accepted for publication, December 1st, 1987)

ABSTRACT

An oligosaccharide isolated from crude lactulose syrup, using ion-exchange resins, was shown by n.m.r. spectroscopy to contain a 1,6-dioxaspiro[4.4]nonane structure.

## INTRODUCTION

Lactulose, a synthetic disaccharide of considerable pharmaceutical interest, can be prepared by the alkaline transformation of lactose in the presence of boric acid[1] as a complexing agent that prevents alkaline degradation. Hicks and Parrish[2] utilised a tertiary amine as the basic catalyst for the preparation of lactulose from lactose–boric acid (molar ratio 1:1).

When the effects of pH, temperature, and concentration on the conversion of lactose into lactulose in 1:1 lactose–boric acid were investigated[3], h.p.l.c. showed that the resulting lactulose syrup always contained a by-product. Efforts to remove this by-product by oxidation with bromine water were unsuccessful; since its removal by chromatography would be uneconomical, it became necessary to determine its structure and origin in order to assess the scope for preventing its formation. We now report on the isolation and the structure of the by-product (**1**) and its pentadeca-acetate.

*Author for correspondence.

0008-6215/88/$ 03.50 

RESULTS AND DISCUSSION

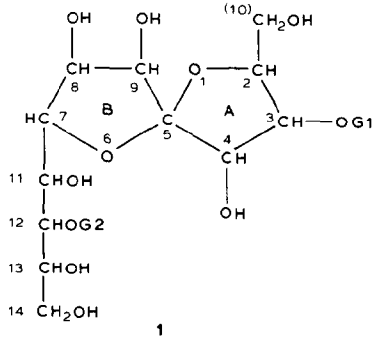
The by-product **1** was isolated crystalline from the crude lactose–lactulose mixture after repeated anion-exchange chromatography on columns first in the borate form and second in the bisulfite form, and characterised as the pentadeca-acetate (**2**). The $^{13}$C-n.m.r. spectrum indicated **2** to contain 15 acetate groups and 24 skeletal carbons [4 $-CH_2O-$, 2 $-CH(-O-)_2$ (acetal), 1 $>C(-O-)_2$ (ketal), and 17 >CH–O–]. Hence, **1** may be regarded as an oligosaccharide.

From the $^1$H,$^1$H-COSY[4] (including correlations for OH proton signals) and $^{13}$C,$^1$H-COSY[4] (across one-bond coupling) data and the deuterium isotope effect[5], four carbon chains were identified in **1**, namely, G1, G2, A, and B. Gl: 6 carbons [C-1/6(G1)] from the acetal $-CH(-O-)_2$ carbon ($\delta$ 103.9) through three >CHOH groups and one >CH–O– group to the terminal $-CH_2OH$ group ($\delta$ 61.1); G2: 6 carbons [C-1/6(G2)] from the second acetal $-CH(-O-)_2$ carbon ($\delta$ 104.9) through three >CHOH groups and one >CH–O– group to the terminal $-CH_2OH$ group ($\delta$ 60.9); A: 4 carbons [C-1/4(A)] $-CH(OH)CH(O-)CH(O-)CH_2OH$; and B: 7 carbons [C-1/7(B)] $-CH(OH)CH(OH)CH(O-)CH(OH)CH(O-)CH(OH)CH_2OH$.

By comparison with the spectra of methyl $\beta$-D-galactopyranoside and its tetra-acetate, the chains G1 and G2 were identified as $\beta$-D-galactopyranosyl groups.

Because of fast carbon relaxation, INADEQUATE[4] experiments failed to show how the chains A, B, G1, and G2 and the remaining ketal carbon were connected. Heteronuclear chemical-shift correlations through long-range couplings ($^{13}$C,$^1$H-COSY[4] and COLOC[6]) and selective proton decoupling when recording $^{13}$C-n.m.r. spectra[7] were then investigated. The COLOC experiment revealed a significant cross-peak between C-1(G2) and H-5(B), thus proving this $\beta$-D-galacto-pyranosyl unit to be linked to C-5(B). $^{13}$C,$^1$H-COSY experiments utilising long-range couplings showed two cross-peaks for the ketal carbon, the first one due to interaction with H-1(A) and the second with H-2(B). Since the ketal carbon must be bonded to two carbon atoms and as there are only C-1(A) and C-1(B) left for C–C bonding, the first cross-peak is due to a two-bond interaction and reveals the connectivity between the ketal carbon and C-1(A). The second cross-peak must be due to a three-bond coupling ($^{13}$C-$^1$H), which confirms indirectly bonding between the ketal carbon and C-1(B). Seemingly in contrast with this connectivity [C-1(A)–C–C-1(B)], only one deuterium $\gamma$-isotope effect was observed on the ketal carbon. This observation, however, agrees with the suggested structure, as only very small (or nil) $\gamma$-isotope effects (0–17 p.p.b.)[5,6] were found in some fructofuranose derivatives.

Which of the remaining oxygen atoms [O-2(A), O-3(A), and O-3(B)] bears the G1 chain and which are bonded to the ketal carbon was deduced as follows. Attachment of G1 to C-3(B) and O-2(A) and O-3(A) to the ketal carbon atom leads to an unlikely 3,5-dioxabicyclo[2.1.1]hexane structure of chain A. Hence, O-3(B) is bonded to the ketal carbon and G2 is attached either to O-2(A) or O-3(A). The latter can be excluded because it would require the presence of an unstable oxetane ring. Consequently, **1** can have only the structure shown.




1

Additional support for this structure is provided by comparison of the $^{13}C$ chemical shift data for **1** and its pentadeca-acetate **2** (Tables I and II). The difference in the chemical shifts is very small if the carbon atom is subject simultaneously to an $\alpha$-acetylation effect (+2–3 p.p.m.) and two $\beta$-effects (−0.5 to −1 p.p.m.) from the neighbouring groups, the two effects partially compensating each other. When only an $\alpha$-effect occurs (*e.g.*, C-4, C-10, C-11) according to the constitutional formula, a positive shift difference +0.5–2 p.p.m. was observed, whereas, when only one or two $\beta$ groups affect the shift (*e.g.*, C-2, C-3, C-5, C-7, C-12), negative shift differences were observed.

Although compounds containing 1,6-dioxaspiro[4.4]nonane structures have been found in Nature[9,10], there are not sufficient data on similar compounds available which would permit the assignment of configuration from the observed $^3J_{H,H}$ values. Even in simple furanose rings, assignments of configuration are difficult because of the flexibility of the ring and may be made safely only when $^3J_{H,H}$ is <3.5 Hz (*trans* protons)[11]. The smallest $^3J_{H,H}$ values associated with the spirononane skeleton involve H-7,8 (3.8 Hz in **1**, 4.4 Hz in **2**) and H-3,4 (4.5 Hz in **2**). Although these values are larger than the above limit, it is assumed tentatively that the involved protons are *trans*.

The structure **1** results from the dehydration condensation of two molecules of lactose or lactulose and since the two galactopyranosyl units are attached to carbons $\beta$ to $CH_2OH$ groups of the chains A and B, **1** was formed without glycolysis and most likely with retention of configuration at all carbons with the possible exception of the sites of reaction (C-5, C-7, and C-9). Accordingly, the substituents on C-4,3,2 and C-11,12,13 are assigned the configurations of C-3,4,5 in the D-fructose residue of lactulose (or in the D-glucose residue of lactose). The reported[12,13] $^3J_{H,H}$ values for various $\alpha$- and $\beta$-D-fructofuranose derivatives cover the ranges 1.4–9.7 Hz (H-3,4) and 2.8–8.8 Hz (H-4,5), which include the values found here for the couplings H-2,3 and H-3,4.

Another feature of **1** is the apparent symmetrical disposition of C-4 and C-9 in the two spiro-condensed furanose rings. According to the direct additivity of carbon chemical shifts[14], the resonances of these two carbons should have essentially the same chemical shifts except for steric effects and the substituent

TABLE I

N.M.R. PARAMETERS FOR THE OLIGOSACCHARIDE **1** AND ITS PENTADECA-ACETATE **2**[a]

| *Compound* | *Parameter*[b] | *Group number* | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *2* | | *3* | *4* | *5* | *7* | *8* | *9* | *10* | | *11* | *12* | | *13* | *14* | | | | |
| **1** | $\delta(^{13}C)$ | 79.0 | | 83.8 | 79.4 | 112.0 | 83.9 | 70.2 | 69.9 | 60.2 | | 70.7 | 82.6 | | 72.0 | 62.5 | | | | |
| | $\delta(^{1}HC)$ | 3.84 | | 3.84 | 4.09 | | 4.06 | 3.85 | 4.00 | 3.52 | 3.64 | 3.66 | 3.66 | | 3.71 | 3.49 | | 3.56 | | |
| | $^{1}J(^{13}C^{1}H)$ | 146 | | 156 | 145 | | 150 | [c] | 151 | [c] | | [c] | 144 | | [c] | [c] | | | | |
| | $^{3}J(^{1}HCC^{1}H)$ | 8.0 | | 8.0 | | | 3.8 | 3.8 | | −12.3[d] | −12.3[d] | | [c] | | [c] | −12.3[d] | | −12.3[d] | | |
| | | 3.7 | 2.4 | 6.7 | 6.7 | | 3.8 | 6.7 | 6.7 | 3.7 | 2.4 | 3.8 | | | 5.0 | 5.0 | | 5.0 | | |
| | $^{3}J(^{1}HOC^{1}H)$ | | | | 4.1 | | | 6.4 | 9.3 | 4.8 | 7.0 | 5.1 | | | 4.6 | 4.8 | | 4.8 | | |
| | $\delta(^{1}HO)$ | | | | 5.03 | | | 4.52 | 4.16 | 4.84 | | 4.10 | | | 4.65 | 4.85 | | | | |
| **2** | $\delta(^{13}C)$ | 78.5 | | 83.2 | 81.6 | 110.8 | 78.9 | 70.9 | 69.2 | 62.3 | | 71.2 | 79.4 | | 71.8 | 63.0 | | | | |
| | $\delta(^{1}H)$ | 4.50 | | 4.23 | 5.90 | | 5.01 | 5.41 | 5.69 | 4.59 | 4.25 | 5.67 | 4.47 | | 5.75 | 4.83 | | 4.40 | | |
| | $^{1}J(^{13}C^{1}H)$ | 150 | | 150 | 158 | | 152 | [c] | [c] | [c] | | 140 | 143 | | 150 | [c] | | | | |
| | $^{3}J(^{1}HCC^{1}H)$ | 7.6 | | 7.6 | | | 4.4 | 4.4 | | −12.0[d] | −12.0[d] | 5.5 | 5.5 | 2.6 | 7.0 | −12.4[d] | | −12.4[d] | | |
| | | 4.0 | 4.0 | 4.5 | 4.5 | | 2.9 | 6.4 | 6.4 | 4.0 | 4.0 | 2.9 | 4.5 | | 4.5 | 2.6 | | 7.0 | | |

[a]For the structure and group numbering, see formula **1**. [b]Chemical shifts on the $\delta$ scale, $J$ in Hz. [c]Not determined. [d]Geminal coupling constants.

TABLE II

N.M.R. PARAMETERS FOR THE β-D-GALACTOPYRANOSYL GROUPS (G1 AND G2) IN THE OLIGOSACCHARIDE **1** AND ITS PENTADECA-ACETATE **2**[a]

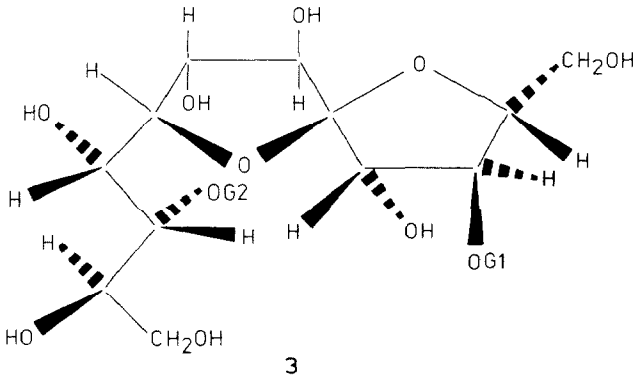
| *Group* | *Compound* | *Parameter*[b] | *Group number* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *1* | *2* | *3* | *4* | *5* | *6* | |
| G1 | **1** | $\delta(^{13}C)$ | 103.9 | 70.7 | 73.5 | 68.6 | 75.7 | 61.1 | |
| | | $\delta(^{1}HC)$ | 4.14 | 3.32 | 3.30 | 3.60 | 3.42 | 3.51 | 3.56 |
| | | $^{3}J(HCCH)$ | 7.5 | 7.5 | 3.2 | 3.2 | 4.5 8.0 | 4.5 | 8.0 |
| | | | | 9.5 | 9.5 | 1.2 | 1.2 | −10.9[c] | −10.9[c] |
| | | $^{3}J(^{1}HOC^{1}H)$ | | 4.3 | 5.5 | 4.6 | | 5.5 | 5.5 |
| | | $\delta(^{1}HO)$ | | 4.87 | 4.69 | 4.40 | | 4.46 | |
| | **2** | $\delta(^{13}C)$ | 101.2 | 69.0 | 71.1 | 67.3 | 71.3 | 61.1 | |
| | | $\delta(^{1}H)$ | 4.62 | 5.51 | 5.26 | 5.56 | 3.85 | 4.19 | 4.23 |
| | | $^{3}J(^{1}HCC^{1}H)$ | 8.0 | 8.0 | 3.4 | 3.4 | 6.3 | 6.3 | 6.3 |
| | | | | 10.5 | 10.5 | 0.5 | 0.5 | −11.0[c] | −11.0[c] |
| G2 | **1** | $\delta(^{13}C)$ | 104.9 | 71.5 | 73.5 | 68.6 | 75.8 | 60.9 | |
| | | $\delta(^{1}HC)$ | 4.24 | 3.32 | 3.30 | 3.61 | 3.38 | 3.50 | 3.53 |
| | | $^{3}J(^{1}HCC^{1}H)$ | 7.4 | 7.4 | 3.2 | 3.2 | 5.6 6.7 | 5.6 | 6.7 |
| | | | | 9.4 | 9.4 | 1.2 | 1.2 | −11.0[c] | −11.0[c] |
| | | $^{3}J(^{1}HOC^{1}H)$ | | 3.9 | 5.5 | 4.6 | | 5.6 | 5.6 |
| | | $\delta(^{1}HO)$ | | 5.06 | 4.67 | 4.38 | | 4.62 | |
| | **2** | $\delta(^{13}C)$ | 102.9 | 69.5 | 71.1 | 67.5 | 71.4 | 61.7 | |
| | | $\delta(^{1}H)$ | 4.72 | 5.53 | 5.23 | 5.53 | 3.82 | 4.32 | 4.24 |
| | | $^{3}J(^{1}HCC^{1}H)$ | 7.8 | 7.8 | 3.4 | 3.4 | 7.0 6.8 | 7.0 | 6.8 |
| | | | | 10.4 | 10.4 | 0.8 | 0.8 | −11.0[c] | −11.0[c] |

[a]See formula **1** for the positions of G1 and G2; CH groups are numbered according to IUPAC nomenclature. [b]Chemical shifts on the δ scale, *J* in Hz. [c]Geminal coupling constants.

effect of G1 on the shielding of C-4. According to the available data[13,15], the β-glycosidation effect is in the range 0–4 p.p.m., but the observed difference is +10 p.p.m. (C-4 being shielded less than C-9). The difference must be assigned to an exceptionally large steric effect only on C-9.

Of four possible configurations at C-5 and C-9, only the configuration shown in **3** can account for the large upfield shift observed for the resonance of C-9. The structure **3** corresponds to an α-D-fructofuranose configuration of the ring A and further support for this conclusion is provided by the $^{13}C$ chemical shift (δ 112) of the resonance C-5; the corresponding carbon atom in methyl α-D-fructofuranoside resonates at δ 109.1, and at δ 104.7 for the β isomer[16].

The all-*trans* arrangement of protons in ring B is tentative and is based on the small value of the couplings for H-7,8 and on the absence of any $^{13}C$ complexation shifts when the n.m.r. spectra of **1** were measured in admixture with $B_2O_3$. Downfield complexation shifts are expected to be observed for vicinal carbons with *cis*-hydroxyl groups[17]; such complexation shifts of 0.4–1.2 p.p.m. were observed for some of the galactosyl carbons in the same mixture. The single deuterium γ-isotope effect (+24 p.p.b.) observed on C-8 is not convincing for the assignment of the

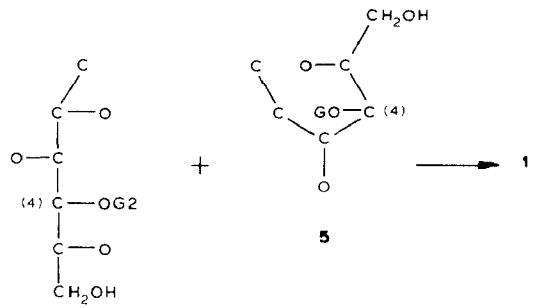



3

configuration of the hydroxyl groups on C-8 and C-9, as there are not sufficient data for the needed comparison according to Reuben's rule[18] ($\gamma$-effect is larger for vicinal *trans*-hydroxyl groups[18]). It is assumed that the configuration of the parent lactose is retained at C-11,12,13.

The reactions known for monosaccharides under alkaline conditions[19,20] allow speculation about the way of formation of **1** from lactose or lactulose.

The $\alpha$-D-fructofuranose form of lactulose, which was found to be a minor component of lactulose both in solution and in the solid state[21,22], would be the source of ring A of **1**. Ring B could then be formed by an aldol-type condensation (**5** + **4**) either with another molecule of lactulose or with lactose as shown.




4 5

EXPERIMENTAL

*General.* — Melting points were determined on a Büchi 510 capillary apparatus and are not corrected. Optical rotations were measured with a Perkin–Elmer 241 automatic polarimeter. H.p.l.c. was performed on a Knauer liquid chromatograph (Model 5200), employing a column (300 × 7.8 mm) of Bio-Rad Aminex HPX 37, elution with 0.005M sulphuric acid at 0.5 mL/min, and refractometric detection (model 8700 dual detector).

N.m.r. data were obtained with a Bruker AM 500 spectrometer operating at 500.13 ($^1$H) and 125.77 MHz ($^{13}$C) on solutions in $(CD_3)_2SO$ (for **1**) or $C_6D_6$ (for **2**). The spectra were referenced to the lines of solvents {$^{13}$C, $\delta[(CD_3)_2SO]$ 39.7 and $\delta(C_6D_6)$ 128.0; $^1$H, $\delta[(CD_3)_2SO]$ 2.52 and $\delta(C_6D_5H)$ 7.29}. $^1$H-N.m.r. spectra were analysed after the hydroxyl protons had been exchanged conventionally for deuterium. Standard spectrometer software was employed in the measurements of 2D-n.m.r. spectra of the following types: $^1$H,$^1$H-COSY[4], $^{13}$C,$^1$H-COSY[4] correlating the signals according to $^1J$ or $^2J$ and $^3J$ couplings between $^{13}$C and $^1$H nuclei, $^{13}$C,$^1$H-COLOC[6], and INADEQUATE[4]. Isotope effects were measured as described by Reuben[5].

*Isolation of the oligosaccharide* **1**. — *(a) Separation of lactulose and lactose.* Two columns (90 × 3.5 cm) of Kastel A-101 resin (2 × 600 mL) were equilibrated with aqueous 4% boric acid. A solution of crude lactulose (570 g, ~155 g of dry substance) was washed through the columns with water at 50 mL/min, to give 5 fractions. The fractionation was monitored by a refractometer (Brix degrees). A drop of Brix-degree below 0.2 indicated completeness of elution. The fractions were analysed by h.p.l.c.; **1** was present in significant amount in fractions 3–5. These fractions from three separations were combined and concentrated under reduced pressure to 730 g of solution (30.0 Brix degree, ~220 g of dry substance), which was re-chromatographed 8 times by the above procedure to yield a fraction that was shown by h.p.l.c. to be practically free from disaccharides and to contain **1**, galactose, and boric acid. The excess of boric acid which precipitated was filtered off, and the boric acid was removed from the complexes by washing through a column of Amberlite IRA-743 ($H^+$) resin with water. Concentration of the neutral effluent gave 181 g of solution containing ~48 g of dry substance.

*(b) Isolation of* **1**. The neutral effluent from (*a*) was washed through a column packed with 750 mL of Kastel A-300P resin in the bisulfite form at 45° with water[23] at 7 mL/min (50-mL fractions). Fractions 1–6 (analysis by h.p.l.c.) were concentrated to 54 g of solution (containing ~16 g of dry substance). Repetition of this step removed the traces of galactose. Two recrystallisations of the product (11.4 g) from methanol gave 3-β-D-galactopyranosyloxy-7-[2-(β-D-galactopyranosyloxy)-1,3,4-trihydroxybutyl]-4,8,9-trihydroxy-2-hydroxymethyl-1,6-dioxaspiro[4.4]nonane (**1**), m.p. 84–86° $[\alpha]_D^{20}$ +36° (*c* 0.8, water) (Found: C, 42.8; H, 6.53. $C_{24}H_{42}O_{21}$ calc.: C, 43.2; H, 6.35%).

Conventional treatment of **1** (2.5 g, 3.7 mmol) with anhydrous sodium acetate (5 g) and acetic anhydride (75 mL) under reflux for 2 h gave the pentadeca-acetate **2** (4.0 g, 80%), m.p. 95–96° (from 2-propanol), $[\alpha]_D^{20}$ +26° (*c* 1, chloroform) (Found: C, 49.88; H, 5.60. $C_{24}H_{27}O_{21}Ac_{15}$ calc.: C, 50.0; H, 5.70%).

ACKNOWLEDGMENTS

We thank Dagmar Schweitzer and Manfred Willascheck for excellent technical assistance, Mr. P. Denk for critically reading the manuscript, and Miss B. W. Yap for assistance in preparing the manuscript.

REFERENCES

1 J. F. MENDICINO, *J. Am. Chem. Soc.*, 82 (1960) 4975–4979.
2 K. B. HICKS AND F. W. PARRISH, *Carbohydr. Res.*, 82 (1980) 393–397.
3 H. KNOBLOCH, unpublished results.
4 A. BAX, *Two-Dimensional Nuclear Magnetic Resonance in Liquids*, Delft University Press, Delft, 1982.
5 J. REUBEN, *J. Am. Chem. Soc.*, 106 (1984) 6180–6186.
6 H. KESSLER, C. GRIESINGER, J. ZARBOCK, AND H. R. LOOSLI, *J. Magn. Reson.*, 57 (1984) 331–336.
7 L. F. JOHNSON, *Top. Carbon-13 NMR Spectrosc.*, 3 (1979) 2–16.
8 J. REUBEN, *J. Am. Chem. Soc.*, 105 (1983) 3711–3713; 107 (1985) 1747–1755.
9 F. BOHLMANN, P. HERBST, C. ARNDT, H. SCHÖNOVSKY, AND H. GLEINING, *Chem. Ber.*, 94 (1961) 3193–3216.
10 F. BOHLMANN, C. ARNDT, H. BORNOWSKI, K. M. KLEINE, AND P. HERBST, *Chem. Ber.*, 97 (1964) 1179–1192.
11 A. F. CASY, *PMR Spectroscopy in Medicinal and Biological Chemistry*, Academic Press, London, 1971, p. 355.
12 R. D. GUTHRIE, I. D. JENKINS, AND R. YAMASAKI, *Aust. J. Chem.*, 35 (1982) 1019–1029.
13 D. M. CLODE, W. A. LAURIE, D. MCHALE, AND J. B. SHERIDAN, *Carbohydr. Res.*, 139 (1985) 147–160.
14 F. W. WEHRLI AND T. WIRTHLIN, *Interpretation of Carbon-13 NMR Spectra*, Heyden, London, 1976, pp. 37–48.
15 P. A. J. GORIN AND M. MAZUREK, *Carbohydr. Res.*, 48 (1976) 171–186.
16 K. BOCK AND H. THØGERSEN, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–50.
17 E. BREITMAIER AND W. VOELTER, *$^{13}C$ NMR Spectoscopy, Methods and Appliations*, Verlag Chemie, Weinheim, 1974, p. 223.
18 J. REUBEN, *J. Am. Chem. Soc.*, 106 (1984) 2461–2462.
19 J. U. NEF, *Justus Liebigs Ann. Chem.*, 403 (1914) 204–383.
20 W. L. EVANS, *Chem. Rev.*, 31 (1942) 537–560.
21 P. E. PFEFFER, K. B. HICKS, AND W. L. EARL, *Carbohydr. Res.*, 111 (1983) 181–194.
22 A. S. PERLIN, P. H. PENHOAT, AND H. S. ISBELL, *Adv. Chem. Ser.*, 117 (1973) 39–50.
23 Y. TAKASAKI, *Agric. Biol. Chem.*, 36 (1972) 2575–2577.