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SCOPE AND LIMITATIONS OF THE SIMPLE ^{13}C-N.M.R. METHOD OF STRUCTURAL ANALYSIS OF CARBOHYDRATES: GLUCODISACCHARIDES

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ABSTRACT

The scope and limitations of the SIMPLE n.m.r. method (secondary isotope multiplet n.m.r. spectroscopy of partially labelled entities) has been investigated for a series of glucodisaccharides. ^{13}C-SIMPLE n.m.r. measurements have been made on solutions of (1→1)- (α,α-trehalose), (1→2)- (sophorose and kojibiose), (1→3)- (laminaribiose), and (1→6)-linked (gentiobiose and isomaltose) glucodisaccharides in $(CD_3)_2SO$ and the results combined with those previously published for (1→4)-linked analogues (maltose and cellobiose). Each linkage (and substitution) type gives rise to a unique pattern of ^{13}C isotopomers which, in principle, may be used for complete assignment of the spectra and structural analysis of the molecule. The glucodisaccharides are difficult to analyse, compared with other disaccharides, because the presence of two glucose moieties leads to degeneracies of a few isotopic multiplets which cannot be differentiated by the magnitudes of the isotope effects. Assignments in aqueous solutions were obtained by using the DIS (differential isotope shift) n.m.r. method in conjunction with the results from SIMPLE n.m.r. In practice, nearly all of the signals can be assigned unequivocally and the remaining signals are choices between two possible assignments.

INTRODUCTION

Recent reports[1–7] have highlighted the power of the SIMPLE n.m.r. method in providing unequivocal assignment of the ^{13}C-n.m.r. spectra of mono- and oligosaccharides. The method consists of measuring the ^{13}C-n.m.r. spectrum under conditions of slow D,H exchange [*e.g.*, in $(CD_3)_2SO$] for carbohydrates with partially deuterated hydroxyl groups. Under these conditions, resonances due to the separate isotopomers give rise to multiplets for each carbon atom which are analysed in terms of two-bond (β, 89–121 p.p.b.) and three-bond (γ, 0–69 p.p.b.) isotope effects. The number of lines in a multiplet depends on the number of

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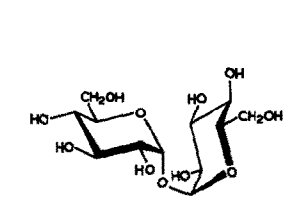
separate isotope effects and their magnitudes, whereas the relative intensities of the lines in the multiplet also depend on the OH:OD ratio. In addition to the number of isotope effects that characterise a given carbon, the magnitudes of γ effects depend on the configuration of the hydroxyl group, making the observation of secondary isotope multiplets of partially labelled entities (SIMPLE) n.m.r. a powerful method for the structural analysis of carbohydrates[1,6,7]. In order to obtain the maximum information from SIMPLE n.m.r., certain procedures have been followed. Signals were assigned to their respective anomers in the normal ^{13}C-n.m.r. spectrum on intensity criteria. Signals arising from the two anomers of the non-reducing unit have chemical shifts closer together than those in the reducing unit[1,8]. The ^{13}C-n.m.r. spectra exhibit three chemical shift regions resulting from the signals for anomeric (88–105 p.p.m.), methylene (60–69 p.p.m.), and the other methine (68–89 p.p.m.) carbons and, within each region, unequivocal assignments may be made from analysis of isotopic multiplets. The SIMPLE n.m.r. method also provides the necessary background information on the magnitudes of β and γ isotope effects that enables the differential isotope shift (DIS) method[9–11] to be used for unequivocal assignment of ^{13}C-n.m.r. signals of molecules in solution in H_2O and D_2O from the results of one experiment. The two methods of analysis (SIMPLE and DIS n.m.r.) are based on the facile exchange of OH by OD groups and provide an important addition to other methods for the assignment of ^{13}C-n.m.r. spectra of carbohydrates[8,12] such as chemical shift criteria and comparison with model compounds[13–15], selective ^{2}H or ^{13}C enrichment[16–18], selective decoupling[19–21], relaxation measurements[22–26], and measurement of ^{1}H–^{13}C coupling constants in 1D and 2D heteronuclear *J*-resolved experiments[27,28].

We have tested the scope and limitations of the SIMPLE n.m.r. method by application to the ^{13}C-n.m.r. assignment of the series of glucodisaccharides in solution in $(CD_3)_2SO$ and to the assignment in aqueous solution by the DIS method; the latter assignment may be checked by results available in the literature[8,12,15,29]. Measurements have been made on (1→1)- (α,α-trehalose), (1→2)- (sophorose and kojibiose), (1→3)- (laminaribiose), and (1→6)-linked (isomaltose and gentiobiose) glucodisaccharides and combined with those available in the literature for the (1→4)-linked analogues (maltose[1] and cellobiose[2]).

RESULTS AND DISCUSSION

The structures of the glucodisaccharides are shown in **1–8**; the numbers of the carbons of the non-reducing residues of **2–8** are primed and those of the reducing residue are unprimed as shown in **3**. Substitution at different positions of the reducing residue gives rise to characteristic multiplets for secondary isotope shifts (H/D) of each carbon atom (Table I). Compared to D-glucose, substitution of a hydroxyl group causes the loss of a β effect for the geminal carbon and the loss of a γ effect for a vicinal carbon; thus, Table I can be used for structural analysis of substituted monosaccharides as well as aiding the analysis of oligosaccharides.



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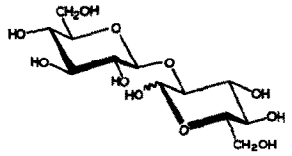

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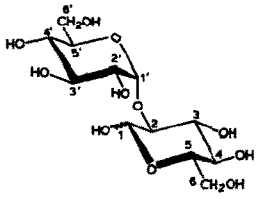
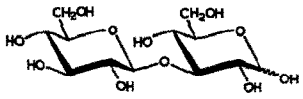
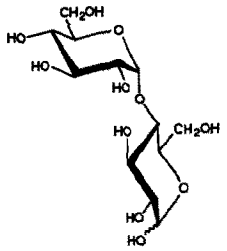


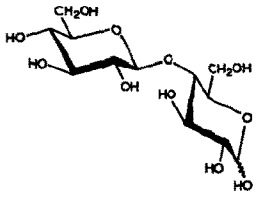
3 kojibiose



4 laminaribiose



5 maltose



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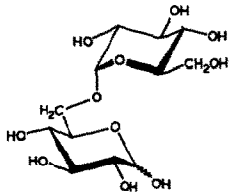
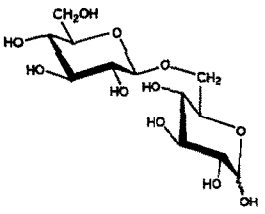


7 isomaltose



8 gentiobiose

TABLE I

ISOTOPE EFFECTS FOR THE REDUCING RESIDUES OF DISACCHARIDES IN THE PYRANOSE FORMS[a]

Linkage	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	
1→1[b]	γ	β+γ	β+2γ	β+γ	2γ	β	Trehalose
1→2	β	2γ	β+γ	β+γ	2γ	β	Kojibiose (α) Sophorose (β)
1→3	β+γ	β+γ	2γ	β	2γ	β	Nigerose (α) Laminaribiose (β)
1→4	β+γ	β+2γ	β+γ	γ	γ	β	Maltose (α) Cellobiose (β)
1→6	β+γ	β+2γ	β+2γ	β+γ	γ	—	Isomaltose (α) Gentiobiose (β)

[a]Different patterns of isotope multiplets are expected for furanose forms. [b]The carbons of the non-reducing residues have the same pattern of multiplets as trehalose.

The carbons of the non-reducing residue of each disaccharide have the same pattern of isotopic multiplets represented by that of trehalose, *viz.*, C-1′ (γ), C-2′ ($\beta + \gamma$), C-3′ ($\beta + 2\gamma$), C-4′ ($\beta + \gamma$), C-5′ (2γ), and C-6′ (β). The expected isotope effects (Table I) are the same as the multiplet matrices reported[7] except for the inclusion in this work of the multiplets for the C-1 and C-6 resonances.

In order to aid the assignment of the spectra and to determine the type of linkage, the expected number of resonances with each multiplicity (s, d, dd, *etc.*) and the expected isotope effects (β, γ, $\beta + \gamma$, *etc.*) have been summarised in Table II, together with the corresponding carbons for each type of glycosidic linkage. Except for those containing (1→2) and (1→3) linkages, the disaccharides give rise to different patterns of expected multiplicities that can be used to aid the assignment of the spectra. For the (1→2)- and (1→3)-linked compounds, it can be seen that, although the number of signals with similar multiplicities is the same, these linkages might be differentiated by the isotope effects on C-1 [(1→2), β effect;

TABLE II

NUMBER AND TYPE OF ISOTOPE MULTIPLETS EXPECTED FOR CARBON ATOMS OF DISACCHARIDES IN THE PYRANOSE FORMS

Linkage	*Resonance multiplicity and isotope effect*					
	s	*d* γ	*dd or t* 2γ	*d* β	*dd* $\beta+\gamma$	*ddd* $\beta+2\gamma$
1→1	0	1 C-1	1 C-5	1 C-6	2 C-2 C-4	1 C-3
1→2	0	1 C-1′	3 C-2 C-5 C-5′	3 C-1 C-6 C-6′	4 C-2′ C-3 C-4 C-4′	1 C-3′
1→3	0	1 C-1′	3 C-3 C-5 C-5′	3 C-4 C-6 C-6′	4 C-1 C-2 C-2′ C-4′	1 C-3′
1→4	0	3 C-1′ C-4 C-5	1 C-5′	2 C-6 C-6′	4 C-1 C-2′ C-3 C-4′	2 C-2 C-3′
1→6	1 C-6	2 C-1′ C-5	1 C-5′	1 C-6′	4 C-1 C-2′ C-4 C-4′	3 C-2 C-3 C-3′

(1→3), $\beta + \gamma$ effect] and, possibly, confirmed by the different behaviour of C-2,3,4. The situation for α,α-trehalose [(1→1) linkage] is trivial in that only six signals are expected for glucodisaccharides with a centre of symmetry. The (1→6) linkage is unique in providing the only example of a signal with no isotope effects, *i.e.*, the signal for the linkage carbon (C-6) is a singlet. The assignment of spectra of molecules containing (1→6) linkages can be confirmed by other unique features such as two signals with one γ effect (C-1′ and C-5) and three signals with three isotope effects ($\beta + 2\gamma$ for C-2, C-3, and C-3′). Similarly, the (1→4) linkage gives rise to a unique pattern of multiplets with, for example, three signals with one γ effect (C-1′, C-4, and C-5) and only two signals with one β effect (C-6 and C-6′).

The ^{13}C-n.m.r. spectra of the reducing glucodisaccharides may be made more complex by anomerisation in solution, producing 24 signals rather than the 12 expected for the dimer. Fortunately, the anomers of glucodisaccharides usually occur in different proportions at equilibrium for solutions in $(CD_3)_2SO$, thus enabling the two sets of signals to be differentiated in a normal proton noise-decoupled spectrum. The normal ^{13}C-n.m.r. experiment is an important prelude to ^{13}C SIMPLE measurements because it provides a preliminary assignment of resonances to the reducing and non-reducing residues. Previous work on α and β anomers has shown that the chemical shifts of the carbon signals of the non-reducing residues are similar to each other, whereas those of the reducing residue are significantly different from each other, with those of the β anomer often downfield of those of the corresponding α anomer[8,12]; this method of assignment is referred to as the anomerisation experiment. When the difference in proportions of anomers is large, the separate signals can also be distinguished in the SIMPLE n.m.r. spectrum, despite the decrease in sensitivity relative to the normal ^{13}C-n.m.r. spectrum.

α,α-Trehalose [*α-Glc*p-*(1→1)-α-Glc*p]. — There are only six multiplets in the 100-MHz SIMPLE ^{13}C-n.m.r. spectrum (Fig. 1) of α,α-trehalose (**1**; OH:OD, 41:59) because of the molecular symmetry. Most carbons have a unique pattern of isotopomers (Table II) and immediate assignment was possible for C-1 (γ), C-3 ($\beta + 2\gamma$), C-5 (2γ), and C-6 (β). The C-2 and C-4 signals were distinguishable from the 4-line ($\beta + \gamma$) multiplets, but the signals could not be assigned unequivocally by SIMPLE n.m.r. because the magnitudes of the β and γ effects were similar for both carbons. In this work, the C-2 and C-4 signals were assigned by an INADEQUATE n.m.r. experiment[30].

The spectrum in Fig. 1 is similar to that observed[1] for methyl α-D-glucopyranoside and is characteristic of all 1-substituted glucopyranosides in solution in $(CD_3)_2SO$. As the signals for C-2 and C-3 of α,α-trehalose are significantly downfield of that for C-4, it was assumed that this criterion may be applied to assign the C-2 and C-4 signals of the non-reducing groups of all the glucodisaccharides.

Sophorose [*β-Glc*p-*(1→2)-Glc*p]. — Previous ^{1}H-n.m.r. measurements indicated that the α anomer preponderates[31] in solutions in $(CD_3)_2SO$, although signals from both anomers (α:β, 80:20) were observed by ^{13}C-n.m.r. measurements

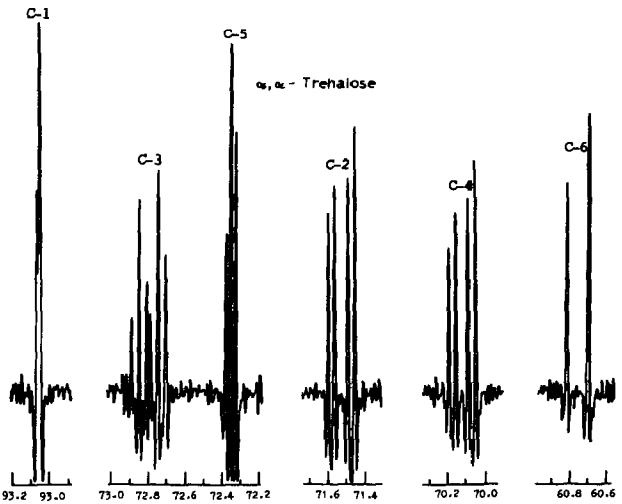


Fig. 1. 100-MHz SIMPLE ^{13}C-n.m.r. spectrum [$(CD_3)_2SO$] of α,α-trehalose (**1**; OH:OD, 41:59).

in this work (Table III). The 100-MHz SIMPLE ^{13}C-n.m.r. spectrum of sophorose (**2**; OH:OD, 49:51) is shown in Fig. 2. Inspection of Table II shows that C-1′ (γ) and C-3′ ($\beta + 2\gamma$) could be assigned immediately since they had unique patterns of isotope effects, although the signal for C-3′β was difficult to observe due to overlap with the multiplets for C-3′α and C-3β. The signal for C-1 (β effect) in the anomeric region was readily differentiated from those of C-6 and C-6′ by virtue of its chemical shift. Six carbons were expected and observed to have 2γ effects (C-2, C-5, and C-5′) and, of these, four multiplets exhibited γ magnitudes of 27 or 28 p.p.b. and so were assigned to C-5 and C-5′, whereas the other signals were assigned to C-2. The remaining signals (C-2′,3,4,4′) each exhibited 2 isotope effects with the same ($\beta + \gamma$) multiplet pattern and, as the magnitudes of corresponding β and γ effects were expected to be similar, differentiation between these signals in sophorose was not possible by SIMPLE n.m.r., and other criteria were required. The anomerisation experiment indicated that the signals at 73.8 and 70.1 p.p.m. belong to the non-reducing residue, whereas those at 71.6 and 70.2 p.p.m. belong to the reducing residue. The former carbons (C-2′ and C-4′) could be differentiated by chemical shift criteria, but the latter pair (C-3 and C-4) had isotope effects of such similar magnitudes (γ, 36–47 p.p.b.) that they might only be differentiated by a ^{1}H–^{13}C heteronuclear COSY[27,28] or a similar experiment.

Kojibiose [*α-Glcp-(1→2)-Glcp*]. — The patterns of isotope effects for kojibiose (**3**) were the same as those for sophorose (Table II), although some changes in magnitudes might be expected for α-(1→2)- compared to β-(1→2)-linked glucodisaccharides as well as changes in chemical shifts in both the reducing and non-reducing residues. For kojibiose, resonances due to both anomers (α:β, 46:54) were clearly visible in the 100-MHz SIMPLE ^{13}C-n.m.r. spectrum (OH:OD, 49:51) (Fig. 2). Differentiation between the signals for α and β anomers was

TABLE III

CHEMICAL SHIFTS OF THE GLUCODISACCHARIDES IN SOLUTION IN $(CD_3)_2SO$[a]

Disaccharide	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α,α-Trehalose (**1**) (1→1)	93.08	71.60	72.90	70.20	72.39	60.83
Sophorose (**2**) (1→2)						
α non-reducing unit	104.92	73.81	76.34	70.10	76.73	61.17
reducing unit	91.37	82.07	71.64[b]	70.17[b]	71.72	60.09
β non-reducing unit	103.92	74.16	76.25[g]	70.07[g]	76.66	61.17
reducing unit	95.10	83.50	76.03[c]	69.95[c]	76.92	60.98
Kojibiose (**3**) (1→2)						
α non-reducing unit	96.90	72.45	73.47	70.28	72.47[d]	60.97
reducing unit	88.62	77.60	71.63[e]	70.65[e]	72.02[d]	61.26
β non-reducing unit	98.25	72.22	73.60	70.25	72.30	60.95
reducing unit	96.30	80.50	75.30[f]	70.59[f]	76.74	61.19
Laminaribiose (**4**) (1→3)						
α non-reducing unit	104.01	73.79	76.19	70.14	76.84	60.95
reducing unit	91.75	70.89	84.94	68.56	71.80	61.05
β non-reducing unit	103.90	73.79	76.19	70.14	76.82	60.93
reducing unit	96.30	73.49	88.16	68.65	76.17	61.08
Maltose (**5**) (1→4)						
α[h] non-reducing unit	100.85	72.68	73.47	70.11	73.51	61.04
reducing unit	92.22	72.00	73.03	80.39	70.40	60.77
β[h] non-reducing unit	100.81	72.57	73.45	70.07	73.51	61.02
reducing unit	96.89	74.53	76.57	79.86	75.16	60.88
Cellobiose (**6**) (1→4)						
α non-reducing unit	101.69	73.48	74.44	69.99[g]	76.53	60.99
reducing unit	91.99	72.05	71.37	80.96	69.77	60.49
β[i] non-reducing unit	103.09	73.28	74.44	70.01	76.73	60.99
reducing unit	96.62	74.47	75.01	80.70	74.69	60.55
Gentiobiose (**8**) (1→6)						
α non-reducing unit	103.11	73.51	76.81	70.08	76.73	61.07
reducing unit	92.14	72.21	73.02	70.59	70.48	69.00
β non-reducing unit	103.11	73.47	76.82	70.04	76.73	61.04
reducing unit	96.76	74.75	76.60	70.18	75.13	68.81
Isomaltose (**7**) (1→6)						
α non-reducing unit	98.66	71.94	73.08	70.17	72.35	60.88
reducing unit	92.17	72.15	73.00	70.86	70.25	67.32
β non-reducing unit	98.72	71.91	73.13	70.17	72.37	60.86
reducing unit	96.80	74.68	76.63	70.32	74.80	67.29

[a]Chemical shifts of the protio isomer were measured in p.p.m. relative to $\delta_{(CD_3)_2SO}$ 39.5. [b–f]Tentative assignment obtained using chemical shift criteria. Pairs of assignments with the same letter may be exchanged. [g]Resonance difficult to observe in the SIMPLE n.m.r. spectrum due to overlapping multiplets, but clearly visible in the normal ^{13}C-n.m.r. spectrum. [h]Ref. 1. [i]Ref. 2.

accomplished from intensity ratios of the signals in the normal ^{13}C-n.m.r. spectrum. Differentiation between reducing and non-reducing residues relied on the similarity of chemical shifts of α and β anomers for the non-reducing residue compared to the reducing residue (*e.g.*, C-2 v C-2′, C-3 v C-3′, and C-6 v C-6′).

Many signals for kojibiose were assigned by inspection of multiplets in the same way as for sophorose (*i.e.*, C-1, C-1′, C-2, C-3′, C-6, and C-6′). A similar limitation of the SIMPLE n.m.r. method for the unequivocal assignment of signals

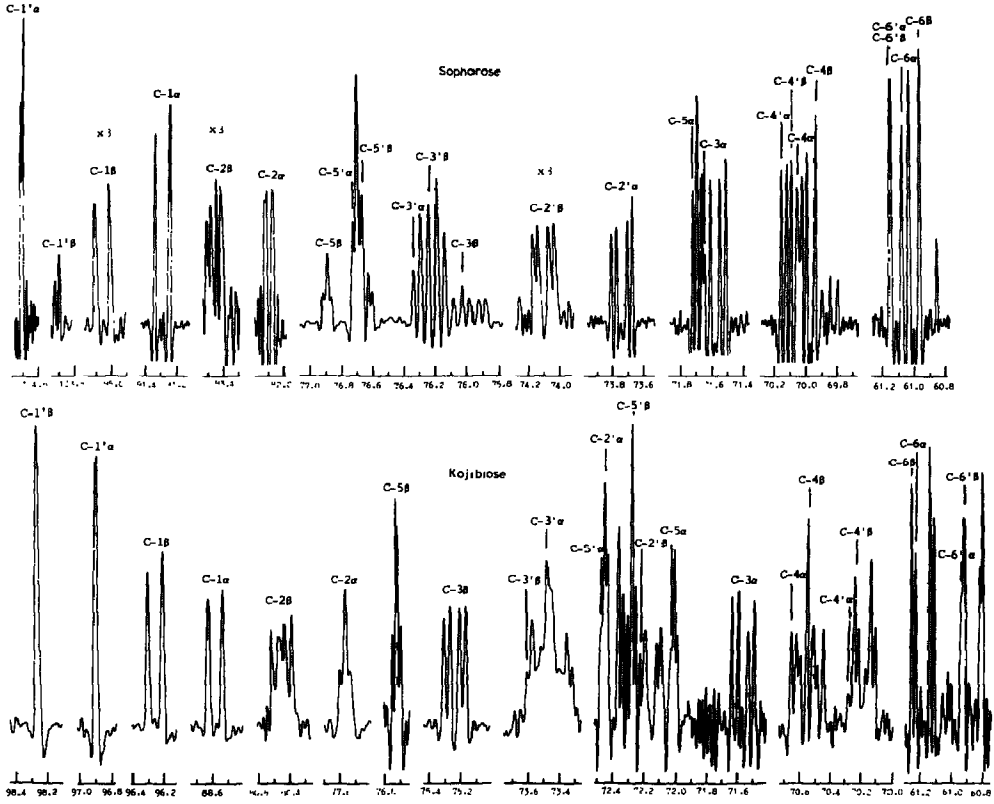


Fig. 2. 100-MHz SIMPLE ^{13}C-n.m.r. spectra [$(CD_3)_2SO$] of sophorose (**2**; α:β, 80:20, OH:OD, 49:51) and kojibiose (**3**; α:β, 46:54, OH:OD, 49:51).

was exposed for the four carbons with (β + γ) isotope effects (*i.e.*, C-2′, C-4′, C-3, and C-4) in kojibiose as found for sophorose. The presence of both α- and β-forms should enable the C-2′ and C-4′ signals to be differentiated from those for C-3 and C-4. For this molecule, three pairs of closely spaced resonances were observed (72.2 and 72.4, 70.6, and 70.3 p.p.m.). The pair that had the most widely separated chemical shifts (76.0 and 71.6 p.p.m.) was assigned to C-3 as, of the carbons considered, it is the closest to the anomeric centre and followed the behaviour found in sophorose. The signal for C-2′ was assigned by using chemical shift criteria, and those for C-4 and C-4′ were assigned using the anomerisation experiment. The signal for C-5β was distinguished from that for C-5′β, using the anomerisation experiment, but the signals for C-5α and C-5′α had similar chemical shifts and could be assigned only tentatively.

Laminaribiose [*β-Glcp-(1→3)-Glcp*]. — The 100-MHz SIMPLE ^{13}C-n.m.r. spectrum of laminaribiose (**4**; OH:OD, 52:48) in solution in $(CD_3)_2SO$ (Fig. 3) showed isotopic multiplets for both α and β anomers. Measurements from the C-1 signals in the normal ^{13}C-n.m.r. spectrum indicated an α:β-ratio of 53:47 which

was sufficient to assign signals of both anomers from the intensities of analogous signals. Inspection of Table II indicates that the C-1′ (γ) and C-3′ ($\beta + 2\gamma$) signals could be assigned from the unique patterns of their isotope effects and that the C-4, C-6, and C-6′ signals could be recognised by a single β effect for each anomer. The C-4 signals (68.6 p.p.m.) could be differentiated from those of C-6 and C-6′ by using chemical shift criteria. Assignment of the up-field signals to C-6 and C-6′ was based on an anomerisation experiment. There are three carbons (C-3, C-5, and C-5′) expected to have 2γ effects. Chemical shift criteria were used to differentiate the C-5 and C-5′ signals from the C-3 signal, which is observed down-field of the other (84–88 p.p.m.) due to substituent effects. The magnitudes of γ_{32} and γ_{34} (22–25 p.p.b.) were smaller than those (~40 p.p.b.) normally associated[1] with these magnitudes in glucose derivatives.

Of the resonances expected to show ($\beta + \gamma$) effects, the anomeric C-1 signal is readily identified for both α (91.8 p.p.m.) and β (96.4 p.p.m.) anomers, whereas signals for the non-reducing residue (C-2′ and C-4′) have similar chemical shifts for both α and β anomers and the signals could be assigned only tentatively using criteria based on relative chemical shifts. The remaining signals corresponded to C-2β (73.5 p.p.m., γ_{21} 64 p.p.b.) and C-2α (70.9 p.p.m., γ_{21} 24 p.p.b.), confirmed by the relatively large magnitudes of γ_{21} for the β compared to the α anomer as found previously for maltose and melibiose[1].

Maltose [α-*Glc*p-(*1→4*)-*Glc*p] *and cellobiose* [β-*Glc*p-(*1→4*)-*Glc*p]. — The assignments for maltose[1] (**5**) and cellobiose[2] (**6**) have been accomplished previously by SIMPLE ^{13}C-n.m.r. measurements, using criteria based on numbers and distribution of isotope effects as summarised in Table II. The magnitudes of the isotope effects have been included in Tables IV and V in order to complete the series. The spectrum of maltose was notable for the extra isotope effect observed

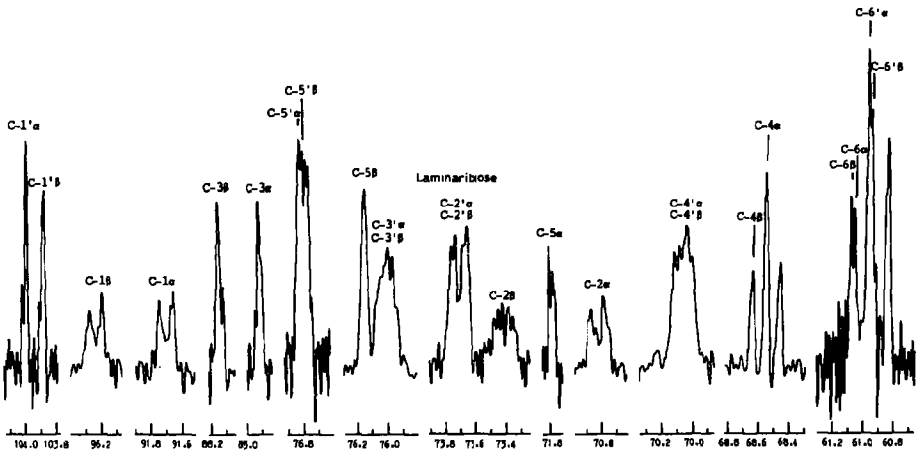


Fig. 3. 100-MHz SIMPLE ^{13}C-n.m.r. spectrum [$(CD_3)_2SO$] of laminaribiose (**4**; α:β, 53:47, OH:OD, 52:48).

TABLE IV

MAGNITUDES OF β-ISOTOPE EFFECTS IN THE GLUCODISACCHARIDES[a]

Disaccharide		β_1	β_2	β_3	β_4	β_6
α,α-Trehalose (1→1)		—	107	105	104	119
Sophorose (1→2)						
α	non-reducing unit	—	99	96	103	119
	reducing unit	93	—	100	102	114
β	non-reducing unit	—	100	106[b]	103[b]	119[b]
	reducing unit	95	—	96	100	107
Kojibiose (1→2)						
α	non-reducing unit	—	92[b]	93	103	117
	reducing unit	90	—	100	99	117
β	non-reducing unit	—	101	99	104	117
	reducing unit	95	—	101	103	115
Laminaribiose (1→3)						
α	non-reducing unit	—	97	95	89	117
	reducing unit	95	100	—	96	121
β	non-reducing unit	—	97	95	89	112
	reducing unit	94	91	—	93	108[b]
Maltose (1→4)						
α[c]	non-reducing unit	—	100	103	102	115
	reducing unit	96	100	95	—	112
β[c]	non-reducing unit	—	99	103	102	115
	reducing unit	105	100	93	—	108
Cellobiose (1→4)						
α	non-reducing unit	—	104[b]	97[b]	97[b]	113[b]
	reducing unit	91	90	101	—	115
β[d]	non-reducing unit	—	104	97	97	113
	reducing unit	104	105	99	—	110
Gentiobiose (1→6)						
α	non-reducing unit	—	99	90	104	119
	reducing unit	99	103	101[b]	102[b]	—
β	non-reducing unit	—	99	99	102	116
	reducing unit	104	103	100	105	—
Isomaltose (1→6)						
α	non-reducing unit	—	108	103[b]	101[b]	117[b]
	reducing unit	104	105	92	98	—
β	non-reducing unit	—	105	103	101	113
	reducing unit	101	100	104	97	—

[a]Magnitudes were measured in p.p.b. All isotope effects are negative. The effects are tabulated on the basis that the tentative assignments in Table III are correct. [b]Slight inaccuracies in these magnitudes may occur due to overlapping multiplets. [c]Ref. 1. [d]Ref. 2.

on C-2′ which was attributed to the effect of an HO-3 · · · HO-2′ intramolecular hydrogen-bond as found in cyclomaltoheptaose[1].

Gentiobiose [*β-Glc*p-*(1→6)-Glc*p]. — The 100-MHz SIMPLE ^{13}C-n.m.r. spectrum of gentiobiose (**8**; OH:OD, 49:51) in solution in $(CD_3)_2SO$ (Fig. 4) showed isotopic multiplets for both anomers. Measurements of the C-6 signal, which exhibited no isotope effects, indicated that the α:β-ratio was 37:63, so that the spectra of both anomers could be readily differentiated and analysed.

TABLE V

MAGNITUDES OF γ-ISOTOPE EFFECTS IN THE GLUCODISACCHARIDES[a]

Disaccharide		γ_{12}	γ_{21}	γ_{23}	γ_{32}	γ_{34}	γ_{43}	γ_{54}	γ_{56}
α,α-Trehalose (1→1)		15	—	34	41[b]	41[b]	38	25[b]	25[b]
Sophorose (1→2)									
α	non-reducing unit	17	—	34	48[b]	48[b]	53	27[b]	27[b]
	reducing unit	—	9[c]	47	—	40	36	28[b]	28[b]
β	non-reducing unit	22	—	35	53[b]	53[b]	47	28[b]	28[b]
	reducing unit	—	59	30	—	43	47	27[b]	27[b]
Kojibiose (1→2)									
α	non-reducing unit	[c]	—	28	32[b]	32[b]	30	21[b]	21[b]
	reducing unit	—	34	34	—	40	36	21[b]	21[b]
β	non-reducing unit	[c]	—	27	32[b]	32[b]	30	26[b]	26[b]
	reducing unit	—	62	31	—	40	40	26[b]	26[b]
Laminaribiose (1→3)									
α	non-reducing unit	25	—	21[d]	42	54	37	22[b]	22[b]
	reducing unit	20[d]	24[d]	—	22[b]	22[b]	—	17[bd]	17[bd]
β	non-reducing unit	31	—	21[d]	42	51	37	22[b]	22[b]
	reducing unit	22[d]	64	—	25[b]	25[b]	—	18[bd]	18[bd]
Maltose (1→4)									
α[e]	non-reducing unit	20	—	29	45[b]	45[b]	33	27[b]	27[b]
	reducing unit	16	50[b]	50[b]	42	—	40	—	20
β[e]	non-reducing unit	40	—	36	38[b]	38[b]	38	22[b]	22[b]
	reducing unit	22	66	53	43	—	34	—	22
Cellobiose (1→4)									
α	non-reducing unit	[c]	—	29	45[b]	45[b]	33	27[b]	27[b]
	reducing unit	17	[c]	45	38	—	20[d]	—	[c]
β[f]	non-reducing unit	20	—	29	45[b]	45[b]	33	27[b]	27[b]
	reducing unit	21	65	40	43	—	17[d]	—	22
Gentiobiose (1→6)									
α	non-reducing unit	22	—	36	45[b]	45[b]	33	20[b]	20[b]
	reducing unit	14	38[b]	38[b]	40[b]	40[b]	34	31	—
β	non-reducing unit	22	—	34	47[b]	47[b]	34	20[b]	20[b]
	reducing unit	21	68	35	46[b]	46[b]	34	29	—
Isomaltose (1→6)									
α	non-reducing unit	17	—	23[d]	35[b]	35[b]	35	30[b]	30[b]
	reducing unit	28	35[b]	35[b]	46[b]	46[b]	31	32	—
β	non-reducing unit	9[c]	—	27[d]	52[b]	52[b]	35	27[b]	27[b]
	reducing unit	26	69	33	46[b]	46[b]	32	32	—

[a]Magnitudes were measured in p.p.b. All isotope effects are negative. The effects are tabulated on the basis that the tentative assignments in Table III are correct. [b]Averaged value due to degeneracies. [c]Effect too small to be clearly resolved. [d]Inaccuracies in magnitudes may occur due to signal overlap or poor signal-to-noise. [e]Ref. 1. [f]Ref. 2.

Inspection of Table II shows that three carbons had a unique pattern of isotope effects, making unequivocal assignment straightforward for C-6 (no effects), C-6′ (one β effect), and C-5′ (2 γ effects). In addition, two carbons had only one γ effect and the down-field signal in the anomeric region was readily assigned to C-1′ (103.1 p.p.m., γ_{12} 14 p.p.b.), whereas the up-field signals were assigned to C-5β (75.1 p.p.m., γ_{54} 29 p.p.b.) and C-5α (70.5 p.p.m., γ_{54} 31 p.p.b.)

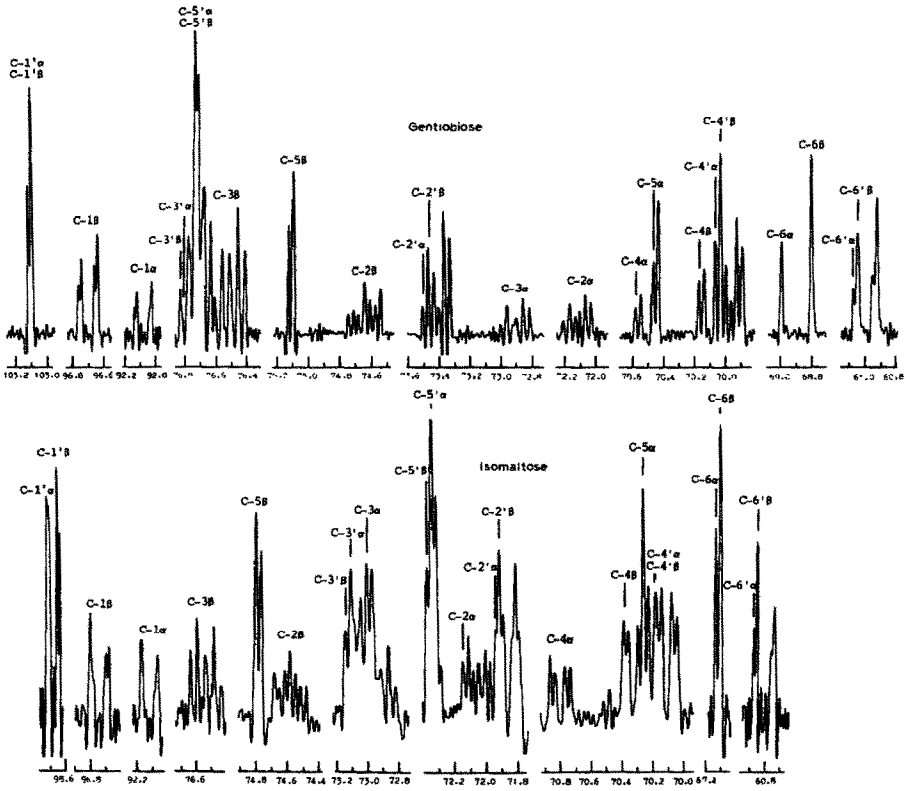


Fig. 4. 100-MHz SIMPLE ^{13}C-n.m.r. spectra [$(CD_3)_2SO$] of gentiobiose (**8**; α:β, 37.63. OH:OD, 49:51) and isomaltose (**7**; α:β, 43:57, OH:OD, 52:48).

on chemical shift and signal intensity criteria. Although four carbons (C-1,2′,4,4′) were expected to show four lines from (β + γ) effects, the signal of the anomeric carbon C-1 was significantly down-field from those of the others (C-1β 96.8, C-1α 92.2 p.p.m.) and the C-2′ and C-4′ signals were distinguished by the similarity of chemical shifts of their different anomers. This left the C-4 signals at 70.6 and 70.2 p.p.m., which could be readily assigned to the α and β anomers, respectively, because of the large difference in their intensities. Three carbons were expected to show three (β + 2γ) isotope effects (Table II) and, of those, the signals for C-3′ had very similar chemical shifts (76.81 and 76.82 p.p.m.) for the α and β anomers, respectively, whereas those for C-2 and C-3 were expected to exhibit different chemical shifts for the α and β anomers. The major signals corresponded to the α anomer and occurred at 76.6 and 74.8 p.p.m. The up-field signal exhibited one large γ effect (68 p.p.b.) and so was assigned to C-2β by analogy with the large γ_{21} magnitudes found for maltose and melibiose[1] and with reference to the observation[3] that $\gamma_{trans} > \gamma_{cis}$. The corresponding C-2$\beta$ resonance (72.2 p.p.m.) exhibited γ_{21} and γ_{23} magnitudes of ~38 p.p.b. similar to those observed[1] for the analogous signal in maltose and melibiose. The remaining C-3β (76.6 p.p.m.) and C-3α (73.0 p.p.m.)

signals were readily assigned by considerations of signal intensities and magnitudes of appropriate β and γ isotope effects.

The SIMPLE ^{13}C-n.m.r. spectrum of isomaltose (7) in solution in $(CD_3)_2SO$ (Fig. 4) exhibited isotope multiplet signals from both anomers (α:β, 43:57) that were essentially the same as those found for gentiobiose, and the assignment of the spectrum was accomplished in the same manner as for gentiobiose. The OH:OD ratio was 52:48. The spectra of isomaltose and gentiobiose were similar, except for changes in chemical shifts that were most apparent for the signals of C-1',2',3',5'.

Assignment in aqueous solution using DIS n.m.r. — Such assignments have been reported[9-11,17] for some of the glucodisaccharides. When used in conjunction with results obtained by SIMPLE n.m.r. for solutions in $(CD_3)_2SO$, DIS n.m.r. becomes a powerful tool for unequivocal assignment of carbon signals of molecules in aqueous solutions.

DIS n.m.r. spectra display two peaks for each resonance, one for the sample in D_2O and one for the sample in H_2O. The difference between the two peaks corresponds to the sum of the isotope effects on the resonance concerned plus the bulk magnetisation effect of the D_2O that surrounds the H_2O. The bulk magnetisation effect is usually ~+30 p.p.b. (in the direction opposite to the isotope effect) for a 5-mm tube in a 10-mm tube and affects all resonances equally. The bulk magnetisation effect is calculated for each sample by averaging over all signals, according to the following formula.

$$\text{Bulk magnetisation} = \frac{\text{Total DIS effects} - \text{Total SIMPLE effects}}{\text{Total number of carbons}}$$

Spectra of samples in aqueous solution were assigned initially by direct comparison with the assignments for solutions in $(CD_3)_2SO$. Some resonances appeared close together (within ~1 p.p.m.), giving rise to the possibility of a reversal of assignment between solvents. Comparison of the observed DIS effect (minus the bulk magnetisation effect) with the total of the expected magnitudes of the β and/or γ effects observed by SIMPLE n.m.r. of the resonances of the glucodisaccharides always led to an unequivocal assignment of the resonances for solutions in D_2O and H_2O based on the assumption that the assignments for solutions in $(CD_3)_2SO$ solution are correct. The chemical shifts (Table VI) obtained by DIS n.m.r. are consistent with previous assignments[15], although accurate to an extra order of magnitude.

CONCLUSIONS

Thus, the combination of the normal ^{13}C- and the SIMPLE ^{13}C-n.m.r. experiments provide an unequivocal assignment for ~93% of the signals in the glucodisaccharides, and the remaining signals are choices between 2 alternatives.

TABLE VI

CHEMICAL SHIFTS OF THE GLUCODISACCHARIDES IN SOLUTION IN D_2O[a]

Disaccharide	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α,α-Trehalose (1→1)	93.78	71.72	73.27	70.38	72.73	61.25
Sophorose (1→2)						
α non-reducing unit	104.54	74.07	76.42	70.22	76.42	61.58
reducing unit	92.53	81.45	72.48	70.22	71.82	61.25
β non-reducing unit	103.30	74.47	76.42	70.22	76.42	61.58
reducing unit	95.34	82.14	76.42	70.39	76.79	61.25
Kojibiose (1→2)						
α non-reducing unit	96.69	71.79	73.36	69.92	72.27	61.38
reducing unit	89.94	79.03	71.74	70.27	71.74	61.61
β non-reducing unit	98.61	71.92	73.36	69.86	72.10	61.32
reducing unit	96.67	76.35	74.97	70.13	76.18	61.84
Nigerose (1→3)						
α[b] non-reducing unit	98.8	71.8	73.1	70.3	71.8	60.8
reducing unit	92.1	70.3	79.8	69.6	71.2	60.8
β[b] non-reducing unit	98.8	71.8	73.1	70.3	71.8	60.8
reducing unit	96.0	73.1	82.2	69.6	75.6	60.8
Laminaribiose (1→3)						
α non-reducing unit	103.65	74.68	76.50	70.45	76.78	61.58
reducing unit	92.86	71.86	83.25	68.99	72.03	61.44
β non-reducing unit	103.56	74.68	76.50	70.45	76.78	61.58
reducing unit	96.53	74.35	85.51	69.11	76.30	61.44
Maltose (1→4)						
α non-reducing unit	100.49	72.68	73.85	70.29	73.50	61.45
reducing unit	92.78	72.25	74.13	77.84	70.78	61.54
β non-reducing unit	100.46	72.59	73.83	70.26	73.50	61.45
reducing unit	96.66	74.98	77.10	77.63	75.41	61.67
Cellobiose (1→4)						
α non-reducing unit	103.20	74.98	75.10	70.28	76.70	61.42
reducing unit	92.61	72.06	72.17	79.46	70.82	60.75
β non-reducing unit	103.20	74.98	75.10	70.28	76.70	61.41
reducing unit	96.54	74.78	76.38	79.46	75.57	60.89
Gentiobiose (1→6)						
α non-reducing unit	103.24	73.77	76.38	70.31	76.47	61.46
reducing unit	92.79	72.14	73.42	70.15	71.05	69.27
β non-reducing unit	103.28	73.77	76.38	70.31	76.51	61.46
reducing unit	96.64	74.78	76.40	70.19	75.77	69.41
Isomaltose (1→6)						
α non-reducing unit	98.78	72.55	74.05	70.33	72.62	61.42
reducing unit	93.08	72.40	74.00	70.41	70.84	66.53
β non-reducing unit	98.78	72.55	74.05	70.33	72.62	61.42
reducing unit	97.96	75.07	76.91	70.47	75.11	66.65

[a]Chemical shifts were measured in p.p.m. relative to $\delta_{1,4\text{-dioxane}}$ 67.4. Assignments are based on the assumption that those for $(CD_3)_2SO$ are correct. [b]Previously reported assignments[15] adjusted for $\delta_{1,4\text{-dioxane}}$ 67.4.

SIMPLE n.m.r. is therefore a powerful tool for assignment in such difficult circumstances. However, 10% of the assignments were based on the results of an

INADEQUATE experiment on α,α-trehalose. The magnitudes of isotope effects were consistent with those previously reported for compounds similar to those discussed here. For oligosaccharides that are more complex than the glucodisaccharides, especially where the reducing terminal is blocked, the role of 2D experiments in assignment would be greater. The SIMPLE n.m.r. experiment would still be useful because it would remove the necessity for carrying out extra, less-sensitive 2D experiments. When SIMPLE n.m.r. is combined with the DIS n.m.r. method, all the ^{13}C signals assigned for solutions in $(CD_3)_2SO$ can be assigned for solutions in D_2O and H_2O. When used in this way, DIS n.m.r. enables more accurate and reliable assignments for aqueous solutions than those assignments previously reported. With just three 1D-n.m.r. experiments, the methods described here provide an assignment that is equivalent to the application of a variety of 1D- and 2D-n.m.r. experiments which are more difficult to obtain and process.

EXPERIMENTAL

The glucodisaccharides were commercial samples and were deuteriated by lyophilising from D_2O, and dissolved in $(CD_3)_2SO$. The OH:OD ratio was adjusted by adding very small amounts of H_2O and D_2O as necessary. The extent of deuteriation was determined by ^1H-n.m.r. measurements of the areas of H-1 and residual OH multiplets, or from the ratio of the areas of the HOH and HOD peaks. The OH:OD ratio may also be determined from appropriate ^{13}C resonances.

Natural abundance, 100-MHz (Bruker WH400) SIMPLE ^{13}C-n.m.r. spectra were obtained at 310 K under proton noise-decoupling conditions. Normal ^{13}C-n.m.r. spectra were obtained at 50 MHz (JEOL FX200) under the same conditions. 50-MHz DIS ^{13}C-n.m.r. spectra were obtained for solutions in H_2O in a 5-mm tube inside a 10-mm tube containing the sample in D_2O. Depending on the experimental conditions, between 5,000 and 100,000 transients were recorded for concentrations of 50 mg/cm^3. The sweep-width was set to record all the peaks of interest and, for solutions in $(CD_3)_2SO$, to fold the $(CD_3)_2SO$ signal onto a section of the spectrum free of other signals. Zero-filling up to 64k points led to a digital resolution of ~1.6 p.p.b. per point. Spectra of solutions in $(CD_3)_2SO$ were referenced with respect to $\delta_{(CD_3)_2SO}$ 39.5, and spectra of aqueous solutions were referenced to $\delta_{1,4\text{-dioxane}}$ 67.4. The α:β-ratio was calculated from the ratio of the peak heights of the appropriate ^{13}C signals or from ^1H-n.m.r. spectra.

The notation used for isotope effects (β, γ) denotes the ^{13}C signal being observed with a numerical subscript (*e.g.*, β_3) and, when appropriate, a second numerical subscript for the hydroxyl group which gives rise to the isotope effect (*e.g.*, γ_{32} and γ_{34} correspond to the 3-bond isotope effects observed on the C-3 signal resulting from deuteriation of HO-2 and HO-4, respectively). All isotope effects reported are negative, *i.e.*, substitution by the heavier isotope leads to an up-field chemical shifts. All isotope effects are quoted in p.p.b. (*i.e.*, 10^{-3} p.p.m.).

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