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ASSIGNMENT OF SIGNALS OF THE CARBON-13 MAGNETIC RESONANCE SPECTRUM OF A SELECTED POLYSACCHARIDE: COMMENTS ON METHODOLOGY*

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ABSTRACT

The mannan from *Rhodotorula glutinis* contains alternate (1→3)- and (1→4)-linked β-D-mannopyranose residues (**1**) and its carbon-13 magnetic resonance spectrum displays 12 signals. These were assigned in terms of the positions of their parent nuclei in the sugar rings [but not whether the signals arose from a (1→3)- or (1→4)-linked residue] by preparation of D-mannans from specifically deuterated D-glucoses and observation of α- and β-deuterium isotope-effects. Individual assignments could then be made for carbon atoms of each unit by using the spectra of known oligo- and polysaccharides. The signal displacements of certain ^{13}C nuclei observed on *O*-methylation were compared with those obtained on *O*-mannosylation in order to determine whether methyl ethers could be used as model compounds for signal assignments in spectra of mannose-containing polysaccharides. The displacements observed were in the same direction and of a similar order of magnitude. An assessment is made of the use of the various techniques in assigning signals of polysaccharides and their possible interpretation in terms of chemical structure.

INTRODUCTION

Most polysaccharides give well-defined carbon-13 magnetic resonance (^{13}c.m.r.) spectra and an increasing number of reports on the subject have been appearing recently in the literature. The spectra have been used to determine whether overall repeating sequences of sugar units exist[1–3], and also signals have been assigned to ^{13}C nuclei in specific chemical structures[1–9]. Conversely, as the chemical shifts of the ^{13}C signals are apparently consistent, it should be possible to postulate chemical structures on the basis of the shift value. However, to do this successfully, reliable methods for signal assignment are necessary. In order to test possible methods, the mannan from *Rhodotorula glutinis* was selected as it gives rise to a ^{13}c.m.r. spectrum showing 12

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signals[3,7] (Fig. 1) and is sufficiently complex that a number of useful, but still unambiguous, assignments can be made. Structurally, the mannan consists of alternate (1→3)- and (1→4)-linked β-D-mannopyranose residues[10] (**1**).

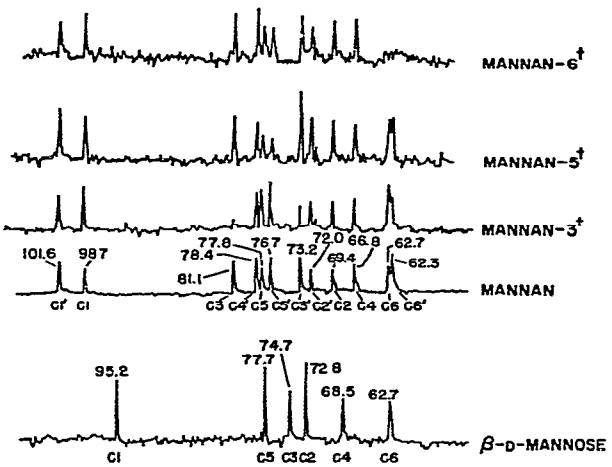
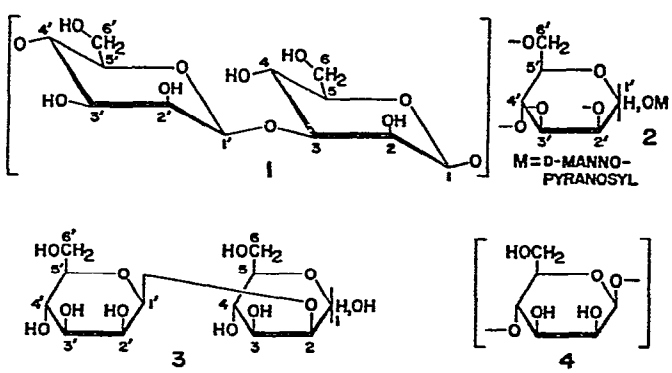


Fig. 1. Carbon-13 magnetic resonance spectra of β-D-mannose and mannans of *Rhodotorula glutinis*, recorded in D_2O at 70°. †Mannans-3, -5, and -6 are those prepared from D-[3-^{2}H], [5-^{2}H], and [6-^{2}H]glucose, respectively.

The two low-field signals at δ_c 101.6 and 98.7 are readily assignable to C-1 signals, as are the two high-field signals (δ_c 62.7, 62.3) to C-6 nuclei. However, in order to assign some of the other 8 signals with confidence, it was necessary to prepare three deuterated mannans starting from D-[6-$^2H_2$]glucose, and the [5-^{2}H], and [3-^{2}H] anologues. Incorporation of the 6-$^2H_2$ derivative into the mannan occurred with little rearrangement of the carbon skeleton. The C-6 signals (δ_c 62.7,



62.3) were absent[11] and the C-5 signals (δ_c 77.8, 76.7) could be assigned, as they underwent an upfield shift of 0.10 p.p.m. because of the β-carbon deuterium isotope-effect[12]. These assignments were confirmed, as the ^{13}c.m.r. spectrum of mannan obtained from D-[5-^{2}H]glucose showed relatively small C-5 signals (Fig. 1). Some rearrangement of the D-glucose skeleton also occurred when the 3-^{2}H derivative was used as precursor, but the size of signals of the *O*-mannosylated C-3 atom at relatively low field[4] (δ_c 81.1) and C-3′ (δ_c 73.2) were both diminished. Confirmation of assignments utilizing the β-carbon deuterium isotope-effect could not be made in the last two experiments, as the expected upfield shifts of 0.06 p.p.m.[12] are close to the half-height line-width of the signals. It follows from these assignments that the signal at δ_c 78.4 arises from the *O*-mannosylated C-4′ nucleus, as it is shifted 11.6 p.p.m. downfield from that of the unsubstituted C-4, which is at δ_c 66.8. The two remaining signals at δ_c 71.9 and 60.4 should arise from nuclei of the two C-2 signals.

Assignments of the relative positions of a number of pairs of signals (Fig. 1), those of C-1 and C-1′, C-2 and C-2′, C-5 and C-5′, and C-6 and C-6′ were made. The C-2 and C-2′ signals may be differentiated because *O*-mannosylation of a ^{13}CHOH group causes a strong upfield shift of the signal of the adjacent ^{13}C nucleus if it is attached to an axial hydroxyl group[7,8]. It is therefore probable that the higher-field signal at δ_c 69.4, rather than that at δ_c 71.9, arises from C-2, because of the adjacent (1→3)-substituent (**1**).

The assignment of pairs of C-1 and C-5 signals required a somewhat more detailed investigation. In a polysaccharide, it appears that the chemical shift of a given ^{13}C nucleus depends on the nature of the groups in close proximity to it. From previous data on C-1 signals obtained in the mannan series[7], the shifts vary according to the position of substitution (**2**; O-2′ to O-6′) and whether the *O*-mannosyl substituent has the α- or β-configuration. The shift at C-1′ is also affected by the configuration at C-1′ and the position of substitution on the attached mannopyranosyl residue, M. Only in one instance, that of (1→2)-substitution (**3**), is the configuration of unit M important and this is presumably due to the relative proximity of its anomeric center, C-1 to C-1′. This involves a difference in stereochemistry at a carbon center separated from the appropriate ^{13}C nucleus by three chemical bonds. Displacements have not yet been observed at distances greater than this.

By using these considerations, and the spectra of saccharides previously assigned (for assignments see the following Section), the signals of each C-1 and C-5 nucleus of *R. glutinis* mannan were identified. Little difference was observed between the chemical shifts of C-1 signals of β-D-mannose (δ_c 95.2) and 4-*O*-β-D-mannopyranosyl-β-D-mannose (δ_c 95.4) on the one hand and α-D-mannose (δ_c 95.6) and 3-*O*-β-D-mannopyranosyl-α-D-mannose (δ_c 95.2) on the other. Rather, the major effect depends on whether the appended unit M (**2**) is substituted on its 3- or 4-hydroxyl group. In 4-*O*-β-D-mannopyranosyl-β-D-mannose, C-1′ of the non-reducing end group gives[7] a signal at δ_c 101.7, whereas that of C-1′ of 3-*O*-β-D-mannopyranosyl-α-D-mannose, although it cannot be assigned with certainty, is at higher field (δ_c 100.7, 99.1, or 98.4). Another consideration is that the 4-*O*-linked β-D-

mannopyranan **4** has its C-1 signal at δ_c 101.7. Therefore, in mannan **1**, C-1 of (1→3)-substituted residues, which are linked to O-4′, corresponds to the signal at δ_c 101.5, and C-1′ to the signal at δ_c 98.7.

The assignment of C-5 nuclei of **1** is aided by comparison of the C-5 resonance of β-D-mannose (δ_c 77.7) with those of 4-*O*-β-D-mannopyranosyl-D-mannose. Mannosylation at OH-1 of β-D-mannose results in little change of the chemical shift of the C-5′ signal (δ_c 78.0), whereas *O*-mannosylation at OH-4 causes an upfield displacement of the C-5 signal to δ_c 76.5. It therefore appears that, in mannan **1**, the C-5′ atom adjacent to the 4-*O*-mannosyl substituent should give rise to the upfield C-5 signal at δ_c 76.7. Unfortunately, effective comparisons of the C-5′ signals of mannan **1** with that of the (1→4)-linked β-D-mannopyranan **4** cannot be made as the latter dissolves only in aqueous alkali, a solvent that causes changes[7] in the relative resonances of signals of mannan **1** of up to 1.7 p.p.m. (see Experimental Section).

As the preparation of oligosaccharides and interpretation of their ^{13}c.m.r. spectra is relatively difficult, it would be helpful if the signal displacements obtained on *O*-mannosylation of the mannose residues were similar to those produced on *O*-methylation. Such a similarity might be expected by analogy with the results of Usui *et al.*[8], who compared ^{13}C chemical shifts of glucose oligosaccharides with structurally analogous *O*-methylglucoses and observed that "the shift due to glycoside formation is of almost the same magnitude as the methylation shift". Thus the ^{13}c.m.r. spectra of the readily prepared 2-*O*-, 3-*O*-, 4-*O*-, and 6-*O*-methyl derivatives of β-D-mannose were compared with that of β-D-mannose, and the signal displacements were determined. The signals of the β-anomers were distinguishable from those of the α-anomer in each spectrum, as they were generally much smaller. As the assignments could not be made with certainty, they were checked by examination of the spectral displacements in the α-anomeric series, which were almost equal in magnitude. The results are summarized in Table I. The more-marked shifts arising on *O*-methylation are as follows. Methylation of an OH group results in a downfield displacement of 7–10 p.p.m. of the signal of the appended ^{13}C nucleus[13,14]. *O*-Methylation causes an upfield shift of the signal of the adjacent ^{13}C nucleus, provided that its C–O bond is axial. The shift occurs if OCH_3 substitution is at an equatorial[13] or axial[15] COH group and is generally about 4 p.p.m., except in the cases of 1-*O*-substitution when it is 0.8 and 0.9 p.p.m., and not especially prominent. The shift of −2.6 p.p.m. occurring with the C-1 signal of 2-*O*-methyl-α-D-glucopyranose has been attributed[8] to hydrogen-bond formation between OH-1 and OMe-2. However, bonding of this kind does not explain the shift of the C-1 signal of 2-*O*-methyl-α-D-mannopyranose (−3.2 p.p.m.), as its OH-1 and OMe-2 groups are diaxial and cannot form a mutual hydrogen bond. Methylation of OH-4 and OH-6 groups causes upfield shifts of 0.9–1.4 p.p.m. of the C-5 signals. Finally, a shift of approximately 1 p.p.m. is observed for the C-4 signal (−1.2 p.p.m.) on 3-*O*-methylation.

A somewhat more tentative assignment of the C-6 signals of **1** can be made. As the C-6 signal of 4-*O*-methyl-β-D-mannose is at 0.92 p.p.m. higher field than that of the 3-*O*-methyl derivative, it follows therefore that the mannan signal at δ_c 62.3 arises

TABLE I

DISPLACEMENTS OF ^{13}C.M.R. SIGNALS ON MONO-*O*-SUBSTITUTION OF *β*- AND α-D-MANNOPYRANOSE (RUNS AT 33°)

Mono-O-methyl derivative	*Displacements of ^{13}C signal in p.p.m.*[a]					
	C1	*C2*	*C3*	*C4*	*C5*	*C6*
β-D-*Mannose (33°)*						
1-*O*-	*+7.5*	*−0.9*	−0.1	+0.1	+0.2	+0.1
2-*O*-	+0.4	*+10.3*	+0.4	+0.2	+0.3	0
3-*O*-	+0.1	*−4.2*	*+9.1*	*−1.2*	+0.1	−0.1
4-*O*-	0	−0.2	−0.2	*+9.9*	*−0.9*	*−0.3*
6-*O*-	+0.1	0	0	0	*−1.4*	*+9.9*
α-D-*Mannose (33°)*						
1-*O*-	*+6.9*	*−0.8*	+0.4	−0.1	+0.2	+0.2
2-*O*-	*−3.2*	*+9.9*	−0.3	+0.3	−0.1	0
3-*O*-	0	*−4.4*	*+9.5*	*−1.2*	0	−0.1
4-*O*-	−0.1	+0.2	−0.2	*+9.9*	*−1.0*	*−0.3*

Displacements on O-mannosylation of mannose (70°)

(a) at *O*-1′ in *β*-D-Man*p*-(1→4)-α,*β*-D-Man: C-1′, +6.5; C-5′, +0.3
(b) at *O*-4 in *β*-D-Man*p*-(1→4)-α,*β*-D-Man: C-4, +9.8; C-5, −1.2
(c) at *O*-3 in *β*-D-Man*p*-(1→3)-α-D-Man: C-3, +6.6; C-4, −1.2

$\Delta\delta_c$ between signals of mannan **1** *and β-D-mannose (70°)*

(a) *(1→3)-Substituted residue*
C-1, +3.4; C-2, −3.4; C-3, +6.3; C-4, −1.7; C-5, +0.1; C-6, 0.
(b) *(1→4)-Substituted residue*
C-1′, +6.3; C-2′, −0.9; C-3′, −1.5; C-4′, +9.9; C-6′, −0.4.

[a]Accuracy is to ±0.04 p.p.m.

from C-6′ of the 4-*O*-substituted residue and the one at δ_c 62.7 from C-6 of the 3-*O*-substituted residue.

It can be seen from Table I that the signal-displacement values observed on *O*-methylation of mannose on any specific OH group are in the same direction, and often of a somewhat larger magnitude, than those observed on *O*-mannosylation of mannose to give mannobioses. A similar comparison between the difference of shifts ($\Delta\delta_c$) between *β*-D-mannose, mannan **1** (which can be considered as containing 1,4 and 1,3-di-*O*-mannosylated *β*-D-mannoses), and methyl 3-*O*- and methyl 4-*O*-methyl-*β*-D-mannopyranoside could not be made because synthesis of the glycosides is difficult. However, an approximate estimate of $\Delta\delta_c$ values for signals of the glycosides and *β*-D-mannose can be made by adding the $\Delta\delta_c$ values observed for methyl *β*-D-mannopyranoside and 4-*O*-methyl-*β*-D-mannose. The values are quite close to those observed for mannan **1** (compare Sections 1 and 4 in Table I).

ASSIGNMENT OF SIGNALS IN MODEL COMPOUNDS

The ^{13}c.m.r. spectrum of 4-*O*-β-D-mannopyranosyl-α,β-D-mannose has six signals that could be those of C-5 nuclei. Examination of the 6,6′-dideuterated derivative, prepared by partial hydrolysis[10] of the appropriate deuterated mannan, showed that two of these, at δ_c 78.0 and 76.5, underwent an upfield β-carbon deuterium isotope-shift. The minor signal (δ_c 76.5) corresponded to C-5 of the reducing end of the β-anomer and the major one to C-5′ of the α,β-anomeric mixture. The other shifted signal (δ_c 72.0) is that of C_α-5.

3-*O*-β-D-Mannopyranosyl-α,β-D-mannose, obtained by partial acetolysis of mannan **1**, could not be separated from its 4-*O*-β-isomer and consequently ^{13}c.m.r. spectral examination was carried out on the mixture. The C-1 region showed a signal at δ_c 95.2 probably arising from the reducing end of the α-anomer, and three other signals at δ_c 100.7, 99.1, and 98.4.

The assignments for α,β-D-mannose were those previously determined on the basis of the α- and β-carbon deuterium isotope-effects[12]. The spectrum of methyl α-D-mannopyranoside shows C-2 and C-3 signals that are very close, but the one at lower field was assigned to the latter as it underwent a β-carbon deuterium isotope-effect in methyl α-D-[2-^{2}H]mannopyranoside.

COMMENTS ON METHODOLOGY

By using the mannan **1** as a model, it appears that deuterated D-mannans can be prepared from suitable specifically deuterated D-glucoses without marked rearrangement of the labelling. One possible exception was not examined, that of D-[2-^{2}H]glucose, whose stereochemistry at C-2 differs from that of the mannan. The deuterated ^{13}C nuclei, by analogy with the monosaccharides, did not give rise to signals, and were thus easily recognizable. The less prominent β-carbon deuterium isotope-effect was only observed with the 6,6′-deuterated mannan, when it was larger than the widths of the C-5 signals. Obviously, however, any observation of this effect in other polysaccharides depends on the line width of their signals.

The yield of mannan was sufficiently good, from 5–10%, so that only one gram of deuterated glucose was required for each run. In other systems, yielding less polysaccharide, the preparation of 5-^{2}H and 6-$^2H_2$ derivatives of D-glucose can be conveniently scaled up. However, problems have been encountered in this laboratory in preparing D-[3-^{2}H]glucose (see Experimental Section).

Assignments of the C-2 and C-5 signals of the mannan may be made using the displacements of >1.0 p.p.m. observed with readily interpretable signals of 3-*O*-methyl-β-D-mannose and 4-*O*-methyl-β-D-mannose, respectively. However, the assignments of the C-6 signals of the mannan, which are based on the ^{13}C spectrum of 4-*O*-methyl-β-D-mannose, are more tentative, as they are only 0.4 p.p.m. apart. It can be seen that the "long-range" displacements observed on mono-*O*-methylation of α- and β-D-mannose (Table I) are of almost the same order.

The foregoing data, coupled with those previously observed with other mannans[7], support the concept that a number of structural factors affect the chemical shift of a signal, so that the observed shift-value is characteristic of a given chemical structure.

EXPERIMENTAL

Carbon-13 magnetic resonance spectroscopy. — ^{13}C.m.r. spectra were obtained on samples containing a natural abundance of ^{13}C by using a Varian XL-100-15 spectrometer with Fourier transform having an 8K memory. The sugars (~50 mg) were dissolved in D_2O (2 ml) in a 12-mm diameter × 20 cm n.m.r. tube fitted with a Teflon vortex plug. Mannose and *O*-methylmannose solutions were examined at 33° and mannose, oligo- and polysaccharides at 70°. Usually, the spectral width used was 5000 Hz, the acquisition time 0.4 sec, the pulse width 50 μsec, and the number of transients 20,000–150,000, depending on the spectral resolution. For experiments in which the β-carbon deuterium isotope-shift was measured, these values were, respectively, 1000 Hz, 2 sec, 90 μsec, and 20,000 transients. The chemical shifts are expressed in δ_c, relative to external Me_4Si, whose shift relative to D_2O was obtained in a separate experiment.

^{13}C.m.r. signals of R. glutinis *mannan in D_2O (70°) and in alkali in D_2O (33°).* — As depicted in Fig. 1, the signals of the mannan in D_2O are assigned as follows: 101.6 (C-1′), 98.7 (C-1), 81.1 (C-3), 78.4 (C-4′), 77.8 (C-5), 76.7 (C-5′), 73.2 (C-3′), 71.9 (C-2′), 69.4 (C-2), 66.8 (C-4), 62.7 (C-6), and 62.3 (C-6′). In aqueous alkali[7] they are, respectively, 101.7, 98.0, 81.2, 78.8, 77.1, 75.4, 73.4, 72.3, 68.9, 66.9, 62.9, and 62.0.

Preparation of Sugars. — *Mannobioses.* 4-*O*-β-D-Mannopyranosyl-D-mannose and its 6,6′-deuterated derivative* were prepared by partial acid-hydrolysis of appropriate *R. glutinis* mannans[10].

A mixture of 3-*O*- and 4-*O*-β-D-mannopyranosyl-D-mannose was obtained from *R. glutinis* mannan (6 g) by partial acetolysis according to the method of Lee and Ballou[16], except that half the proportion of sulfuric acid was used and the duration of the cleavage was one day. The resulting mixture of sugars was fractionated on a cellulose column. Acetone–water (7:1 v/v) eluted D-mannose, and acetone–water (4:1 v/v) eluted the mixture of mannobioses (0.39 g).

Methyl α-D-[2-^{2}H]mannopyranoside. This was prepared by treating mixed D-mannose and D-[2-^{2}H]mannose[17] with refluxing 3% hydrogen chloride in methanol for 3 h. Neutralization (silver carbonate), followed by filtration and evaporation, gave the glycoside. ^{13}C.m.r. spectroscopy showed that the C-3 signal underwent a β-carbon deuterium isotope-effect of −0.08 p.p.m. The assignments for the undeuterated glycoside are as follows (in δ_c): 101.9 (C-1), 73.7 (C-5), 71.8 (C-3), 71.0 (C-2), 68.0 (C-4), 62.1 (C-6), and 55.9 (OMe). The relative positions of the C-3 and C-2 signals are opposite to those for α-D-mannose[12].

*In order to obtain sufficient disaccharide, it was necessary to hydrolyze 400 mg of mannan.

Deuterated D-*glucoses*. The 6-$^2H_2$ and 5-^2H derivatives of D-glucose were prepared according to references 12 and 18, respectively.

The 3-^2H derivative was prepared as follows: oxidation of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose with dimethyl sulfoxide–phosphorus pentaoxide gave 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose[19], which was purified by column chromatography on silicic acid (eluant: chloroform–hexane, 3:2 v/v) in order to obtain a good yield of crystalline material. This was converted into D-[3-^2H]glucose by the method of Koch and Perlin[20].

Deuterated mannans. Growth of *R. glutinis* on a medium containing deuterated D-glucoses (1 g) provided exocullular mannans (80 mg) that were isolated by precipitation with ethanol and purified via their insoluble copper complexes[10].

Methyl ethers of D-*mannose*. The 2-, 4- and 6-methyl ethers were prepared as previously described[21], as was the 3-methyl ether[22].

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REFERENCES

1 H. J. Jennings and I. C. P. Smith, *J. Amer. Chem. Soc.*, 95 (1973) 606.
2 A. S. Perlin, N. M. K. Ng Ying Kin, S. S. Bhattarcharjee, and L. F. Johnson, *Can. J. Chem.*, 50 (1973) 2437.
3 J. F. T. Spencer and P. A. J. Gorin, *Biotechnol. Bioeng.*, 15 (1973) 1.
4 P. A. J. Gorin and J. F. T. Spencer, *Can. J. Microbiol.*, 18 (1972) 1709.
5 D. R. Bundle, H. J. Jennings, and I. C. P. Smith, *Can. J. Chem.*, 51 (1973) 3812.
6 T. Usui, M. Kobayashi, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto, *Tetrahedron Lett.*, (1973) 3397.
7 P. A. J. Gorin, *Can. J. Chem.*, 51 (1973) 2375.
8 T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto, *J. Chem. Soc.*, (1973) 2425.
9 P. A. J. Gorin and M. Mazurek, *Can. J. Chem.*, 51 (1973) 3277.
10 P. A. J. Gorin, K. Horitsu, and J. F. T. Spencer., *Can. J. Chem.*, 43 (1965) 950.
11 H. Spiesecke and W. G. Schneider, *J. Chem. Phys.*, 35 (1961) 731.
12 P. A. J. Gorin, *Can. J. Chem.*, 52 (1974) 458.
13 D. E. Dorman, S. J. Angyal, and J. D. Roberts, *J. Amer. Chem. Soc.*, 92 (1970) 1351.
14 A. S. Perlin, B. Casu, and H. J. Koch, *Can. J. Chem.*, 48, (1970) 2596.
15 P. A. J. Gorin and M. Mazurek, *Carbohyd. Res.*, 27 (1973) 325.
16 Y.-C. Lee and C. E. Ballou, *Biochemistry*, 4 (1965) 257.
17 P. A. J. Gorin, *Can. J. Chem.*, 51 (1973) 2105.
18 W. Mackie and A. S. Perlin, *Can. J. Chem.*, 43 (1965) 2921.
19 K. Onodera and N. Kashimura, *Methods Carbohyd. Chem.*, 6 (1972) 331.
20 H. J. Koch and A. S. Perlin, *Carbohyd. Res.*, 15 (1970) 403.
21 S. S. Bhattacharjee, R. H. Haskins, and P. A. J. Gorin, *Carbohyd. Res.*, 13 (1970) 235.
22 G. O. Aspinall and G. Zweifel, *J. Chem. Soc.*, (1957) 2271.