A Method to assign ^{13}C N.M.R. Signals of Compounds with Exchangeable Hydrogen Atoms using Two and Three Bond Isotope Effects: Cellobiose

John C. Christofides and David B. Davies*
Department of Chemistry, Birkbeck College, Malet St., London WC1E 7HX, U.K.

Partial deuteriation of exchangeable hydroxy-protons in carbohydrates produces characteristic multiplets in ^{13}C n.m.r. spectra observed under slow exchange conditions, enabling most carbon signals to be assigned unequivocally in one experiment by analysis of two and three bond isotope effects.

Assignment of the ^{13}C n.m.r. signals of oligosaccharides such as cellobiose [D-glucopyranosyl-(β1→4)-D-glucopyranose, β-structure in Figure 1] may be made by chemical substitution and empirical correlations,[1] by selective spin labelling using ^2H

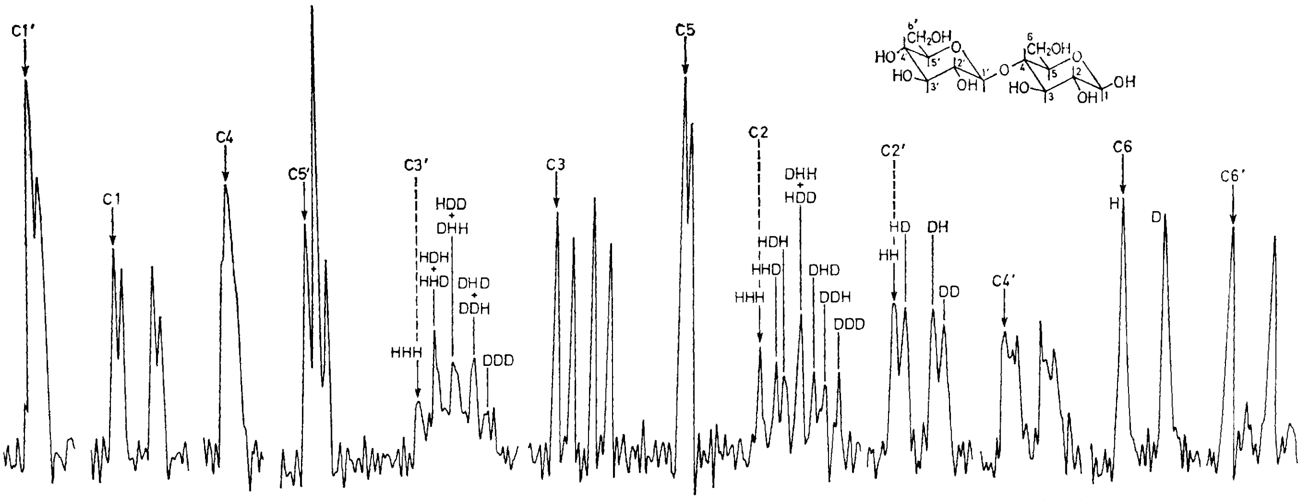

Figure 1. The 100 MHz proton noise-decoupled ^{13}C n.m.r. spectrum of β-cellobiose (OH : OD, *ca.* 1 : 1) in $(CD_3)_2SO$ solution at 295 K calculated at a data resolution of 0.25 Hz pt^{-1}. Also shown are the structure of cellobiose, the expected β and γ isotope effects on each carbon atom and the origins of H and D isotopomer resonance signals for one (C6), two (C2′), and three (C2 and C3′) isotope effects. Chemical shifts are given with respect to Me_4Si {$= \delta[(CD_3)_2SO] + 39.5$ p.p.m.}.

or ^{13}C,[2–4] by deuterium induced isotope shifts,[2–6] by selective homonuclear decoupling,[2] or, more recently, by homo- and hetero-nuclear 2D n.m.r. spectroscopy.[7] One method of assignment is based on observation of isotope shifts when OH groups are partially replaced by OD enabling hydroxy-bearing carbon atoms in carbohydrates to be identified using ^{13}C n.m.r. measurements under conditions of slow exchange.[6] In this work we demonstrate that under these experimental conditions the ^{13}C n.m.r. signals appear as a series of multiplets in which all the potential isotopomers resulting from two bond (β) and three bond (γ) isotope effects are observed. These measurements enable all carbon signals to be assigned and the signs and magnitudes of β and γ isotope effects to be determined.

The 100 MHz proton noise-decoupled ^{13}C n.m.r. spectrum of cellobiose (OH : OD, *ca.* 1 : 1) in $(CD_3)_2SO$ solution at 295 K consists of 12 separate carbon multiplets with between 1 and 7 resonance signals. After lyophilisation from an H_2O–D_2O solution the sample of cellobiose (Lancaster Synthesis Co.) was found by ^1H n.m.r. spectroscopy to be predominantly the β-anomer. Assignment of signals of the glucose residue at the reducing (C1–C6) and non-reducing end (C1′–C6′) of the molecule was made by consideration of the expected β and γ isotope effects† appropriate to each carbon atom as summarised in Table 1. For carbon atoms with only one isotope effect the observed doublets for C1′ ($\gamma_{12} = -0.020$), C6 ($\beta_6 = -0.110$), and C6′ ($\beta_6 = -0.113$ p.p.m.) are readily analysed in terms of H and D isotopomers in which negative isotope effects‡ correspond to lower frequencies by substitution with the heavier isotope as shown for the C6 signal in Figure 1.

For carbon atoms with two possible isotope effects (C1, C3, C2′, C4′, C5′) four resonance signals are expected corresponding to HH, HD, DH, and DD isotopomers as shown for the C2′ signal in Figure 1. If the two isotope effects have different magnitudes, all four resonance signals are observed (C1, C3, C2′) and the magnitudes of the separate isotope effects can be determined from appropriate line separations, *i.e.* C1, β_1 -0.104, γ_{12} -0.021; C3, β_3 -0.099, γ_{32} -0.043; C2′, β_2 -0.104, γ_{23} -0.029 p.p.m. The intensity ratios of the four lines are given by the probabilities of occurrence of the four isotopomers at a particular OH : OD ratio. It should be noted that the signs and magnitudes of the secondary isotope effects on each carbon atom can be determined by analysis of signal multiplicities and intensities at OH : OD ratios close to but not exactly equal to 1 : 1. If the two isotope effects are of similar magnitudes within experimental error, the carbon atom is observed as three signals (C5′, γ_{54} *ca.* γ_{56} *ca.* -0.027 p.p.m.) and the observed signal intensity ratio (1 : 2 : 1) is accounted for by the degeneracy of the HD and DH isotopomers. The C4(γ_{43}) and C4′ ($\beta_4 + \gamma_{43}$) signals should be

Table 1. Characteristics of carbon signals of β-(1→4) glucosides: β-cellobiose.[a]

Carbon atom	Isotope effects	No. of lines	
		Expected	Obs.[a]
C1	$\beta_1 + \gamma_{12}$	4	4
C1′	γ_{12}	2	2
C2	$\beta_2 + \gamma_{21} + \gamma_{23}$	8	7[b]
C2′	$\beta_2 + \gamma_{23}$	4	4
C3	$\beta_3 + \gamma_{32}$	4	4
C3′	$\beta_3 + \gamma_{32} + \gamma_{34}$	8	5[c]
C4	γ_{43}	2	1[d]
C4′	$\beta_4 + \gamma_{43}$	4	4[d]
C5	γ_{56}	2	2
C5′	$\gamma_{54} + \gamma_{56}$	4	3[e]
C6	β_6	2	2
C6′	β_6	2	2

[a] Observations by n.m.r. spectroscopy in this work. [b] DHH and HDD degenerate because $(\gamma_{21} + \gamma_{23}) = \beta_2$ and $\gamma_{21} \neq \gamma_{23}$. [c] Degeneracies caused by $(\gamma_{21} + \gamma_{23}) = \beta_2$ and $\gamma_{21} = \gamma_{23}$. [d] Broad signal due to presence of extra effect. [e] Degeneracy caused by $\gamma_{54} = \gamma_{56}$.

---

† The notation for β and γ isotope effects describes the carbon signal being observed (*e.g.* C3′) by a numerical subscript (*e.g.* β_3) and, when appropriate, a second numerical subscript is used for the hydroxy-group which gives rise to the isotope effect (*e.g.* γ_{32} and γ_{34}).

‡ The definition of the sign of the isotope effect is consistent with the definition of secondary isotope shifts, $\delta(X^nH) - \delta(X^1H)$.

observed as a doublet and quartet, respectively, whereas both signals appear significantly broadened owing to the presence of another effect (possibly a long range isotope effect or deuterium coupling).

For carbon atoms with three possible isotope effects (C2 and C3′) a maximum of eight lines are expected corresponding to isotopomers with no D substitution (HHH), one D substitution (HHD, HDH, DHH), two (HDD, DHD, DDH), and three D atom substitution (DDD) of hydroxy-groups. The C2 signal is observed as seven lines (Figure 1) corresponding to three isotope effects with different magnitudes (β_2 -0.105, γ_{21} -0.065, γ_{23} -0.040 p.p.m.) in which the DHH and HDD isotopomers are degenerate because $\beta_2 = (\gamma_{21} + \gamma_{23})$. On the other hand, the C3′ signal has three isotope effects (β_3 -0.097, γ_{32} *ca.* γ_{34} *ca.* -0.045 p.p.m.) but is observed as a five line multiplet owing to the degeneracies of the HHD/HDH, the HDD/DHH, and DHD/DDH isotopomers.

The ^{13}C n.m.r. spectrum of cellobiose, for example, can be completely assigned (except for C2′ and C4′) without recourse to comparisons with other molecules or other types of n.m.r. experiments by analysis of the signal multiplicities in Figure 1 and from a knowledge of which signals belong to the reducing and non-reducing glucose residues. The latter information was determined by warming a solution of cellobiose to form a mixture of α- and β-anomers; this mixture was manifested by the appearance of a second set of ^{13}C signals in which those from the non-reducing residues have similar chemical shifts whereas those from the reducing residues have different chemical shifts. The signal assignment of cellobiose in $(CD_3)_2SO$ solution (Figure 1) agrees with that previously published for the same solvent[2] and with the assignment in D_2O solution[2,7] which was checked by observing chemical shift changes of signals utilising a series of $(CD_3)_2SO$–D_2O mixtures.[2]

The variation of isotopomer multiplicities of ^{13}C n.m.r. signals of molecules with partially deuteriated hydroxy-groups together with the variations of the magnitudes of γ isotope effects (-0.020 to -0.066 p.p.m. in cellobiose which may be related to the stereochemistry of the hydroxy-groups) provides the basis of an important method of assignment of signals and for investigation of isotope effects in molecules with exchangeable hydrogen atoms such as carbohydrates, nucleosides and peptides.

We thank the S.E.R.C. for providing a studentship (J. C. C.) and n.m.r. facilities (together with the University of London, ULIRS service) and the M.R.C. for providing n.m.r. computing facilities (Birkbeck College).

Received, 20th December 1982; Com. 1451

References

1 D. E. Dorman and J. D. Roberts, *J. Am. Chem. Soc.*, 1970, **93**, 4463.

2 A. Heyraud, M. Rinaudo, M. Vignon, and M. Vincendon, *Biopolymers*, 1979, **18**, 167.

3 P. A. J. Gorin, *Adv. Carbohyd. Chem. Biochem.*, 1981, **38**, 13.

4 G. K. Hamer, F. Balza, N. Cyr, and A. S. Perlin, *Can. J. Chem.*, 1978, **56**, 3109.

5 P. E. Pfeffer, K. M. Valentine, and F. W. Parrish, *J. Am. Chem. Soc.*, 1979, **101**, 1265.

6 D. Gagnaire and M. Vincendon, *J. Chem. Soc., Chem. Commun.*, 1977, 509.

7 L. D. Hall, G. A. Morris, and S. Sukumar, *J. Am. Chem. Soc.*, 1980, **102**, 1745.