Complete $^1$H and $^{13}$C Assignments of Coenzyme B$_{12}$ through the Use of New Two-Dimensional NMR Experiments

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Abstract: Four different types of new two-dimensional (2D) NMR techniques have been used to determine unambiguous $^1$H and $^{13}$C spectral assignments for (5'-deoxyadenosyl)cobalamin (coenzyme B$_{12}$, $M_r$ 1580, 6.5-mg sample in 0.35 mL of $H_2O$). Two-dimensional homonuclear Hartmann-Hahn spectroscopy in combination with 2D spin-locked NOE spectroscopy was used to assign the resonances of all nonexchangeable protons. Sensitivity enhanced $^1$H-detected 2D multiple-quantum NMR then provided the resonance assignments for all protonated $^{13}$C nuclei. The resonance assignments for nonprotonated carbons were determined via $^1$H-$^{13}$C multiple-bond, multiple-quantum spectroscopy. This experiment also confirmed independently our $^1$H and $^{13}$C assignments made with the other methods. The relative intensity of long-range $^1$H-$^{13}$C correlations is related to the magnitude of the $J_{CH}$ coupling involved and therefore provides qualitative structural information. Despite the careful application of classical $^{13}$C NMR assignment techniques in recent reports on coenzyme B$_{12}$, we found that nearly one-third of the $^{13}$C resonances had been assigned erroneously.

Since the early 1970s, cobalamins have been the subject of extensive NMR studies.$^{1-4}$ $^1$H and $^{13}$C resonance assignments have been used in the interpretation of steric and electronic effects and for structural analyses.$^{1,2}$ These resonance assignments were based on comparison with B$_{12}$ analogues and related model compounds, interpretation of $^{13}$C relaxation times, analyses of line-broadening effects in the presence of paramagnetic relaxation agents, pH effects, and heteronuclear scalar couplings. Assignments based on such evidence are susceptible to error; and as we will demonstrate in this paper, this approach can lead to erroneous conclusions for molecules as complex as cobalamins. In fact, despite the careful nature of earlier studies, nearly one-third of the $^{13}$C NMR resonances of (5'-deoxyadenosyl)cobalamin (coenzyme B$_{12}$, Chart I) are reassigned, as reported here.

Two-dimensional NMR methods can greatly simplify the analysis of complex $^1$H and $^{13}$C spectra. However, the low solubility of coenzyme B$_{12}$ makes the use of regular heteronuclear $^1$H-$^{13}$C experiments difficult. It is shown here that this problem can be overcome easily by using $^1$H-detected heteronuclear multiple-quantum coherence via direct coupling (HMQC)$^{9,14}$ or via multiple-bond coupling.$^{15}$ This approach alleviates the $^{13}$C sensitivity problem by at least an order of magnitude. Another problem in the NMR study of coenzyme B$_{12}$ is that nuclear Overhauser effects (NOEs) are very weak at the magnetic field strength employed. This is due to the size of the molecule, which has a molecular tumbling time, $\tau_m$, close to $1/\omega_L$, where $\omega_L$ is the angular proton Larmor frequency. Thus, the 2D NOESY experiment$^{16-19}$ that normally provides valuable "through-space connectivity" often gives poor results for molecules with molecular weights in the range of 1000-2000. In this study, we use the spin-locked NOE experiment, recently proposed by Bothner-By et al.,$^{20}$ to obtain the NOE information in a more effective way. This experiment provides the crucial connection between the J-coupled fragments of the molecule that are not connected via homonuclear $^1$H coupling. Proton resonance assignments within each fragment could then, in principle, be established by using the well-known homonuclear correlated spectroscopy (COSY) method.$^{19-21}$ However, because of the severe spectral overlap in several regions of the $^1$H spectrum, we find the recently developed homonuclear Hartmann-Hahn (HOHAA) method$^{22-25}$ preferable. This sensitive technique provides high-resolution phase-sensitive spectra that display both direct and relayed connectivities.

Combined use of the techniques mentioned above provides a new and reliable procedure for assignment of complex $^1$H and $^{13}$C

NMR spectra. This approach involves (a) assignment of the $^1$H spin-echo difference methods; (b) correlation via $^1$H-$^3$P spin-echo difference; (c) correlation via $^1$H-$^1$H-$^3$P spin-echo difference; (d) correlation via $^1$H-$^1$H-$^1$H-$^3$P spin-echo difference methods; and (c) correlation via $^1$H-$^3$C and $^1$H-$^1$H-$^3$C to provide assignments of nonprotonated carbons and to independently verify $^1$H and $^1$C assignments made with the other methods. All experiments were carried out using a relatively small amount of sample (6.5 mg), requiring special attention to be paid to the optimization of the experimental parameters for the various types of newer 2D NMR techniques.

Assignment of the $^1$H NMR Spectrum

A $^1$H-$^3$P echo difference spectrum

$^1$H

90°, $\Delta$-180°, $\Delta$-Acq. ($\pm$)

$^3$P

90°, 90° Ax decoupled

Figure 1. Part of the regular $^1$H spectrum of coenzyme B$_{12}$ (bottom) and $^1$H-$^3$P spin-echo difference spectrum (top). In the top spectrum, only protons that have a scalar interaction with the $^3$P nucleus are present.

Figure 2. Part of the phase-sensitive 2D HOHAHA spectrum of coenzyme B$_{12}$, obtained with a 57-ms MLEV-17 mixing period. The J-connectivity patterns for the two ribose rings and the propanolamine protons are indicated in the figure by drawn lines. Other connectivity patterns are indicated in supplementary figures.

is used to identify protons that are coupled to the $^3$P nucleus. Figure 1 shows the difference spectrum obtained for coenzyme B$_{12}$ and identifies protons R3H and Pr2H plus low intensity resonances that will later be identified as Pr3H. Hydrogens are identified by the carbon atom to which they are attached; for magnetically nonequivalent geminal protons, $^1$H and $^2$H refer to the protons with the downfield and upfield chemical shift, respectively. The R3H and Pr2H protons provide a simple starting point for further assignments which then can be made on the basis of NOE and scalar connectivity.

Homonuclear Hartmann–Hahn Spectroscopy. The recently developed HOHAHA method is used for defining the scalar-coupled networks. The sequence used here is

$90°, t_1 -$SL$_x$(MLEV-17)$_x$SL$_x$-Acq.$(t_2)$

where SL$_x$ denotes a short (2.5 ms) spin lock field applied along the $x$ axis to defocus magnetization transverse to the $x$ axis, and the phase $\phi$ is cycled along all four axes as described elsewhere. The MLEV-17 sequence is repeated an integer number of times, $N$. We prefer use of the MLEV-17 version over other types of propagation schemes for the following reasons. (a) A substantial bandwidth ($>$ 4 kHz) can be covered by using a modest rf field strength (7 kHz). (b) The apparent $T_1$ can be prolonged by up to a factor of 2.25. This makes the method attractive for larger molecules that generally have short $T_2$ and $T_2^*$ values. (c) The MLEV mixing sequence attenuates magnetization transfer that can occur via spin-locked NOE.18,31 This magnetization transfer is attenuated because during the MLEV sequence the “spin-locked” magnetization is aligned approximately 50% of the time along the static magnetic field. For molecules that are not in the

The connectivity networks for the corrin ring side chains are indicated in supplementary figures. All observed HOHAHA connectivities are presented in Table I. To assign the networks to a particular side chain, the spin-locked NOE experiment is used.

One disadvantage of the HOHAHA method is that it is sometimes difficult to determine whether a cross peak represents direct or relayed connectivity. To make this distinction one may also have to record a COSY spectrum with a shorter mixing time. For a mixing time of intermediate duration, as used for the spectrum of Figure 2, the assignments are largely unambiguous, however. Consider, for example, the adenosyl ribose ring. The anomic proton, A11H, shows connectivity to A12H and A13H, but only A13H shows relayed connectivity to the A15 protons, distinguishing A12H and A13H unequivocally. As is the case with most homonuclear shift correlation methods (COSY, NOESY, and variations thereof), the intensities of two cross peaks at coordinates \((\delta_A, \delta_B)\) and \((\delta_B, \delta_A)\) can be different if (a) the acquisition times in the two time domains are different; (b) different digital filtering procedures are used in the two dimensions; or (c) if the longitudinal relaxation times, \(T_1\), of spins A and B are different and the delay time between scans is much shorter than the relaxation times of the spins concerned. To minimize measuring time, a delay time of 1.5 times the average \(T_1\) value of the protons is commonly used in homonuclear correlation methods. A delay time much shorter than this introduces artifacts and reduces sensitivity. Because the ribose protons R3H and R4H relax faster than anomic proton R1H, weak relay peaks from R3H and R4H to R1H are observed, whereas the relay peaks from R1H to R3H and R4H fall just below the contour level (Figure 2). In addition, attenuation of spectral intensities close to the edges of the 2D spectrum in the \(F_2\) dimension can be caused by the audiofrequency filters.

**Spin-Locked NOE Spectroscopy.** The spin-locked NOE experiment is employed for providing through-space connectivity extreme narrowing limit, the regular NOE is much smaller (or of opposite sign) than the spin-locked NOE, and therefore the average NOE observed during the MLEV sequence is strongly attenuated. (d) The rate at which magnetization is transferred (and sensitivity) will be much lower than for relatively short mixing periods, magnetization from one proton will be redistributed over (and sensitivity) will be much lower than for relatively short mixing periods, magnetization from one proton will be redistributed over

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*Primes and double primes denote downfield and upfield 1H signals, respectively, of geminal methylene protons; if absent, cross peaks include both proton signals. 
* C18 and C60 proton signals overlap. 
* C26 and C35 proton signals overlap. 
* A14 and C26 proton signals overlap.

**Table I.** Summary of Observed NMR Connectivities in (5'-Deoxyadenosyl)cobalamin with the Homonuclear Hartmann-Hahn (HOHAHA), Spin-Locked NOE, and Heteronuclear Multiple-Bond Correlation (HMBC) Methods.

between the various networks of coupled protons. At a 11.75-T magnetic field strength, the regular 2D NOE experiment\(^{16,17}\) resulted in very weak cross peaks due to the fact that the molecular tumbling time, \(t_\text{r}\), for coenzyme B12 is close to the reciprocal of the angular Larmor frequency, \(\omega_L\).\(^{18}\) At lower temperature (4°C) due to the fact that the molecular tumbling was slower, the regular 2D NOE experiment\(^{16,17}\) resulted in very weak cross peaks and are therefore readily distinguished from direct NOEs, which are inverted relative to the diagonal. A complete analysis of this effect will be presented elsewhere.\(^{31}\) These effects can be minimized by using a relatively weak rf field and positioning the carrier frequency in the downfield region of the spectrum. For more details, see the Practical Suggestions section.

Figure 4 shows the spin-locked NOE spectrum, obtained with a mixing time of 200 ms. Nearly one hundred NOE connectivities can be identified in this spectrum and are presented in Table I. Below, we will briefly outline the use of the NOE connectivities to complete the assignment of the \(^1\)H spectrum. In the HOHAHA spectrum, two benzimidazole protons at 7.16 ppm (outside the spectral region shown in Figure 2) and 6.24 ppm show scalar connectivity to two overlapping methyl groups at 2.19 ppm. The downfield signal shows NOE cross peaks with the R1 and R2 protons and is assigned to B7H. The other benzimidazole proton, which does not exhibit cross relaxation with the ribose ring but has cross peaks with corrin side-chain protons, is assigned to B4H. The signal at 6.95 ppm shows NOE connectivity with the R1 and R4 protons and is assigned to B2H. The A8 proton (8.00 ppm) is identified by the cross peaks with sugar protons A11H, A12H, and A13H. The most downfield proton signal (8.19 ppm) shows no NOE cross peaks and is therefore assigned to A2H. The remaining downfield singlet (5.93 ppm) is then assigned to C10H since this is the only remaining proton at a conjugated site in the molecule and also because it is the only remaining single proton which lacks J coupling to other protons.
Table II. \(^1\)H and \(^{13}\)C NMR Chemical Shifts and Signal Assignments for (5'-Deoxyadenosyl)cobalamin

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<th>Assignment(^b)</th>
<th>(^{13})C NMR</th>
<th>(^1)H NMR</th>
<th>Assignment(^b)</th>
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<td>C18 (C18)</td>
<td>42.5</td>
<td>2.65</td>
<td>A4 (A4)</td>
<td>151.8</td>
<td></td>
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<tr>
<td>C37 (C26)</td>
<td>45.3</td>
<td>2.19, 1.72</td>
<td>A2 (A2)</td>
<td>156.0</td>
<td>8.19</td>
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<tr>
<td>C26 (C37)</td>
<td>46.2</td>
<td>2.41</td>
<td>A6 (A6)</td>
<td>158.7</td>
<td></td>
</tr>
<tr>
<td>Pr1 (Pr1)</td>
<td>47.8</td>
<td>3.54, 3.16(^d)</td>
<td>C6 (C6)</td>
<td>166.6</td>
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<tr>
<td>C12 (C12)</td>
<td>49.5</td>
<td></td>
<td>C14 (C14)</td>
<td>167.2</td>
<td></td>
</tr>
<tr>
<td>C2 (C2)</td>
<td>49.5</td>
<td></td>
<td>C9 (C9)</td>
<td>173.1</td>
<td></td>
</tr>
<tr>
<td>C7 (C7)</td>
<td>53.1</td>
<td></td>
<td>C11 (C11)</td>
<td>177.6</td>
<td></td>
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<tr>
<td>C13 (C13)</td>
<td>55.8</td>
<td>2.89</td>
<td>C38 (C37)</td>
<td>177.9</td>
<td></td>
</tr>
<tr>
<td>C8 (C8)</td>
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<td>3.29</td>
<td>C57 (C38)</td>
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<tr>
<td>C3 (C3)</td>
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<td>C16 (C16)</td>
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<tr>
<td>C17 (C17)</td>
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<td>63.4</td>
<td>3.88, 3.74(^d)</td>
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<td>72.0</td>
<td>4.23(^d)</td>
<td>C27 (C27)</td>
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<tr>
<td>A12 (A13)</td>
<td>75.6</td>
<td>4.54</td>
<td>C42 (C43)</td>
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<tr>
<td>Pr2 (R2)</td>
<td>76.0(^d)</td>
<td>4.33(^d)</td>
<td>C32 (C32)</td>
<td>181.1</td>
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</tr>
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</table>

\(^a\)The difference between \(^{13}\)C shifts observed here (referenced to TSP) and reported earlier (referenced to neat Me\(_2\)Si) is 2.9 ppm. \(^b\)Previous assignments (ref 1) are given in parentheses. \(^c\)Referenced to TSP. \(^d\)Previously assigned (ref 2).

*\(^1\)J_{\text{C-H}} = 5.83 Hz. \(^1\)J_{\text{C-H}} = 9.10 Hz.

(which will be shown to correspond to C25) exactly overlap. A cross peak between these two overlapping methyl group signals and a resonance at 2.65 ppm is tentatively assigned as the cross peak between these two overlapping methyl group signals. The difference between \(^1\)C shifts observed here (referenced to TSP) and reported earlier (referenced to neat Me\(_2\)Si) is 2.9 ppm. Previous assignments (ref 1) are given in parentheses. Referenced to TSP. Previously assigned (ref 2).

Assignment of the \(^{13}\)C NMR Spectrum

With the \(^1\)H assignment complete, assignment of the \(^{13}\)C spectrum can be obtained straightforwardly by using 2D \(^1\)H-\(^{13}\)C shift correlation methods. The low solubility of coenzyme B\(_{12}\), however, causes rather poor \(^{13}\)C NMR sensitivity and makes the recording of a conventional one-bond 2D shift correlation spectrum time consuming, unless very large sample volumes are used. As we will demonstrate below, a convenient alternative approach is to use the large sensitivity enhancement that can be obtained by using the \(^1\)H-detected heteronuclear multiple-quantum coherence (HMQC) for correlation of \(^1\)H and \(^{13}\)C chemical shifts.

Detect Heteronuclear Multiple-Quantum Coherence.

Indirect detection of low-\(^\gamma\) nuclei, by monitoring their effect on the more intense \(^1\)H signals, can provide a large enhancement in sensitivity relative to direct detection methods. A number of experiments with different advantages and disadvantages have been proposed for this purpose. 5,11,13,14,23,24 We find the following sequence most convenient for practical use

\[ \text{\(^1\)H NMR: 90°, −\text{\(^1\)H}, 180°, −\text{\(^1\)H}, 90°, +, 90°, −, 90°, −, 90°, } \]

\[ \text{where } \Delta \text{ is chosen to be } 1/(2J_{\text{C-H}}). \text{ It has been shown recently}^{14} \text{ that this scheme provides a very rapid and effective way for correlating } \(^1\)H \text{ and } \(^{13}\)C \text{ chemical shifts. The first part of the sequence, a BIRD pulse,}^{15,16} \text{ inverts all protons not coupled to } \(^{13}\)C \text{ and leaves protons coupled to } \(^{13}\)C \text{ unaffected. A time } \tau \text{ later, the actual HMQC experiment is started. The value for } \tau \text{ (in our case 300 ms) is adjusted to minimize signals from protons not coupled to } \(^{13}\)C, \text{ facilitating the suppression of these unwanted signals and alleviating dynamic range problems. The phase, } \phi, \text{ is cycled } x, y, x−y, \text{ while the receiver phase is } +, −, +, −, −, \text{ in consecutive scans. The data acquired for odd- and even-numbered scans are stored in separate memory locations, and the data are processed in the standard fashion}^{29,30} \text{ to obtain phase-sensitive absorption mode spectra. In principle, the observed } \(^1\)H \text{ signals are modulated} \]

\[ \text{BIRD} \]

\[ \text{"A} \]

\[ \text{90°, −; 180°, −; 90°, +; 90°, −; } \]

\[ \text{30 ms} \]

\[ \text{decoupling} \]

\[ \text{BIRD} \]

\[ \text{"A} \]

\[ \text{90°, +; 90°, −; } \]

\[ \text{BIRD} \]
enhancements that are obtainable by using 'H-detected hetero-
guities.

earlier)

Coherence.

multiple-bond JcH coupling can be used to resolve these ambi-

this way are tabulated in Table 4290

spectrum, some of the I3C resonances (e.g., B10 and B11 or C25

strength, sufficient to decouple an 8-kHz I3C bandwidth

strength, yielding a factor of 10^1/2 in sensitivity enhancement

for larger molecules, however, transverse relaxation times of the

often requires unacceptably large data matrices.

Therefore, in practice, different decay constants do not influence

the sensitivity dramatically. For CH3 and NH3 groups, only

one-half of the total proton magnetization is transferred to the

low-y nucleus in the conventional 2D experiment. For methyl

groups, only one-third of the 'H magnetization results in net 13C

magnetization. In contrast, the HMQC experiment utilizes the

full proton magnetization of NH2, CHI, and CH, groups, pro-

viding extra enhancement factors of 2, 2, and 3, respectively.

For direct detection of low-y nuclei, large sample volumes can be

conveniently, yielding significant increases in sensitivity. For the

'H-detected HMQC experiment, large sample volumes do not

necessarily increase sensitivity because of the resulting severe
dynamic range problems and practical problems of decoupling

the low-y nucleus. In conclusion, if sample quantities are the main

factor limiting sensitivity, the HMQC experiment provides a gain

of about 16 or 100 for 13C or 15N detection, respectively, relative
to the conventional 2D experiment. An extra factor of 2 applies

for NH3 and CH3 groups, and an extra factor of 3 applies for

methyl groups.

'H-Detected Multiple-Bond Heteronuclear Multiple-Quantum

Coherence. The HMQC experiment described above does not

provide assignment information for nonprotonated carbons. Also,

for the case of exactly overlapping 'H resonances, the HMQC

experiment does not provide an unambiguous 13C resonance as-

signment. Here we show how JcH and JcN couplings can be used
to generate multiple-bond heteronuclear multiple-quantum

coherence which then provides the equivalent of a heteronuclear

shift correlation spectrum via long-range couplings.13 This HMCB

eXPERIMENT can be considered a more sensitive alternative to

the H2C-C-LOCOC experiment.18 The pulse sequence of the HMCB

experiment is

'H 90°,-Δτ1,- Δτ2, -t1,-2/180°, -t1,-2/2,- -Δq.(t2,ν)

13C 90°a, 90°a, 90°a

The first 90°a pulse, applied at time Δ1 (= 1/2JcH) after the first

90° proton pulse, serves to suppress one-bond correlations

in the 2D spectrum.99 This 90° 13C pulse generates heteronuclear

multiple-quantum coherence for protons directly attached to

13C, which is removed from the spectrum by alternating the phase

of the pulse along the z axis, thus negating the effect of the

receiver. The second 90° 13C pulse, applied at time Δ2 later, pro-

vides the multiple-bond, multiple-quantum coherence of interest.

In principle, the optimum choice of Δ2 is 1/2JcCH, where JcCH is

the long-range coupling constant of interest. However, in practice,
decay of the 'H magnetization occurs due to transverse relaxation

Figure 5. Part of the high-field absorption mode 'H-detected 1H--13C

shift correlation spectrum of coenzyme B12. The F2 coordinate of a

resonance indicates the 'H chemical shift, and the F1 coordinate indicates

the shift of the 13C nucleus directly attached to this proton. The

conventional 1D 'H spectrum corresponding to the displayed region of

the 2D spectrum is shown at the top of the figure. The total measuring

time was 5 h. For further experimental details, see the Experimental Section.

Direct connectivity between the protons and the carbons downfield from

80 ppm are shown in a supplementary figure.

not only in amplitude by the 13C shifts, but also slowly in phase

by the homonuclear scalar 'H coupling. For acquisition times in

the τ2 dimension that are much shorter than 1/JHH, the line

shapes will not be affected seriously. We typically use 20–30-ms

acquisition times in the τ1 dimension which normally provide more

than adequate resolution in the 13C dimension.

13C decoupling during data acquisition is employed by using

WALTZ-16 modulation.22 For our probe, the highest safe 13C
decoupling power level (12 W) produces a 3.0-kHz rf field

on our 500-MHz spectrometer. To cover the entire I3C spectral

width two experiments were performed, one ranging from 15 to

85 ppm (Figure 5) and one ranging from 85 to 155 ppm (sup-

plementary figure). From expanded displays of these spectra, the

chemical shifts of all 13C nuclei attached to protons (assigned

earlier) can be measured directly. Resonance assignments obtained

this way are tabulated in Table II. Due to the overlap in the

'H spectrum, some of the 13C resonances (e.g., B10 and B11 or C25

and C54) cannot be assigned unambiguously on the basis of the

HMQC spectra alone. As will be shown later, correlation via

multiple-bond JcH coupling can be used to resolve these ambi-

guities.

'Sensitivity of 'H-Detected Heteronuclear Multiple-Quantum

Coherence. There has been some confusion about the sensitivity

enhancements that are obtainable by using 'H-detected hetero-
nuclear shift correlation. Here, we compare two heteronuclear

shift correlation experiments: the conventional experiment with

direct detection of the low-y nucleus, and the analogous 'H-de-

tected HMQC scheme discussed above. Neglecting rf loss factors,

the sensitivity of a nucleus is proportional to γ^2. The ratio of the

magnetoigric constants of the proton (γH) and the low-y nucleus

(γL) is defined as ε (ε = γH/γL). In the conventional 2D ex-

periment 'H spin polarization is transferred to the low-y nucleus,

enhancing the sensitivity by a factor of ε. This reduces the ad-

vantage of the 'H-detected experiment to a factor of ε^2. A com-

plication of the comparison of sensitivities of the two experiments

is caused by the different decay constants of the signals acquired

during data acquisition. For the 'H-detected experiment, optimum

sensitivity is often obtained by using a rather short data acquisition

time in the τ2 dimension (about 1/2JHHi) and strong digital filtering

with a Gaussian function to avoid appearance of homonuclear J

coupling, which would distribute the intensity of one proton over

all homonuclear multiplet components. The effective data ac-

quisition time is therefore only about 50 ms in the HMQC ex-

periment. In the conventional experiment, the decay constant of

the low-y nucleus can be up to 10 times larger, intrinsically

yielding a factor of 10^{1/2} in sensitivity enhancement for the

conventional experiment. For larger molecules, however, transverse

relaxation times of the low-y nuclei are often rather short, and

moreover, a long data acquisition time in the conventional ex-

periment often requires unacceptably large data matrices.

Therefore, in practice, different decay constants do not influence

the sensitivity dramatically. For CH3 and NH3 groups, only

one-half of the total proton magnetization is transferred to the

low-y nucleus in the conventional 2D experiment. For methyl

groups, only one-third of the 'H magnetization results in net 13C

magnetization. In contrast, the HMQC experiment utilizes the

full proton magnetization of NH2, CH3 and CH, groups, pro-

viding extra enhancement factors of 2, 2, and 3, respectively.

For direct detection of low-y nuclei, large sample volumes can be

conveniently, yielding significant increases in sensitivity. For the

'H-detected HMQC experiment, large sample volumes do not

necessarily increase sensitivity because of the resulting severe

dynamic range problems and practical problems of decoupling

the low-y nucleus. In conclusion, if sample quantities are the main

factor limiting sensitivity, the HMQC experiment provides a gain

of about 16 or 100 for 13C or 15N detection, respectively, relative

to the conventional 2D experiment. An extra factor of 2 applies

for NH3 and CH3 groups, and an extra factor of 3 applies for

methyl groups.
and unresolved homonuclear couplings, and a somewhat shorter value for $\Delta_2$ (50–80 ms) is found to be optimal. In contrast to the HMQC experiment discussed earlier, the dephasing due to homonuclear J coupling is generally quite large during this long delay period, which makes obtaining absorptive 2D spectra impossible. The use of purge pulses and z filters, as recently proposed by Frey et al., degrades the sensitivity unacceptably in this application and also makes the suppression of proton signals not coupled to $^{13}C$ more difficult. Therefore, the experiment is most easily executed by using artificial phase modulation,\(^{(4,5)}\) by cycling the phases $\phi$ and $\psi$ simultaneously along the $x$, $y$, $-x$, and $-y$ axes.

The spectrum is then displayed in the absolute value mode.\(^{(4)}\)

At the beginning of the detection period, the magnetization originating from multiple-bond, multiple-quantum coherence is in antiphase. A sine bell or convolution difference filter in the $t_1$ dimension provides close to matched filtering, optimizing sensitivity. We use a relatively short data acquisition time in the $t_1$ dimension (20–40 ms) with little or no digital filtering. At least one "zero fill" in the $t_1$ dimension is used prior to Fourier transformation. The resulting truncation artifacts in the $F_1$ dimension do not constitute a problem at the low signal-to-noise levels typically encountered. Since the presence of a $^{13}C$ nucleus does not significantly affect the longitudinal relaxation rate of a proton two or more bonds removed, the repetition rate of the experiment is determined by the $T_1$ relaxation rate measured in a conventional experiment. We prefer to use a relatively short repetition rate, equal to about twice the $T_1$ of the methyl protons in the molecule. As for the HMQC experiment, methyl protons give signals of much higher intensity than other protons.

In the large variety of compounds we have studied so far with the HMBC method, we always find connectivity to the carbon adjacent to the methyl group and all carbons that are three bonds removed from the methyl protons. Most difficult to observe are the long-range connectivities to protons that have a broad, poorly resolved multiplet structure, as often encountered for nonequivalent geminal protons that are also coupled to a number of other protons.

In an earlier communication,\(^{(5)}\) we presented the multiple-bond correlation spectrum for the high-field proton and the high-field carbon resonances of coenzyme B$_{12}$. A similar spectrum, recorded under slightly different conditions, is shown in Figure 6. This spectrum shows the connectivity between the C54 methyl protons and C18. Connectivity between C18H and the C54 carbon is also observed, outside the spectral region displayed in Figure 6, removing the ambiguity in the NOE data mentioned earlier. Also visible in Figure 6 is the connectivity between C13H and C46, whereas no connectivity was observed between C13H and C47. This indicates that C13H has a larger coupling with C46 than with C47, in agreement with the cis configuration between C46 and C13H and the gauche configuration between C13H and C47.

$^1H$ correlation with the downfield carbon signals (below 90 ppm) is shown in Figure 7. As mentioned above, the methyl resonances provide intense correlations via long-range couplings but also present the highest level of incompletely suppressed signal from protons not coupled to $^{13}C$, resulting in severe ridges of $t_1$ noise. Plots with different contour levels are needed to display both the connectivity to methyl groups and connectivity to other protons that resonate in the same upfield region of the $^1H$ spectrum. Most nonprotonated carbons can be easily assigned from such a correlation spectrum. Consider, for example, the adenoyl ring. The relevant correlations are shown in Figure 7a. A8H and A11H show connectivity to the same carbon resonance at 151.8 ppm, which then must be A4. A8H also shows connectivity to a resonance at 121.8 ppm, which then must be A6. Assignment of the benzimidazole carbons follows in a similar fashion. Because R1H does not show connectivity to B8, distinction between the B8 and B9 carbon resonances is not immediately obvious. However, since two-bond J$^{13}C_H$ couplings in aromatic systems are usually quite small,\(^{(5,6)}\) whereas three-bond couplings are usually

\(^{(45)}\) Bax, A. Two-Dimensional Nuclear Magnetic Resonance in Liquids; Radel: Boston, 1982; pp 59-61.

\(^{(46)}\) Hansen, P. E. Prog. NMR Spectrosc. 1981, 14, 175.

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**Figure 6.** Part of the absolute value mode $^1H$-$^{13}C$ multiple bond shift correlation spectrum of coenzyme B$_{12}$. The $^1$H spectrum (obtained with a 50- to 150-ppm sample on a 400-MHz spectrometer) and the $^1$H spectrum are shown along the top and left side of the spectrum, respectively. Correlation peaks are due to multiple bond connectivity between $^{13}C$ nuclei (labeled with the peak in the 2D spectrum) and protons (labeled in the $^1$H spectrum). For example, the three peaks near the top of the 2D spectrum indicate long-range coupling between methyl protons C30 and carbons C1, C19, and C2. The lowest contour level for the part of the spectrum below the drawn line in the spectrum is 3 times lower than for the region above the drawn line. Resonances folded in the $^1$C dimension are labeled "F."
resonance at 178.7 ppm, which then has to be C4. Methyl protons C53H3 and C54H3 are also coupled to a carbon at 178.7 ppm which then is assigned to C16. Similarly, C11 is identified by correlation with methyl protons C46H3 and C47H3. An expansion of the downfield $^{13}$C region of Figure 7b, presented at lower contour levels, is shown in Figure 8. In this figure, protons C60H2 and C18H show connectivity to C61. Protons C26H2 show connectivity to C27, and protons C42H2 and C41H2 correlate with C43. C57 is identified via coupling with both R1 protons (Figure 7b) and also shows connectivity to C55H2. Proton C37H2 shows an intense correlation with a carbon at 177.9 ppm, which is then assigned to C38. The only unassigned carbon resonance, at 181.1 ppm, has an intensity corresponding to two carbons in the one-dimensional spectrum. Consequently, it must correspond to C32 and C50. The protons that are expected to show connectivity to these carbonyls (C49H2, C31H2, C48H2, and C30H2) also have nearly degenerate multiplets, but an intense correlation between the overlapping protons C31H2 and C49H2 and a carbon resonance at 181.1 ppm are observed in Figure 8. Moreover, three-bond connectivity is observed between this carbonyl resonance and protons C48 and C30. No other unidentified correlations remain, completing the $^{13}$C spectral assignment. Assignments have been summarized in Table 11.

Experimental Section

Coenzyme B$_12$ (6.5 mg) (Sigma) was lyophilized once and dissolved in 0.35 mL of 99.96% D$_2$O (Merck) containing 10 mM phosphate buffer, pH 7.0. Experiments were performed by using a modified Nicolet NT-500 spectrometer, equipped with a Cryomagnet Systems 45 IH probe with a broad-band ($^{15}$N-3'$^3$P) decoupling coil for irradiation of $^{13}$C. The temperature was 20 °C, and all experiments were carried out without sample spinning. All proton shifts are referenced to internal sodium 3-(trimethylsilyl)propionate (TSP). $^{13}$C shifts are relative to external TSP in D$_2$O, as measured using a 50-mg sample in a 10-mm sample tube (2.5-mL sample volume) on a JEOL GX400 spectrometer (100.5-MHz $^{13}$C frequency). For the $^1$H-detected $^{13}$C experiments, an approximate ($\pm$0.1 ppm) $^{13}$C scale was obtained by multiplying the $^1$H frequency that corresponds to 0 ppm by 0.251 449 54 (the $^{13}$C/$^1$H frequency ratio in TSP).

$^1$H-$^{31}$P Spin-Echo Difference Spectroscopy. The spectrum of Figure 1 results from 128 scans, preceded by two dummy scans. A 75-ms delay between the 90° and 180° pulses and 3-Hz Gaussian line broadening were used. Four watts of $^{31}$P decoupling power, corresponding to a 100-ns 90° pulse width, was used, and Waltz-16 $^{31}$P decoupling$^{17}$ was employed during 1H observation.

HOHAHA Spectroscopy. The spectrum of Figure 2 results from a 2 × 350 × 1024 data matrix size, with eight scans per $t_1$ value. The delay

Figure 8. Expanded region of the spectrum of Figure 7b, displaying connectivity between protons and the carbonyl region of the $^{13}$C spectrum. The lowest contour level is 5.5 times lower than that used in the right half of Figure 7b.
time between scans was 1.8 s, and the total measuring time was 1.5 h. An MLEV-17 mixing sequence of 52 ms preceded and followed by 2.5- 
ms trim pulses,21 was used. Six watts of rf power provided a 53-μs 90° pulse width. Gaussian line broadening (6 and 9 Hz) was used in the 
t1 and t2 dimensions, respectively, to avoid truncation artifacts.

Spin-Locked NOE Spectroscopy. The spin-locked NOE spectrum resulted from a 2 × 300 × 1024 data matrix size, with 64 scans per t1 v
alue and a delay time between scans of 1 s, including the 300-m s period and a 100-m s acquisition period. The total measuring
ine was 5 h. Twelve watts of 13C rf power was used to provide a 3.6-kHz 13C rf field (84-μs 90° pulse width), sufficient to decouple an 8-kHz 13C bandwidth (64 ppm). Two separate experiments, differing only in the 13C rf carrier position, were carried out to cover the entire spectral range from 18 to 155 ppm. A 90°-180°-270°, composite 180° pulse was used for the carbon pulse at the center of the BIRD pulse unit.

HMQC Spectroscopy. The multiple-bond 1H-13C shift correlation spectra resulted from 300 × 2048 data matrix sizes, with 128 scans (preceded by two dummy scans) per t1 value and a delay time between scans of 1.5 s. Acquisition times were 24 and 220 ms in the t1 and t2 dimensions, respectively. The total measuring time was 16 h per spectrum. Fifty watts of 13C power was used to provide a 3.3 kHz 13C rf field, and 70-μs 13C pulse widths (corresponding to 82° rather than 90° flip angles) were used. Two separate experiments were carried out with the 13C carrier positioned at 144 and 61 ppm, and the 13C spectral width was 99.5 ppm in both experiments. Resonances outside the selected 13C spectral window were also excited by the relatively short 13C pulse widths. The mixing time (spin lock period) was kept constant at 1.5 s through time 2, which gives rise to weak resonances that were folded in the 13C dimension. In both experiments Δp and Δq, durations of 3.4 and 55 ms, respectively, were used. In the t2 dimension, a sine bell filter and 4-Hz exponential line broadening were used prior to Fourier transformation. No digital fil-
tering was used in this dimension.

Practical Suggestions

In this section we present some practical guidelines for using the newer types of 2D NMR techniques discussed in this paper. Of course, the ease of implementation of the various new tech-
niques depends on the versatility of the particular spectrometer used. All experiments reported here were carried out on one of the earliest available Nicolet 500-MHz spectrometers, which required extensive hardware modification for implementation of the techniques discussed here. Some newer types of spectrometers may place special requirements at all, however. We will not mention the instrumental aspects that are critical for particular experiments and suggest simple procedures to check correct functioning of the pulse sequences.

HOAHAA Spectroscopy. To avoid probe damage, it is recom-
manded that this experiment be executed with relatively low 
rf power. A 2-F-Hz rf field strength should be sufficient to cover an F-Hz spectral width, provided the carrier is positioned at the center of the F-Hz spectral window. A high-power power 
transmitter is not desirable since it may lead to attenuator damage or droop of pulse power during the relatively long mixing period. Correct functioning of the experiment is verified by first executing a simple spin lock experiment: 90° -SL-Aqc.(±), where SL is a regular spin lock, generated by defining a long pulse (50–200 ms) along the y axis. The phase of the observed spectrum should be independent of the length of the spin lock period (over the range of 5–300 ms). If the spin lock experiment does not function properly, this may be caused by an automatic rf shut-off device. The second step is to replace the SL spin lock by an MLEV-17 spin lock.22 Again, the phase of the spectrum should be independent of the number of times that one cycles through the MLEV-17 sequence, and the decay of the signal should be as slow as possible; their experimental results are interpreted. If the locking spin lock experiment functions properly but the MLEV-17 ex-
periment does not, the problem may be inaccurate 90º phase shifts or unbalanced rf power for the various phases. We use a tuned

class A Henry radio amplifier (maximum power 10 W) for pro-
viding the observe power. Tuned diodes (500-MHz) and a 500-
MHz band-pass filter are used at the output of this amplifier, and an extra 76.76-MHz 2H band-pass filter is used in the lock circuit to avoid perturbation of the lock by the 1H irradiation.

Spin-Locked NOE Spectroscopy. As mentioned above, one first has to verify whether a regular spin lock experiment functions properly. The duration of the mixing time for molecules of interest, i.e., with ωt1/2 = 1, is chosen between 100 and 300 ms. A 100-ms mixing time makes quantification of the NOE effect easier, but a 300-ms mixing time usually will result in cross peaks closer to their maximum value and therefore provides close to optimum sensitivity for observing the presence of an NOE. As is the case for the regular NOESY experiment, the spin-locked NOE ex-
periment should be used in the phase-sensitive mode, yielding absorptive spectra. Since some of the NOE peaks are quite small, care has to be taken that they are not swamped by baseline distortions of nearby intense resonances. We take the following precautions to avoid baseline problems. First, the time between the end of the spin lock period and the start of data acquisition is set to 20 μs. During the first half of this 20-μs period, the receiver electronics are gated off. Second, the audiofrequency filter bandwidth (3-dB attenuation) is set to about 1.4 times the spectral width. This causes the first couple of data points of the FID to be closer to their true value and therefore also minimizes baseline problems, at the expense of some extra noise near the edges of the spectrum. Incorrect functioning of the Fourier transform will result in a severe baseline distortion on most commercial spectrometers, but fortunately, this problem is not present in Nicolet software.

Resonances at opposite sides and equidistant from the carrier frequency will experience the same magnitude of the spin lock field. If these resonances correspond to scalar coupled protons, this will lead to a homonuclear Hartmann–Hahn effect during the spin lock period, which gives rise to artifacts in the 2D spectrum.21 To minimize these effects, the carrier should be placed about 1 or 2 ppm downfield from the center of the spectrum and a relatively weak rf field employed.21 For a spectral width of F Hz, a 1.5-F-Hz rf field strength is sufficient.

HMQC Spectroscopy. The experiment is most easily imple-
mented by first examining a 13C-labeled compound. If 13C decou-
ing during data acquisition affects the lock signal, this will give rise to poor cancellation of the protons attached to 13C nuclei. We use two 12C bandpass filters in series after the 13C power amplifier and two 1H band-pass filters in series between the probe and the 1H preamplifier to remove this problem. In addition, an extra 1H band-pass filter is used between the probe and the 1H preamplifier. We find that a probe with a proton observe coil inside the low-radiation coil gives results that are far better than those obtained by using a regular broad-band probe or a so-called "dual probe".

The dipolar coupling between a proton and a directly coupled 13C nucleus is of about the same magnitude as the coupling be-
tween geminal methylene protons. Therefore, the protons of interest, attached to 13C, relax efficiently, and a rather short delay time between scans can be used.

HMBC Spectroscopy. Since for 13C the HMBC experiment is preferable and is directly couple

duced during data acquisition effects the lock signal, this will give rise to poor cancellation of the protons attached to 13C nuclei. We use two 12C bandpass filters in series after the 13C power amplifier and two 1H band-pass filters in series between the probe and the 1H preamplifier to remove this problem. In addition, an extra 1H band-pass filter is used between the probe and the 1H preamplifier. We find that a probe with a proton observe coil inside the low-radiation coil gives results that are far better than those obtained by using a regular broad-band probe or a so-called "dual probe".

The dipolar coupling between a proton and a directly coupled 13C nucleus is of about the same magnitude as the coupling be-
tween geminal methylene protons. Therefore, the protons of interest, attached to 13C, relax efficiently, and a rather short delay time between scans can be used.
First Observation of Electroluminescence at the p-Type Semiconductor/Electrolyte Interface Caused by Electron Injection: Energetics of Adsorbed Hydrogen at the p-GaAs Electrode

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Abstract: Electroluminescence (EL) due to a band-to-band transition was observed at the p-GaAs/1 M NaOH and p-GaAs/0.5 M H₂SO₄ interfaces just after the potential was pulsed from the strong negative bias to the potential near the flat band potential. The integrated EL intensity increased with the increase of the cathodic pulse width, i.e., the charge passed during the cathodic pulse, but became constant when the pulse width was longer than ca. 2 ms and the cathodic charge was larger than 350 μC/cm². These results and other evidence suggest that the EL of this system is caused by electron injection from the adsorbed hydrogen atom formed during the cathodic pulse as an intermediate of the hydrogen evolution reaction. The quenching of the EL and the increase of hydrogen evolution reaction rate in the dark by the surface treatment by Ru³⁺ mean that the energy level of adsorbed hydrogen at Ru³⁺ treated p-GaAs is below the conduction band edge at the surface.