Comparisons of heteronuclear RELAY spectroscopy, first introduced by Bolton (1, 2), permits the detection of indirect connectivity between heteronuclei. The most common application of this type of experiment is found in the correlation of $^1$H and $^{13}$C chemical shifts. If a proton ($H_a$) is attached to a carbon ($X$), and a second (remote) proton ($H_b$) has a scalar interaction with proton $H_a$, then the RELAY experiment transfers magnetization from $H_a$ via $H_b$ to $X$. The RELAY spectrum thus shows remote connectivity between $H_a$ and $X$, in addition to the direct correlation between $H_a$ and $X$. In cases where proton $H_b$ resonates in a very crowded spectral region, and therefore $X$ cannot be assigned by its one-bond correlation with $H_a$, assignment of $X$ can be made from the RELAY spectrum by correlation with remote proton $H_b$.

A large variety of slightly different versions of the heteronuclear RELAY experiment have been proposed in recent years (3–9). The sensitivity of all RELAY experiments proposed so far is significantly lower (10) than for direct correlation (11–14) of $^1$H and $^{13}$C chemical shifts. The principal reason for this is that in all these experiments transfer of magnetization from $H_a$ to $H_b$ is a rather inefficient process (depending on the particular spin system and the transfer method used). Here we demonstrate that the use of $^1$H-detected heteronuclear RELAY spectroscopy is readily possible, providing an improvement in sensitivity of at least an order of magnitude over experiments that utilize $^{13}$C detection.

Two possible schemes for $^1$H-detected heteronuclear RELAY are shown in Fig. 1. Scheme 1a is the analog of the conventional RELAY experiment (1–5), where magnetization is transferred between coupled protons by means of a $90^\circ$ pulse. A slightly different version of this scheme has been used by Frey et al. (15) to study relayed connectivity between Cd and the cysteine $C_\alpha$H protons in metallothionin. Scheme 1a also shows similarity to a method, developed by Field and Messerle (16), which employs $^1$H detection of magnetization relayed from $^{31}$P in phosphate sugars. However, this interesting method does not provide the maximum possible gain in NMR sensitivity because it relies upon transfer of magnetization from a low-$\gamma$ nucleus to $^1$H, rather than from $^1$H to $^1$H via an intermediary nucleus, as used in the schemes described.
FIG. 1. Pulse schemes for $^1$H-detected heteronuclear RELAY spectroscopy. Magnetization transfer from $^1$H to $^1$H is accomplished (a) by the final 90° $^1$H pulse or (b) by homonuclear Hartmann-Hahn mechanism. To suppress direct correlations, $^{13}$C decoupling is started at a time $2\Delta (=1/J_{ch})$ after the final 90° $^{13}$C pulse. The phase cycling of the basic 8-step cycle is as follows: $\psi = x, y, -x, -y, y, -y, -y, -y; \theta = x, x, x, x, -x, -x, -x, -x$. Acq. $x, x, -x, -x, x, -x, -x, -x$. The phase $\phi$ is incremented by 90° every 32 scans, and each time $\phi$ is incremented, the phase of the receiver should be inverted.

here. Scheme 1b uses the homonuclear Hartmann-Hahn mechanism (17–21) to transfer magnetization between coupled protons and is the $^1$H-detected analog of another recently proposed modification of the RELAY experiment (9).

In both schemes, protons that are not directly attached to a $^{13}$C nucleus can be saturated by the application of a bilinear pulse (22, 23), in the same way as proposed previously (24) for $^1$H detected $^1$H–$^{13}$C correlation via one-bond couplings. This so-called BIRD pulse, applied at about two-thirds of the delay period between consecutive scans (24), inverts the z magnetization of all protons not coupled to $^{13}$C and leaves the z magnetization of protons attached to $^{13}$C virtually unaffected. At the beginning of the actual experiment (the 90° $^{13}$C pulse), protons not attached to $^{13}$C are close to saturation whereas magnetization of protons attached to $^{13}$C approaches the equilibrium value. True saturation of the intense signal from protons not coupled to $^{13}$C greatly alleviates the problem of suppressing this large unwanted signal component by subsequent phase cycling. It also solves the dynamic range problem that often is encountered on high-field spectrometers, even at millimolar sample concentrations.

The phase cycling is identical for schemes 1a and b and is given in the figure caption. Phase cycling of the observe pulses should be minimized in this type of experiment because a change in phase of one proton pulse relative to another creates a new "steady-
state” z magnetization at the beginning of the actual experiment, aggravating the
suppression problem. Even while phase cycling of the 180° pulse in principle does not
affect the steady-state magnetization, in practice the 90° character of such a pulse (25)
can affect seriously the suppression of unwanted signals. We therefore prefer to cycle
the phase of the 180° pulse as little as possible. For example, if 128 scans are performed
per t₁ value, scans 1–32 use 180°, scans 33–64 use 180°, etc. Each time the phase of
the 180° pulse is incremented by 90°, the receiver phase should be changed by 180°.
If 32 or fewer scans per t₁ value provide sufficient sensitivity, we prefer to use no 1H
phase cycling at all. The 90° 13C pulse eliminates signals originating from 13C mag-
netization, present at the start of the sequence. By alternating the phase of this pulse
without changing the phase of the receiver, signals originating from this magnetization
are effectively suppressed. The 90° pulse could be eliminated from both schemes la
and b if the 180° 1H pulse was cycled along all four axes. However, its presence does
not affect the sensitivity of the method and nothing will be gained by removal of
this pulse.

The active part of the RELAY schemes start at the 90° 1H pulse. The transverse
magnetization created by this pulse for a proton H attached to a 13C nucleus is trans-
ferred into zero- and double-quantum coherences by the application of the 90° pulse,
a time Δ(≈0.5/JCH) later. Zero- and double-quantum coherences are interconverted
by the 180° pulse, and the final 90° 13C pulse converts (part of) the multiple-quantum
coherence back into 1H magnetization. The amplitude of this 1H magnetization is
modulated as a function of t₁ by the 13C chemical shift. The application of a 90° pulse
(Fig. 1a) transfers part of this 1H magnetization to its coupling partners (H), whose
magnetization then also will be modulated in amplitude by the 13C frequency. Alter-
natively, the HOHAHA mechanism can be used to transfer magnetization from Hₐ
to H (Fig. 1b). To minimize crowding of the final spectrum, it is desirable to suppress
the often intense correlation via one-bond couplings. To accomplish this, 13C decou-
pling (with WALTZ-16 modulation (26)) is switched on a time 1/JCH after the final
90° 13C pulse. 1H magnetization corresponding to direct connectivity will be in anti-
phase with respect to the polarization of the 13C nucleus at the time 13C decoupling
is switched on. Consequently, direct connectivity is effectively suppressed by this de-
layed decoupling procedure. Note that the amount of suppression is sensitive to the
decoupling delay and complete suppression is only possible for a narrow range of J
values.

Spectra recorded with either scheme can be presented in the phase-sensitive mode.
For scheme 1a, the signals of interest are along the ±x axis at the beginning of data
acquisition. For scheme 1b, both relayed and direct connectivity signals are aligned
along the y axis at the start of data acquisition. Phase parameters for the T₂ dimension
are most easily obtained from a 1H spectrum obtained for a single scan with a long
delay time, T (T > T₁). For scheme 1b, phasing in the F₂ dimension requires the same
corrections as phasing the one-scan spectrum to the absorption mode. For scheme 1a,
phase parameters should be used that correspond to phasing the one-scan spectrum
to the dispersive mode.

To demonstrate the utility of these methods, we apply them to a 3.5 mg sample of
the trisaccharide Neu5Acα(2,3)Galβ(1,4)Glc dissolved in 0.35 ml D₂O. This trisac-
charide is found in human urine and bovine colostrum, and is closely related to typical
O-linked oligosaccharides found in glycoproteins and proteoglycans (27). Berman (28)
Published a complete $^{13}$C assignment of this compound, as determined by deuterium isotope effects and by comparison with lactose derivatives. Haverkamp et al. (30) have published its partial $^1$H assignment based upon their extensive catalog of "structural reporter groups" for related compounds.

Figures 2a and b display a small, crowded region (23 protons resonate between 3.59 and 3.99 ppm) of the two spectra obtained with the schemes of Figs. 1a and b, respectively. Figure 2a results from a $2 \times 330 \times 512$ data matrix, which means that for each of the 330 different $t_1$ durations 2 FIDs of 512 data points each (256 complex data points) were acquired and stored in separate locations. Spectra were phased in the $F_2$ dimension as described above. For very short values of $t_1$, very little magnetization is transferred from the attached proton $H_a$ to remote proton $H_r$. In the $t_1$ dimension, we therefore started with a $t_1$ value of 20 ms, using a 100 $\mu$s $t_1$ increment; i.e., $t_1$ was stepped from 20 to 53 ms. In the $F_1$ dimension, some Gaussian broadening was used and the final spectrum is displayed in the absolute-value mode. Figure 2b resulted from a $2 \times 293 \times 512$ data matrix and a 25 ms MLEV-17 mixing period, flanked by two 1.5 ms trim pulses (20), was used. The spectrum was phase corrected in both dimensions. For both experiments, the delay time between scans, including the time $T$ (350 ms) between scans and the 120 ms data acquisition period, was 1.1 s. 96 scans were recorded per $t_1$ value and the total measuring times for the spectra of Figs. 2a and b were 11 and 10 h, respectively.

Below, we will briefly discuss how the spectrum of Fig. 2b can be used for assignment purposes. From the partial assignment published by Haverkamp et al. (28), we know that the anomeric proton of galactose appears at 4.53 ppm. Correlations at (78.45, 4.53) and (72.34, 4.53) must arise from relayed connectivity from Gal C2 and Gal C3 to Gal H1. The two are distinguishable by the presence of a third cross peak at (78.45, 3.98) corresponding to a relay from Gal C3 to Gal H4. A Gal H3 to C4 relay assigns Gal C4 to the 70.37 ppm resonance. Relays from Gal C5 to H4 and from Gal C4 to H5 were too small to be detected because of a very small H4/H5 scalar coupling.

Complete analysis and spectral assignment of this trisaccharide, based on the above described RELAY experiments and on other recently developed techniques, will be presented elsewhere (30).

The application to a relatively small sample of the trisaccharide clearly demonstrates the improved sensitivity of the relay methods described here. Considering that the glucose ring occurs as a 67%/33% mixture of $\beta$- and $\alpha$-isomers, the actual quantity of the $\alpha$-isomer present in the sample is only 1.2 mg. Nevertheless, most of the possible relay signals for this unit could be identified from the spectrum of which part is shown.

**FIG. 2.** Most crowded regions of the $^1$H-detected heteronuclear RELAY spectra of Neu5Aco(2.3)Galβ(1,4)Glc recorded with (a) the pulse scheme of Fig. 1a and (b) the scheme of Fig. 1b. 3.5 mg of the trisaccharide (Biocarb Chemicals, Lund, Sweden) was dissolved in 0.35 ml D$_2$O, yielding 1.2 mg of the $\alpha$-isomer and 2.3 mg of the $\beta$-isomer. Spectra were recorded at 21°C, pH 6.5. Total measuring times were (a) 11 h and (b) 10 h. Spectrum (a) has been phase corrected in the $F_2$ dimension, but an absolute-value-mode representation is used in the $F_1$ dimension. Artifacts at the $^{13}$C carrier frequency (80 ppm) in spectrum (a) arise from an incomplete steady state after two dummy scans. Spectrum (b) is phase sensitive in both dimensions. In spectrum (b), broken lines indicate part of the connectivity pattern for the galactose unit. Resonance assignments refer to the carbon of a particular sugar unit (N, Neu5Ac; G, Galβ, A, Glc-α ; B, Glicβ); e.g., C3 of Galβ is labeled G3. Protons are numbered analogously to the carbon numbering; assignment of a proton resonance to a particular sugar unit follows from the $^{13}$C label of the resonance.
in Fig. 2b. The sensitivity of the spectrum of Fig. 2b, as measured for some of the more intense RELAY signals, is about a factor of two greater than the sensitivity of the spectrum of Fig. 2a. The levels of the lowest contours in Figs. 2a and b are about 2 and 4 times higher, respectively, than the level where thermal noise starts appearing in the spectra. The relay methods offer information somewhat similar to the HMBC method (31) that provides long range $^1\text{H}-^1\text{C}$ connectivity information. Suppression of signals from protons not involved in the transfer process is much better in the relay methods than with the HMBC experiment and sensitivity also appears to be higher. The HMBC method, however, can offer additional information not available from a relay experiment (31).

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