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Isotope-edited 1D and 2D n.m.r. spectroscopy of ^{13}C-substituted carbohydrates

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ABSTRACT

Three isotope-edited n.m.r. methods have been applied to selectively ^{13}C-substituted monosaccharides and nucleosides to simplify their spectra and/or measure ^{1}H–^{1}H, ^{13}C–^{1}H, or ^{13}C–^{13}C spin-couplings detected *via* the labeled site. 1D INADEQUATE spectra allowed the selective detection of the natural-abundance carbons that are spin-coupled to the labeled carbon, and adjustment of the mixing time permitted further discrimination between one-bond and longer-range ^{13}C–^{13}C coupling pathways. Geminal and vicinal ^{13}C–^{1}H coupling constants were determined from the analysis of ^{1}H–^{1}H COSY cross-peaks for those protons coupled to the labeled carbon. Long-range ^{13}C- (HETCOR) and ^{1}H-detected (HMBC) ^{13}C–^{1}H chemical-shift correlation spectra permitted the selective observation of those protons coupled to the labeled site, and $J_{H,H}$ values were measured from data projections. The implications of these methods for structural studies of more complex systems is briefly discussed.

INTRODUCTION

^{13}C-Substituted carbohydrates have found many conventional applications in studies of chemical and biological systems. For example, ^{1}H-decoupled ^{13}C-n.m.r. and ^{1}H-n.m.r. spectra of ^{13}C-substituted mono- and oligo-saccharides provide precise ^{13}C–^{13}C ($J_{C,C}$) and ^{13}C–^{1}H ($J_{C,H}$) spin-coupling information, respectively, in these molecules that is valuable in assessing their molecular structures and conformations in solution[1–3]. ^{13}C–^{13}C Spin-couplings are often difficult to obtain with natural (unlabeled) compounds, but long-range $J_{C,H}$ values can be measured in unlabeled molecules by several methods[4]. ^{13}C-Substituted carbohydrates are also employed to monitor biological metabolism *in vivo* and *in vitro*[5–7] and have been used to probe a wide range of chemical and enzymic reaction mechanisms[8–10], often in conjunction with n.m.r. spectroscopy and mass spectrometry.

Over the past decade a variety of multipulse 1D and 2D n.m.r. methods have been developed[11,12] to simplify the interpretation of spectra of complex molecules where

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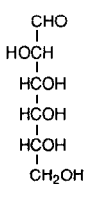
signal overlap can be problematic even at high magnetic fields. Spectral simplification is achieved in 1D spectra by spin editing (*e.g.*, DEPT[13], INADEQUATE[14]), or in 2D spectra by correlating chemical shifts and/or spin-couplings (*e.g.*, COSY[15], HMQC[16], HMBC[16]). Recent developments in three[17,18] and four[19,20] dimensional spectroscopy promise to further reduce signal overlap in complex molecules. Many of these methods are normally applied to natural (unenriched) compounds, and thus it may not be widely appreciated that the power of multipulse n.m.r. spectroscopy can be enhanced significantly when applied to stable isotopically-enriched compounds. The value of this combination has been aptly demonstrated in n.m.r. studies of ^{13}C- and ^{15}N-substituted proteins[21,22], but this methodology has wider potential applications. For example, 1D INADEQUATE and 2D ^{13}C–^{1}H shift correlation spectroscopy of ^{13}C-substituted molecules permits the selective detection of those carbons and protons, respectively, in the molecule that are spin-coupled to the labeled site(s)[1b,23]. Homonuclear 2D J-spectroscopy[24] and ^{1}H–^{1}H COSY applied to selectively ^{13}C-substituted molecules permit the measurement of ^{13}C–^{1}H spin couplings from projected data (^{1}H-decoupled ^{1}H spectrum)[25] or from cross-peaks, respectively. In these applications, the presence of the ^{13}C-label enhances the selectivity of the n.m.r. observation to yield edited spectra containing valuable chemical-shift and spin-coupling information. In this paper, we illustrate the potential of this approach as applied to several model carbohydrates and their derivatives **1–5**, and briefly discuss the implications of these methods for studies of more complex systems.



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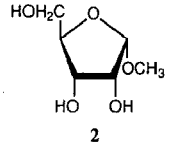

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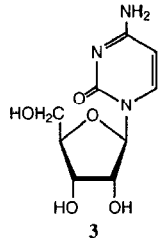

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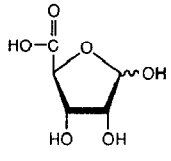

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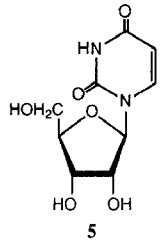

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EXPERIMENTAL SECTION

Materials. — D-(6-^{13}C)Altrose[26a], D-(2-^{13}C)riburonic acid[26b], methyl α-D-(2-^{13}C) ribofuranoside[23], (1′-^{13}C)cytidine[23], and (1′-^{13}C)uridine[23] were prepared by methods reported previously.

Instrumentation. — One dimensional INADEQUATE ^{13}C-n.m.r. spectra[1b,14] in $^{2}H_2O$ were obtained on a General Electric GN-300 300 MHz F.t.-n.m.r. spectrometer equipped with a 5-mm dual ^{1}H/^{13}C probe and operating in the quadrature phase mode. Spectra were collected and processed to give a final digital resolution of 0.075 Hz/pt.

Two-dimensional ^{1}H–^{1}H homonuclear shift correlation (COSY) spectroscopy[15] (absolute value) was conducted on the GN-300 n.m.r. spectrometer using 1024 blocks of 2048 data points collected over a sweep width of ~ 1500 Hz. Two-dimensional long-range ^{13}C–^{1}H chemical shift correlation spectra (CSCMLR)[27] were obtained on the same spectrometer. The pulse sequence included a reverse discrimination TANGO pulse incorporated into the mixing period of the HETCOR experiment to provide selective transfer of magnetization from remote protons to carbon. Blocks (512) of 2048 data points were collected per spectrum over a sweep width of ~ 400 Hz for ^{13}C and ~ 500 Hz for ^{1}H. F.i.d.s were processed with optimized resolution enhancement (double-exponential function) and interferograms were zero-filled twice prior to Fourier transformation in the t_1 dimension.

Heteronuclear multiple-bond coherence (HMBC)[16b] spectroscopy was performed on a Varian VXR-500S 500 MHz F.t.-n.m.r. spectrometer equipped with a 5-mm indirect detection probe. The experiment was optimized for long-range ^{13}C–^{1}H coupling, and the ^{13}C decoupler pulse length was set as 21 μs at a power level of 55 dB. Increments (512) of 2048 data points were obtained over a sweep width of ~ 2000 Hz for ^{13}C and ~ 3000 Hz for ^{1}H. The 2D data were zero-filled to give a final 1K × 4K matrix and processed with sine-bell functions in both t_1 and t_2 dimensions to optimize resolution.

All 2D experiments were conducted with the sample in a non-spin mode.

RESULTS AND DISCUSSION

INADEQUATE ^{13}C-n.m.r. spectra of selectively ^{13}C-substituted compounds. — The INADEQUATE method in either one[14] or two[28] dimensions is applied conventionally to determine carbon–carbon connectivities or to measure ^{13}C–^{13}C spin-couplings in unenriched molecules. When applied to a ^{13}C-substituted molecule, this method permits the selective detection of those natural abundance carbons that are spin-coupled to the labeled carbon. Adjustment of the mixing time in the pulse sequence allows further discrimination of the detectable carbon signals based on the magnitude of $J_{C,C}$.

The application of this editing method shows most promise in studies of complex mixtures and of oligo- or poly-meric compounds. In these cases, ^{13}C-n.m.r. spectra are more complex, and the identification of the spin-coupled carbons is more difficult. For example, aqueous solutions of the aldohexose, D-altrose (**1**), contain four cyclic forms[1b,29,30], α-furanose (*αf*, 17%), β-furanose (*βf*, 13%), α-pyranose (*αp*, 27%), and β-pyranose (*βp*, 43%). The ^{13}C-n.m.r. spectrum of D-(6-^{13}C)altrose (Fig. 1A) is complex, resembling that of a tetrasaccharide. 1D INADEQUATE spectra of D-(6-^{13}C)altrose obtained with different mixing times are shown in Fig. 1B–D. The four C-5 carbons are selectively detected with short mixing times (6 ms) (Fig. 1B) since these carbons

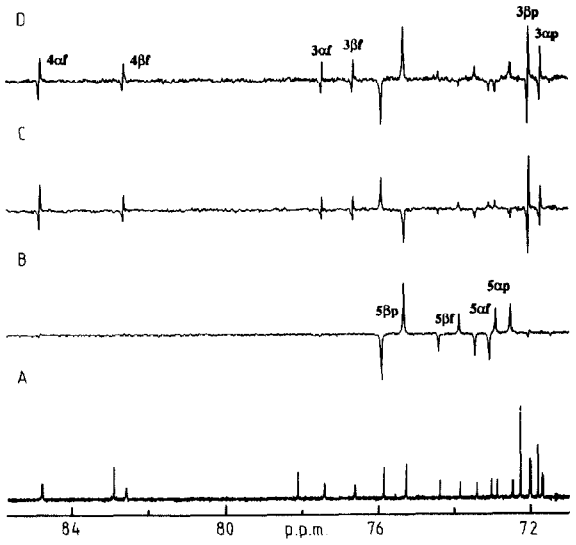

Fig. 1. (A) The partial ^{1}H-decoupled ^{13}C-n.m.r. spectrum (75 MHz) of D-(6-^{13}C)altrose (**1**). (B–D) Partial INADEQUATE ^{13}C-n.m.r. spectra of **1** obtained with 6 (B), 60 (C), and 120 ms (D) mixing times. Short mixing times allow the selective detection of the antiphase C-5 carbon signals of the four anomers (αp, α-pyranose; βp, β-pyranose; αf, α-furanose; βf, β-furanose). Increasing the mixing time results in the suppression of the C-5 signals and the enhancement of the signals of those carbons experiencing long-range ^{13}C–^{13}C coupling to C-6.

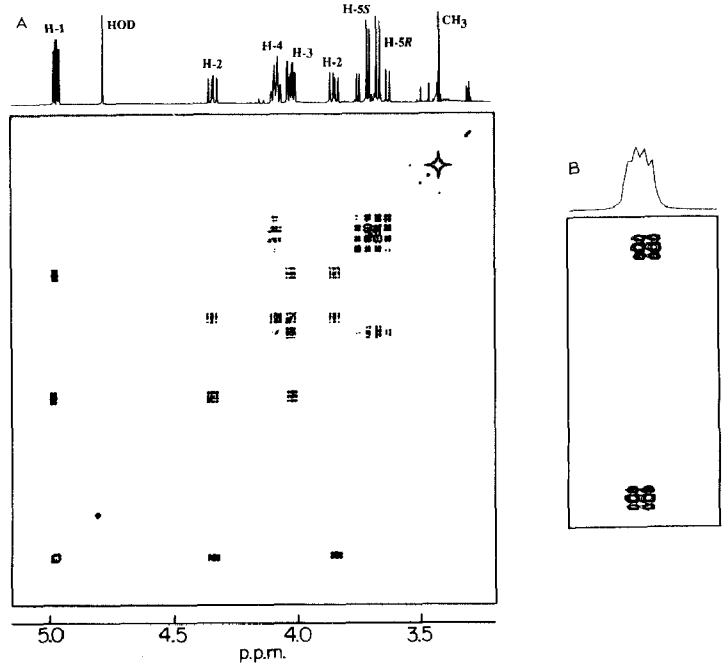

Fig. 2. (A) The ^{1}H–^{1}H COSY spectrum (300 MHz) of methyl α-D-(2-^{13}C)ribofuranoside (**2**) in $^{2}H_2O$ showing signal assignments. The large one-bond $^{1}J_{C\text{-}2,H\text{-}2}$ (149.2 Hz) produces paired H-2 signals in both the 1D and 2D spectrum. (B) The paired cross-peaks for H-1–H-2 are shifted, and projection produces a quartet from which $^{2}J_{C\text{-}2,H\text{-}1}$ (2.2 Hz) was measured.

experience a large one-bond ^{13}C–^{13}C coupling (41.1–42.9 Hz)[26a] to C-6. With longer mixing times (Fig. 1C and D), detection *via* the one-bond pathway is suppressed and detection *via* the smaller $^{2}J_{C,C}$ and $^{3}J_{C,C}$ (0.6–3.2 Hz)[26a] is enhanced, giving ten signals (C-1αp, C-1βp, C-3αf, C-3βf, C-3αp, C-3βp, C-4αf, C-4βf, C-4αp, and C-4βp). The remaining six natural-abundance carbon signals are completely suppressed at all mixing times, since they are not coupled to C-6. Recently, INADEQUATE spectra have also been used to assist in the assignment of signals in the ^{13}C-n.m.r. spectrum of D-(1-^{13}C) talose[31a], and in the simplification of the ^{13}C-n.m.r. spectrum of the disaccharide, sucrose, labeled with ^{13}C at C-2 of the β-D-fructofuranosyl ring[31b].

Effect of selective ^{13}C-substitution on ^{1}H–^{1}H COSY[15] spectra. — ^{1}H–^{1}H COSY spectra are used mainly to establish the ^{1}H–^{1}H coupling network in a molecule and to measure $J_{H,H}$ values through the analysis of off-diagonal elements (cross-peaks). COSY spectra of selectively ^{13}C-substituted compounds, however, may be used in some instances to measure $J_{C,H}$ values, again through the analysis of cross-peaks. The ^{1}H–^{1}H COSY spectrum of methyl α-D-(2-^{13}C)ribofuranoside (**2**) is shown in Fig. 2A. Since H-2 of **2** is directly bonded to the labeled carbon, its signal contains a large $^{1}J_{C\text{-}2,H\text{-}2}$ (149.2 Hz), producing two multiplets along the diagonal. An inspection of the two pairs of cross-peaks correlating the two H-2 signals with H-1 reveals a 2 × 3 pattern for each, with the doublet due to $^{3}J_{H\text{-}1,H\text{-}2}$ (4.3 Hz) and the triplet due to the combined effect of

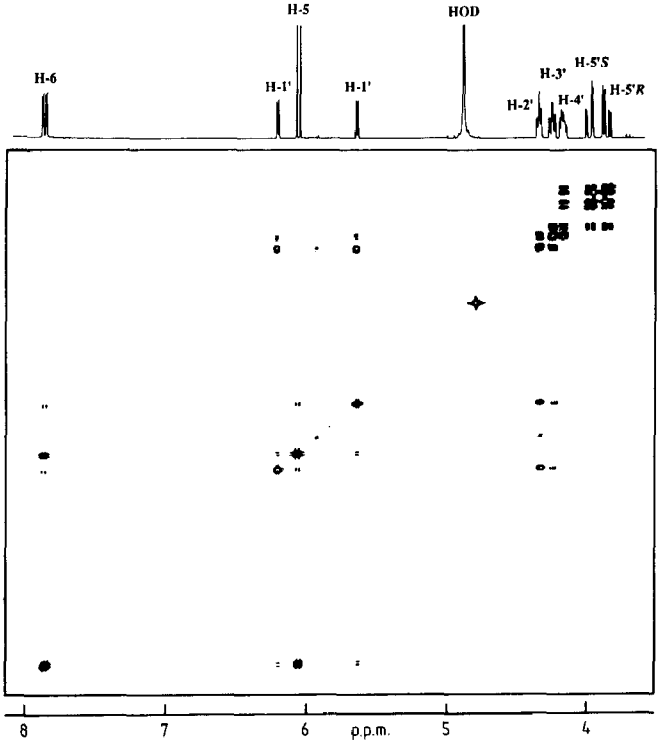

Fig. 3. The ^{1}H–^{1}H COSY spectrum of (1'-^{13}C)cytidine (**3**) in $^{2}H_2O$ showing signal assignments.

$^3J_{H\text{-}1,H\text{-}2}$ and $^3J_{H\text{-}2,H\text{-}3}$ (6.2 Hz). However, these paired cross-peaks are not aligned in the F_1 or F_2 dimension, and their projection produces a quartet (Fig. 2B) that contains two couplings, $^3J_{H\text{-}1,H\text{-}2}$ and $^2J_{C\text{-}2,H\text{-}1}$ (2.2 Hz). A similar analysis of the paired cross-peaks relating H-2 to H-3 permits a determination of $^2J_{C\text{-}2,H\text{-}3}$ (1.1 Hz).

Cross-peak analysis of the ^1H–^1H COSY spectrum of (1′-^{13}C)cytidine (**3**, Fig. 3) indicates the presence of ^{13}C–^1H coupling within the furanosyl ring and across the *N*-glycoside bond[23]. Analysis of the H-1′–H-2′ cross-peaks (Fig. 4A) yields $^2J_{C\text{-}1',H\text{-}2'}$ (1.7 Hz). Interestingly, cross-peaks correlating H-1′ and H-3′ (Fig. 4A), and H-1′ and H-6 (Fig. 4B), indicate the presence of long-range coupling ($^4J_{H,H}$) between these sites in **3**, and the projection of these cross-peaks permits the measurement of $^3J_{C\text{-}1',H\text{-}3'}$ (3.0 Hz) and $^3J_{C\text{-}1',H\text{-}6}$ (2.8 Hz), respectively. Long-range coupling ($^5J_{H,H}$) is also observed between H-1′ and H-5 of **3** (Fig. 3) and the cross-peaks correlating these sites are aligned as expected, since C-1′ is not coupled to H-5[23]. $^4J_{H\text{-}1',H\text{-}6}$ and $^5J_{H\text{-}1',H\text{-}5}$ have been previously observed in pyrimidine nucleosides[31c].

In some cases, $J_{C,H}$ values can be measured from the analysis of a single cross-peak, as illustrated for D-(2-^{13}C)riburonic acid (**4**) in Fig. 5A and B. $^2J_{C\text{-}2,H\text{-}3}$ and $^3J_{C\text{-}2,H\text{-}4}$ for the β-furanose of **4** were determined from an inspection of the cross-peaks correlating H-3 and H-4. The signal shift in the F_1 dimension was used to determine $^3J_{C\text{-}2,H\text{-}4}$ (~ 1.5 Hz), whereas the shift in the F_2 dimension gave $^2J_{C\text{-}2,H\text{-}3}$ (~ 1.5 Hz) (Fig. 5B).

Recently, the H-1′–H-2′ cross-peaks in DQF-COSY spectra were used to measure $^2J_{C\text{-}1',H\text{-}2'}$ values in the A_1 (6.5 Hz) and A_5 (6.6 Hz) residues of the single-stranded DNA

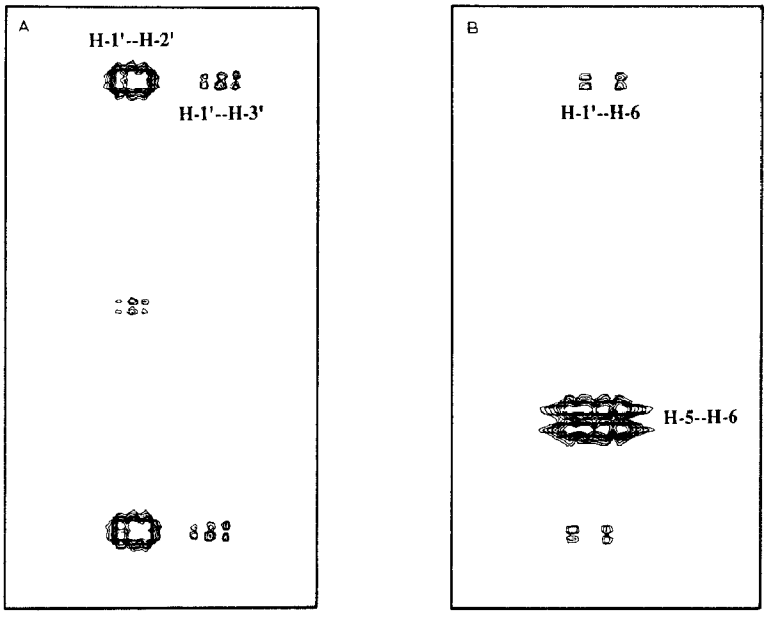

Fig. 4. Expansion of the paired cross-peaks in Fig. 3 for H-1′–H-2′ and H-1′–H-3′ (A), and H-1′–H-6 (B). The shifts for these paired cross-peaks yield $^2J_{C\text{-}1',H\text{-}2'}$ (1.7 Hz), $^3J_{C\text{-}1',H\text{-}3'}$ (3.0 Hz) and $^3J_{C\text{-}1',H\text{-}6}$ (2.8 Hz). The weak signal in the center of the H-1′–H-2′ cross-peaks arises from the small population of molecules not enriched with ^{13}C at C-1′.

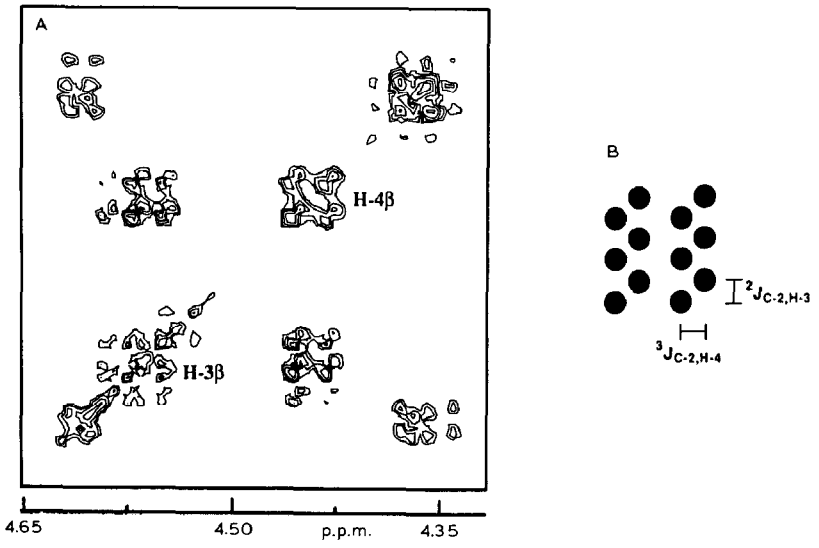

Fig. 5. (A) The partial ^1H–^1H COSY spectrum of D-(2-^{13}C)riburonic acid (**4**) in ^2H_2O (p^2H 1.7) showing the H-3–H-4 cross-peaks for the β-furanose. Each cross-peak consists of two 3 × 2 components that are displaced in both the F_1 and F_2 dimensions. (B) Simulated drawing of the H-3–H-4 cross-peak in (A). $^2J_{C\text{-}2,H\text{-}3}$ (1.5 Hz) and $^3J_{C\text{-}2,H\text{-}4}$ (1.5 Hz) were determined from the shifts in the cross-peaks along F_1 and F_2 as shown.

octamer, d(A_1GCCA_5ATA), which were selectively substituted at C-1′ with ^{13}C, and $^3J_{C\text{-}1',H\text{-}3'}$ (5.4 Hz) was determined in the A_1 residue from the H-1′–H-3′ paired cross-peaks in TOCSY and RELAY-COSY spectra of this labeled octamer, demonstrating that more complex structures can be studied by this method[32]. Furthermore, using a similar strategy, Montelione *et al.*[33] have used NOESY spectra to measure long-range ^{15}N–^1H couplings in a 99% ^{15}N-substituted protein by examining the patterns of the H^N–H^β paired cross-peaks.

^{13}C–^1H Shift correlation spectroscopy of selectively ^{13}C-substituted compounds. — ^{13}C–^1H shift correlation spectroscopy is used routinely to assist in the assignment of ^1H- and/or ^{13}C-n.m.r. signals. The method, however, may be used to edit ^1H-n.m.r. spectra

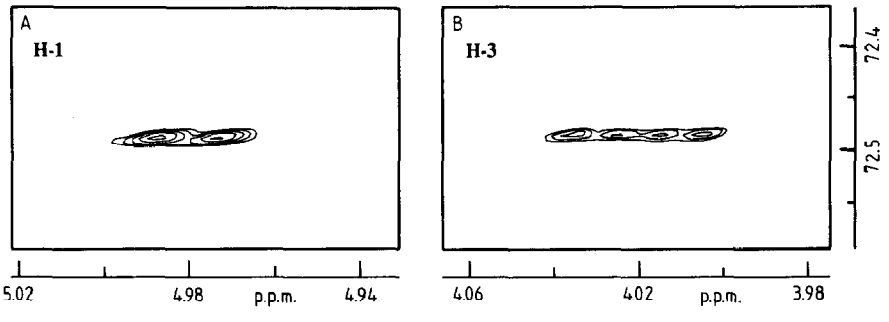

Fig. 6. The selective detection of H-1 (A) and H-3 (B) of methyl α-D-(2-^{13}C)ribofuranoside (**2**) by ^{13}C-detected long-range ^{13}C–^1H shift correlation. $^3J_{H\text{-}1,H\text{-}2}$ (4.3 Hz), $^3J_{H\text{-}2,H\text{-}3}$ (6.1 Hz), and $^3J_{H\text{-}3,H\text{-}4}$ (3.1 Hz) were determined from the H-1 doublet and H-3 quartet obtained by projection.

of molecules that have been selectively labeled with the ^{13}C isotope. Recently, 2D ^{13}C–^{1}H shift correlation spectroscopy (direct detection) optimized for $^{1}J_{C,H}$ was used to selectively detect the H-1′ signal of (1′-^{13}C)uridine[23]. The same experiment optimized for long-range $J_{C,H}$ is illustrated for methyl α-D-(2-^{13}C) ribofuranoside (**2**) in Fig. 6. The 1D ^{1}H-n.m.r. spectrum of **2** (Fig. 2A) showed that H-1 (2.2 Hz), H-3 (1.1 Hz) and H-4 (0.8 Hz) are coupled[23] to C-2′. The long-range correlation map reveals signals correlating C-2′ with H-1 (doublet, Fig. 6A) and H-3 (quartet, Fig. 6B), and an analysis of these multiplets yielded values of $^{3}J_{H\text{-}1,H\text{-}2}$ (4.3 Hz), $^{3}J_{H\text{-}2,H\text{-}3}$ (6.1 Hz), and $^{3}J_{H\text{-}3,H\text{-}4}$ (3.1 Hz) that are identical or close in magnitude to those derived from the 1D spectrum[23]. The H-4 signal was also detected but not well resolved due to the small value of $^{3}J_{C\text{-}2,H\text{-}4}$ and insufficient signal-to-noise. It should be appreciated that the F_1 block size corresponding to the ^{1}H dimension is critical to the success of this experiment and must be large enough to provide sufficient digital resolution to resolve the ^{1}H multiplets.

The sensitivity of this method is enhanced considerably with the use of indirect detection (HMBC)[16b]. This experiment has been applied to (1′-^{13}C)uridine (**5**) allowing

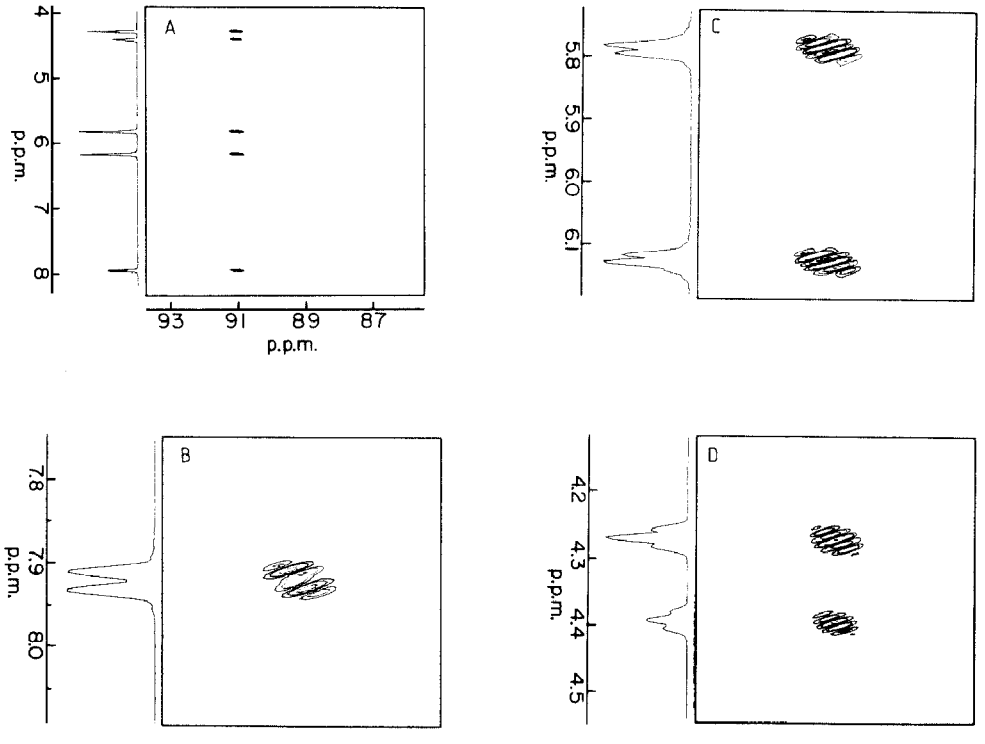

Fig. 7. (A) The ^{13}C–^{1}H multiple-bond coherence (HMBC)[16b] spectrum of (1′-^{13}C)uridine (**5**) showing the selective detection of H-1′, H-2′, H-3′, and H-6 which are spin-coupled to C-1′. The projection of the H-6 (B) and H-1′ (C) signals allows measurement of $^{3}J_{H\text{-}5,H\text{-}6}$ (8.7 Hz) and $^{3}J_{H\text{-}1',H\text{-}2'}$ (4.9 Hz), respectively. $^{3}J_{H\text{-}2',H\text{-}3'}$ (5.9 Hz) and $^{3}J_{H\text{-}3',H\text{-}4'}$ (5.9 Hz) were derived from the projected H-3′ triplet (~ 4.27 p.p.m.) (D). The H-2′ quartet (~ 4.39 p.p.m.) (D) was not well resolved and appears as a triplet due to the combined effect of $^{3}J_{H\text{-}1',H\text{-}2'}$ and $^{3}J_{H\text{-}2',H\text{-}3'}$.

the selective detection of the H-2′ and H-3′ multiplets in the furanose ring and H-6 of the base (Fig. 7A). $^3J_{H\text{-}5,H\text{-}6}$ (8.7 Hz) (Fig. 7B) and $^3J_{H\text{-}1',H\text{-}2'}$ (4.9 Hz) (Fig. 7C) were obtained from the doublets generated by projection. The projection of H-3′ appeared as a triplet (Fig. 7D), from which $^3J_{H\text{-}2',H\text{-}3'}$ and $^3J_{H\text{-}3',H\text{-}4'}$ could be calculated as 5.9 Hz. These couplings are ~ 8% larger than those measured from the 1D ^{1}H-n.m.r. spectrum of **5**[23], and this error may be caused by insufficient digital resolution and/or effects from sine-bell processing of the data.

Implications of isotope-edited n.m.r. methods in studies of complex systems. — Simple monosaccharides and their derivatives were chosen in this study to illustrate the salient features of several ^{13}C-edited n.m.r. methods. It should be appreciated, however, that the full power of these methods will be realized in studies of more complex structures and mixtures, for example, in structural studies of complex carbohydrate-based oligomers such as oligosaccharides and oligonucleotides. In these systems, the use of ^{13}C as a "spy" nucleus to selectively detect carbons or protons spin-coupled to the enriched site(s) may be invaluable in assessing local geometry. The ability to detect specific spins conferred by ^{13}C-edited n.m.r. methods has great potential. This approach has recently been used to selectively detect the histidine C-2 protons in the ^{1}H-n.m.r. spectrum of chloramphenicol acetyltransferase (EC 2.3.1.28), an enzyme of overall molecular mass[34] 75 000. In addition, under proper solution conditions, the ^{1}H-n.m.r. signals of a ^{13}C-substituted carbohydrate substrate bound to a protein (*e.g.*, lectin) may be selectively detected over the more abundant proton signals of the protein using ^{13}C–^{1}H shift correlation spectroscopy conducted in the HMQC[16] mode, in a manner analogous to that recently reported for drug–protein complexes[35]. The identification of unknown metabolites *in vivo* generated from ^{13}C-substituted substrates could be assisted by obtaining complementary ^{1}H chemical-shift and spin-coupling information made available through ^{13}C–^{1}H correlation maps. Finally, it should be appreciated that spectral editing may also be achieved *via* isotopes other than ^{13}C (*e.g.*, ^{15}N), and that the methods discussed in this paper are not confined to carbohydrate-based systems.

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