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Vitamin B_{12} Monocarboxylic Acids: Unambiguous Isomer Assignments by Modern Two-Dimensional NMR Spectroscopy†

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Received January 12, 1989; Revised Manuscript Received April 7, 1989

ABSTRACT: The three cyanocobalaminmonocarboxylic acid isomers known to be produced by the mild acid hydrolysis of the *b*-, *d*-, and *e*-propionamide side chains of vitamin B_{12} have been unambiguously assigned by modern 2D NMR methods. Previously, structural assignments had been made by less definitive NMR methods, and both X-ray and neutron diffraction studies had failed to locate unambiguously the position of the carboxyl group. The b and e isomers were structurally assigned in this study, on the basis of the assignment of the ^{13}C NMR signal of the carboxyl group from HMBC (^1H-detected heteronuclear multiple-bond correlation) spectra. The carboxyl group resonances exhibited the greatest changes in chemical shift between the protonated (pH 2) and deprotonated (pH >7) forms of the acids. The d isomer was assigned by difference. Since the HMBC experiments required the assignments of side-chain CH_2 signals, homonuclear Hartmann–Hahn, 2D homonuclear correlation, 2D nuclear Overhauser effect, ^1H-detected heteronuclear multiple quantum coherence, and HMBC spectroscopies were used to assign completely the ^1H and ^{13}C NMR spectra of the b and e isomers at pH ~7. By comparison with the ^{13}C NMR spectra of the b and e isomers, nearly one-fourth of the resonances of the ^{13}C NMR spectrum of vitamin B_{12} have been reassigned. The sites of incorporation of ^{13}C-labeled precursors in B_{12} biosynthesis found in previous studies have been verified by a comparison of ^{13}C assignments. The results of studies using cobalamins modified at the *b*-, *d*-, and *e*-propionamide side chains in which the incorrect structural assignments were used (before 1980), particularly studies of B_{12}-dependent enzymes, require reinterpretation using the correct structural assignments.

An essential early step in the catalytic cycle of B_{12}-dependent enzymes is the homolysis of the Co–C bond of coenzyme B_{12} (AdoCbl)[1] (Dolphin, 1982). Although several hypotheses have been proposed to account for the roles the enzymes play in promoting homolysis (Finke et al., 1984; Halpern, 1985; Bresciani-Pahor et al., 1985), the exact mechanism is unknown. There is general agreement that an enzyme-induced distortion of the coenzyme leads to the Co–C bond cleavage.

The six amide groups around the periphery of the corrin ring in the coenzyme often play essential roles in these hypotheses. These amide groups can interact with the enzymes (Toraya et al., 1979). Since the molecular masses of the holoenzymes range from 76 to 560 kDa (Dolphin, 1982), it is very difficult to obtain structural information from direct techniques such as NMR spectroscopy, and no X-ray structures of a B_{12} enzyme have been reported. Therefore, indirect methods such as the use of coenzyme B_{12} analogues modified at the pro-

† This work was supported by NIH Grant GM 29225.
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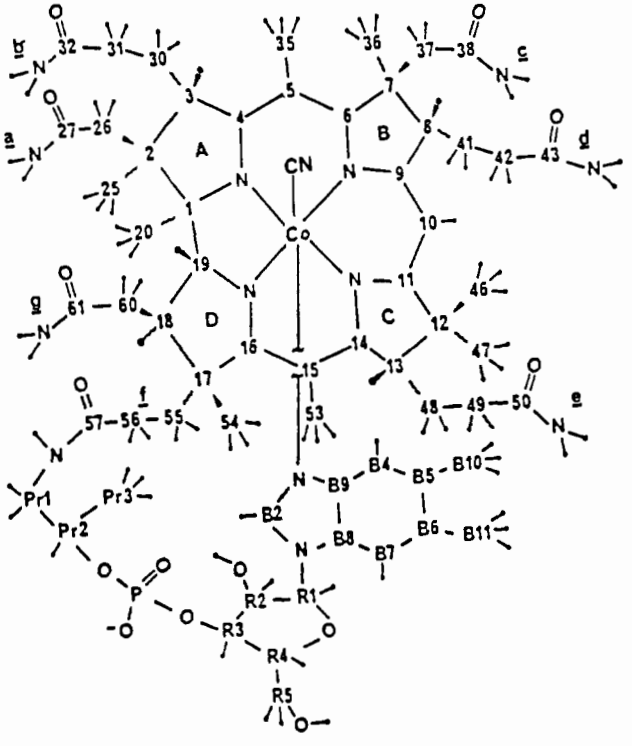
[1] Abbreviations: AdoCbl, (5′-deoxyadenosyl)cobalamin; CNCbl, cyanocobalamin; NMR, nuclear magnetic resonance; 2D, two dimensional; 1D, one dimensional; COSY, homonuclear shift correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; HMBC, ^1H-detected multiple-bond heteronuclear multiple quantum coherence; HMQC, ^1H-detected heteronuclear multiple quantum coherence; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$; ALA, δ-aminolevulinic acid; PBG, porphobilinogen.

0006-2960/89/0428-7213$01.50/0

pionamide side chains have played important roles as probes to obtain information about the B_{12} binding site in the enzymes. For example, in a study on the activity of diol dehydrase with coenzyme B_{12} analogues modified at the propionamide side chains, Toraya et al. (1979) showed that the activity of diol dehydrase decreased for the modified coenzymes. These analogues included the three monocarboxylic acid isomers and their derivatives. The coenzyme B_{12} monocarboxylic acids were synthesized from the corresponding CNCbl monocarboxylic acids prepared by the mild acid hydrolysis of vitamin B_{12} (CNCbl, structure I). Interaction of each peripheral amide group with the enzyme was thought to contribute to the formation of a catalytically active holoenzyme since modification of any one group led to reduced catalytic activity. It was speculated that steric effects and/or lack of hydrogen-bond donation from the analogues formed a "looser" complex with the apoenzyme making the breakage of the Co–C bond less energetically favorable. The interpretation of this and other experiments (Morley et al., 1968; Yakusheva et al., 1977; Kensley et al. 1978; Lien et al., 1974; Ellenbogen, 1975) involving the modification of the propionamide side chains depends on the assignment of the three cyanocobalaminmonocarboxylic acid isomers that are used as a starting point for further modifications.



I

The first attempt to identify the monocarboxylic acid isomers was made by chemical means (Bernhauer et al., 1966). Two of the three isomers were identified from reactions of the carboxylic acid group and the phosphate group. In the acid isomer Bernhauer called E_2, the molecule was slowly hydrolyzed in solution by the acid group with loss of the nucleotide group. From this evidence, he postulated that this isomer contained a carboxylic acid group at the *e*-propionamide side chain. The acid isomer he called E_1 was unchanged under the same conditions and was identified as the b isomer. However, later studies strongly suggested that these identifications were incorrect (Anton et al., 1980; Moore et al., 1984).

X-ray and neutron diffraction studies on the acid isomers would be expected to give definitive assignments, but this has not been the case. In X-ray diffraction studies of molecules this size it is difficult to distinguish between an oxygen and a nitrogen atom and, hence, between a carboxyl and an amide group (Hodgkin, 1984). Neutron diffraction, in which hydrogen atoms can be detected, should prove to be more useful. Also, it is easier to distinguish between a nitrogen and an oxygen atom in neutron diffraction studies (Hodgkin, 1984). However, neutron diffraction studies of the predominant acid isomer led to the identification of the acid as the e isomer at 1.3-Å resolution but led to a contradictory identification of the acid as the b isomer at 1.0-Å resolution (Moore et al., 1984). The authors expressed reservations since the assignment was based on the nonobservation of one hydrogen atom combined with the relative peak heights of two terminal atoms. In addition, the e side chain was found to be disordered even at 1.0-Å resolution.

NMR methods, particularly ^{13}C NMR, offer another means to identify the CNCbl monocarboxylic acid isomers. The first identification of the acid isomers by NMR spectroscopy (Anton et al., 1980) was based on earlier assignments of the ^{13}C NMR spectrum of CNCbl. The earliest assignment of any part of the ^{13}C spectrum of vitamin B_{12} was based on T_1 values and was most detailed for the nucleotide portion of the molecule (Doddrell & Allerhand, 1971). The first detailed assignment of the side-chain methylene and corrin ring carbon resonances was based on ^{13}C-labeled precursors incorporated into the molecule biosynthetically (Scott et al., 1974a). Sites of incorporation were determined from carbon-by-carbon degradation of the molecule biosynthesized from ^{14}C-labeled precursors (Scott et al., 1974b). In some cases, specific assignments and, in other cases, only group assignments of resonances to similar carbons in the molecule were made (Scott et al., 1974a).

The first isomer assignment of the CNCbl monocarboxylic acids by ^{13}C NMR methods (Anton et al., 1980) was based on three factors. First, the ^{13}C chemical shifts of other CNCbl derivatives, cyanocobalamin lactam, cyano-13-epicobalamin, and cyanocobalamin lactone, were compared. The X-ray crystal structure of CNCbl lactam (Hodgkin et al., 1955) shows a ring closure between the *c*-acetamide side chain and C8. CNCbl lactone, also containing a ring closure between the *c*-acetamide side chain and C8, is thought to have a similar structure to the lactam (Bonnett et al., 1957). In cyano-13-epicobalamin, the X-ray crystal structure (Stoeckli-Evans et al., 1972) shows a configurational inversion of the *e*-propionamide side chain. Second, the group assignments of the propionamide side chain methylene carbon resonances from biosynthetic studies (Scott et al., 1974a) were employed. Third, the shifts of the methylene carbon resonances of the monoacids in both the protonated and deprotonated forms were compared to those of CNCbl. Such arguments have been shown recently to lead to incorrect assignments of nearly one-third of the ^{13}C resonances in coenzyme B_{12} (Summers et al., 1986). These signal misassignments cast some doubt on the identifications of the CNCbl monocarboxylic acid isomers, even though the arguments used appear to be totally consistent. However, relatively few of the misassignments involve the b, d, and e side chains. It was, therefore, probable that the isomer assignments (Anton et al., 1980) were correct, even if not all the ^{13}C NMR signal assignments were correct.

Although B_{12}-type compounds are among the most complex nonpolymeric natural products, modern 2D NMR methods (Summers et al., 1986; Bax et al., 1987; Pagano et al., 1989) can be used to give unambiguous ^1H and ^{13}C NMR spectral assignments. Therefore, we decided to perform such studies on the monocarboxylic acid isomers in order to identify the

isomers unambiguously. Assignment of the ^1H resonances and the connectivity patterns of the protons can be made from COSY (Aue et al., 1976; Bax & Freeman, 1981), HOHAHA (Braunschweiler & Ernst, 1983; Davis & Bax, 1985; Bax & Davis, 1985), and NOESY (Jeener et al., 1979; Kumar et al., 1980) spectra. The ^{13}C resonances can then be assigned from HMBC (Bax & Summers, 1986) and HMQC (Müller, 1979; Bax & Subramanian, 1986) spectra. The HMBC experiment allows all the carbon resonances of the side chains to be assigned from long-range ^1H–^{13}C coupling. The carbon signal of the carboxyl group can then be assigned by its change in chemical shift between the deprotonated and the protonated form, identifying the isomer. In this work we present the use of modern 2D NMR methods to provide the unambiguous identification of two of the monocarboxylic acid isomers which have been called peak II and III (Anton et al., 1980), CMS_1 and CMS_2 (Bernhauer et al., 1966), and E_1 and E_2 (Armitage et al., 1953) along with complete ^1H and ^{13}C NMR assignments of both isomers in their deprotonated forms (pH ~7). The greater solubilities of the two monocarboxylic acids allow us to assign the signals of the less soluble isomer and of vitamin B_{12}. These monocarboxylic acid isomers have ^{13}C signals very similar to those of vitamin B_{12}, which has been studied in depth to assess the important biosynthetic pathways for cobalamins. It was of particular interest, therefore, to determine whether the conclusions reached in the biosynthetic studies agreed with our ^{13}C assignments.

Experimental Procedures

A solution of cyanocobalamin-*b*-monocarboxylic acid (15 mg) and a solution of cyanocobalamin-*e*-monocarboxylic acid (15 mg), prepared as previously described (Anton et al., 1980), were lyophilized twice and dissolved in 0.6 mL of 99.8% D_2O (Aldrich), and the pH was adjusted to approximately pH 7 (exact pH was 7.3 and 7.1 for the b and e isomers, respectively) or pH 2.0 with 35% DCl and/or NaOD. All of the 2D NMR experiments were performed on a General Electric GN-500 spectrometer at room temperature (20 °C) without sample spinning. Proton and carbon chemical shifts were referenced to internal TSP. The exact carbon shifts were measured from a 1D spectrum obtained on a General Electric QE-300 instrument.

COSY. The COSY spectra resulted from a 512 × 1024 data matrix size with 16 scans per t_1 value. The delay time between scans was 2.1 s. Gaussian line broadening was used in the t_2 and t_1 dimensions.

HOHAHA Spectroscopy. The HOHAHA spectra resulted from a 512 × 1024 data matrix size with 16 scans per t_1 value. Delay time between scans was 1.0 s. An MLEV-17 mixing sequence of 79.2 ms preceded and followed by 2.0-ms trim pulses was used. Six watts of power provided a 60-μs 90° ^1H pulse width. Gaussian line broadening was used prior to Fourier transformation in both the t_2 and t_1 dimensions.

NOE Spectroscopy (NOESY). The NOESY spectra resulted from a 512 × 1024 data matrix size with 48 scans per t_1 value. Delay time between scans was 2.1 s, and the mixing time was 500 ms. Gaussian line broadening was used before Fourier transformation in both dimensions.

Hypercomplex (Phase Sensitive) NOE Spectroscopy. The hypercomplex NOE spectra resulted from a 512 × 2048 data matrix size with 32 scans per t_1 value. Delay time between scans was 2.1 s, and the mixing time was 250 ms. The spectra were processed with the NMR2 program (New Methods Research, Inc., Syracuse, NY) with an exponential line narrowing of 2 Hz and a Gaussian line broadening of 8 Hz used prior to Fourier transformation in the t_2 dimension. A cosine bell squared filter was used prior to Fourier transformation in the t_1 dimension.

HMQC Spectroscopy. The one-bond ^1H–^{13}C shift correlation spectra resulted from a 512 × 1024 data matrix size with 112 scans per t_1 value (preceded by four dummy scans). Delay time between scans was 1.0 s. Forty-one watts of ^{13}C rf power and a 38-μs 90° pulse width were used. A sine bell filter function was used prior to Fourier transformation in the t_2 and t_1 dimensions.

HMBC Spectroscopy. The multiple-bond ^1H–^{13}C shift correlation spectra resulted from a 512 × 2048 data matrix size with 64 scans (preceded by four dummy scans) per t_1 value and a delay time between scans of 1.0 s. Forty-one watts of power and a 38-μs 90° ^{13}C pulse width were used. Values of Δ_1 (the delay between the first 90° proton pulse and the first 90° ^{13}C pulse) and Δ_2 (the delay between the first and second 90° ^{13}C pulses) were 3.3 and 50 ms, respectively. In the t_2 and t_1 dimensions, a sine bell filter was used prior to Fourier transformation.

Results

The unambiguous structural assignment of the cyanocobalamin-*b*- and -*e*-monocarboxylic acid isomers was based on the assignment of the ^{13}C signal of the carboxyl group. At pH ~7, the most downfield peak in the ^{13}C spectrum should belong to the carboxyl group. Furthermore, from a study of aliphatic carboxylic acids (Hagen & Roberts, 1969), this signal is expected to exhibit the greatest upfield shift difference when the carbonyl group is protonated (pH 2). The HMBC experiment was selected for the assignment on the basis of the connectivity between the signals of the methylene protons of the propionic acid side chain and the signal of the carboxyl group. Since the assignment of the methylene proton signals depends on first assigning some of the other proton signals in the molecule, assignment of the ^1H NMR spectra will be discussed first.

Assignment of the ^1H NMR Spectra. Most of the proton signals of the B_{12}-*b*- and -*e*-monocarboxylic acids were assigned with two-dimensional COSY, HOHAHA, and NOESY experiments. We identify the ^1H nuclei by the carbon atom to which they are attached. For nonequivalent geminal protons, H′ and H″ refer to protons with the downfield and upfield signals, respectively. We designate the ^{13}C nuclei by using the numbers in the scheme. These numbers are preceded by the following designations: C for carbons of the corrin ring and its side chains; B for carbons of the 5,6-dimethylbenzimidazole moiety; R for carbons of the ribose moiety; and Pr for carbons of the propanolamine group (see structure I).

We will first discuss the HOHAHA spectra[2] (Figures 1 and S1) that identified the methylene protons of the *b*-, *d*-, and *e*-propionamide side chains. The use of HOHAHA spectroscopy allows the connectivity networks for the corrin side chains to be seen, as well as the other connectivities seen in a COSY spectrum, since it shows relayed connectivities. Because it sometimes is extremely difficult to distinguish between direct and relayed connectivities in the HOHAHA experiment (Summers et al., 1986), the relayed connectivities allow the protons to be assigned to a specific propionamide side chain for side chains b, d, e, and f, but not necessarily to specific methylene groups. These assignments are based on the corrin methine assignments from NOE spectra (see below). The HOHAHA spectra (Figures 2 and S2) show the

[2] In most cases the spectra of both isomers are very similar, and we show the spectrum of only one of the isomers. The spectrum of the other isomer can be found in the supplementary material.

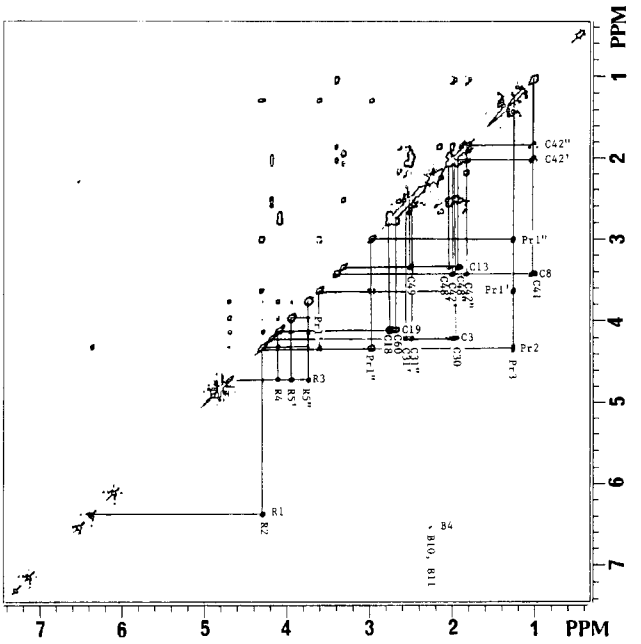


FIGURE 1: 2D HOHAHA spectrum of the e isomer with the connectivity patterns for the ribose ring and propanolamine protons indicated by drawn lines. Connectivities between the corrin ring methine protons and the methylene protons of the corresponding side chains are also shown.

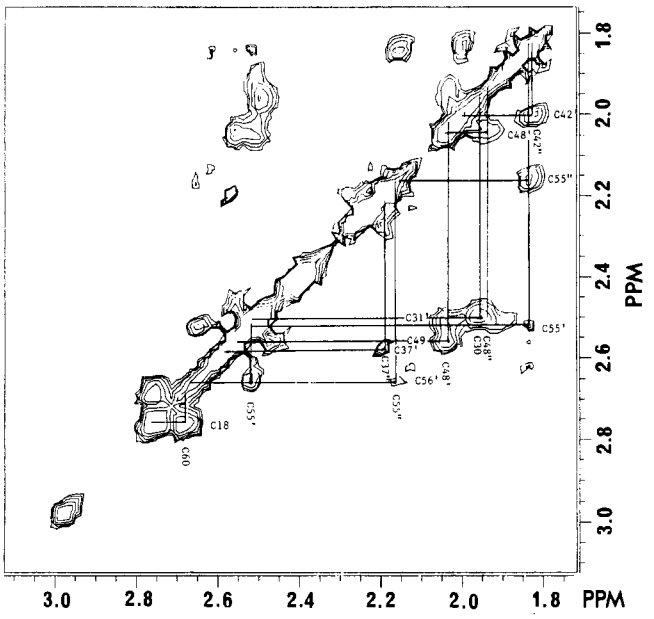


FIGURE 2: Part of the HOHAHA spectrum of the b isomer showing the connectivities between the methylene protons of the side chains.

connectivity between the methylene protons of a given side chain, including the c and f side chains which are attached to the corrin ring at nonprotonated carbons. The HOHAHA spectra (Figure 2 and S2) show the connectivity between C55H′, C55H″, C56H′, and C56H″ of the f side chain. These four signals were assigned to the C55 and C56 methylene groups because the f side chain is the only propionamide side chain attached to the corrin ring at a nonprotonated carbon. Since all the connectivities to these protons in the HOHAHA spectrum are direct connectivities, the four signals can be assigned to the C55 and C56 methylene groups without a more specific assignment. Other experiments (see below) were needed for more specific assignments.

COSY spectra, although not necessary to determine the identity of the B_{12}-monocarboxylic acid isomers, were needed

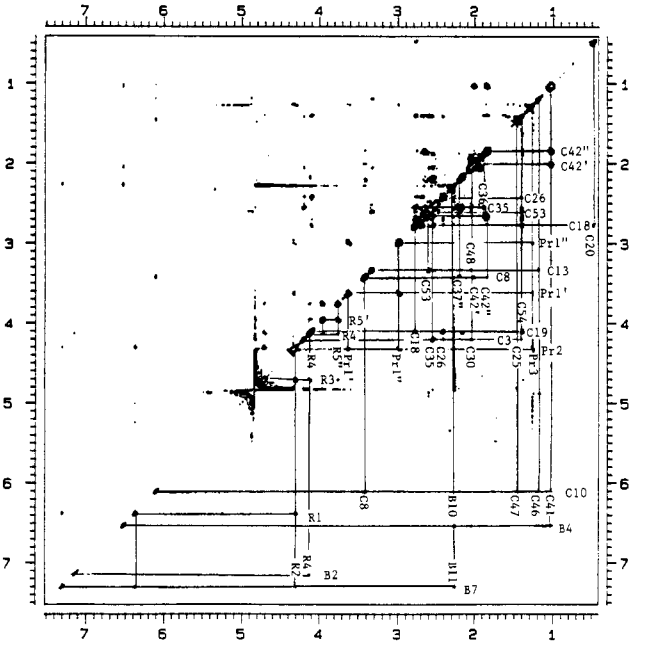


FIGURE 3: Hypercomplex NOE spectrum of the e isomer. Lines drawn indicate the NOE connectivities of the molecule.

for a complete assignment of the ^{1}H NMR spectra. The COSY spectra (Figure S3) were used to assign the ribose protons, the propanolamine protons, and the C30, C41, and C48 methylene protons. The anomeric ribose proton R1H, identified as the only doublet in the downfield region of the ^{1}H NMR spectrum, shows a correlation to R2H. R2H in turn shows a weak correlation to R3H which shows a correlation to R4H. R4H shows a correlation to only one of the R5 protons (R5H″). R5H″ shows a correlation to R5H′, completing the assignment of the ribose protons.

The propanolamine protons were assigned by starting with $Pr3H_3$ which is identified as the doublet corresponding to three protons at 1.26 and 1.27 ppm in the spectra of the b and e isomers, respectively. The only methyl group in the molecule attached to a protonated carbon is $Pr3H_3$. $Pr3H_3$ shows connectivity to Pr2H which in turn shows connectivity to one of the Pr1 protons (Pr1H″). Pr1H″ shows connectivity to Pr1H′, completing the assignment of the propanolamine protons.

The COSY spectra also show correlations between C19H and C18H, C8 and $C41H_2$, C13 and the C48 methylene protons, and C3H and the C30 methylene protons. The assignment of at least one signal in each of these pairs was made from NOESY spectra (see below).

Two types of two-dimensional NOE experiments, NOESY (Figure S4) and hypercomplex (phase sensitive) NOE (Figures 3 and S5), were used to establish through-space connectivities between the various networks of coupled protons. Since there were several cases in which one of the experiments gave an NOE peak that was not seen in the other, a more complete assignment of the proton signals of the B_{12}-monocarboxylic acid isomers was made with the NOEs found from both of these experiments. NOE connectivities were used to complete the assignment of most of the ^{1}H NMR spectrum, starting with the downfield proton signals.

Of the unassigned downfield proton signals, three belong to the dimethylbenzimidazole moiety (B2, B4, and B7). Two of the signals show NOEs to the ribose proton R1H. The third signal (at 6.54 and 6.52 ppm in the spectra of the b and e isomers, respectively) shows scalar connectivity to the two overlapping methyl group signals at 2.27 ppm in the HO-

HAHA spectrum (Figure 1) but no NOEs to any of the ribose protons. This peak is assigned to B4 since it is the only single proton in the benzimidazole moiety that is not near the ribose ring. The peak, at 7.10 and 7.13 ppm in the b and e isomer, respectively, that shows NOE connectivity to R1H, R4H, and some of the corrin protons is assigned to B2H. The other benzimidazole proton (at 7.28 and 7.29 ppm in the b and e isomers, respectively) that shows an NOE to R1H also shows NOE connectivity to R2H and the two overlapping methyl group signals ($B10H_3$ and $B11H_3$) at 2.27 ppm and is assigned to B7H. The remaining proton signal at 6.10 ppm, therefore, can be assigned to C10H since it is the only remaining proton at a conjugated site in the molecule and is the only remaining single proton not coupled to other protons. The C10 proton provides a good starting point to assign the corrin ring protons.

In the NOESY spectra (Figure S4) C10H shows three NOE cross peaks, two of which correspond to methyl protons and two to a single proton. The only single proton attached to a carbon of the corrin ring (as deduced from the chemical shift) that is close enough to C10H to show an NOE is C8H. The two methyl groups close to C10H are $C46H_3$ and $C47H_3$. One of the methyl groups has a more intense cross peak in the NOESY spectra and corresponds to the methyl group that is closer to C10H. There are similar C18H–C19H coupling constants for the B_{12}-monocarboxylic acid isomers (10.0 Hz) and coenzyme B_{12} (10.5 Hz), suggesting that the conformations of the corrin ring are very similar in the two B_{12} derivatives. X-ray crystal structure data of coenzyme B_{12} show that $C47H_3$ is closer to C10H (Finney et al.). Therefore, we assume that $C47H_3$ is closest to C10H for the B_{12}-monocarboxylic acid isomers and assign the peak at 1.45 ppm to $C47H_3$. In the hypercomplex NOE spectra (Figure 3) there is a fourth, less intense NOE to C10H, which is assigned to $C41H_2$. From this starting point, one can progress around the corrin ring and assign most of the resonances, as described previously (Summers et al., 1986; Pagano et al., 1989).

At this point all of the ^1H NMR signals have been assigned unambiguously except for the C55 and C56 methylene protons. There is no way to assign these four proton signals, seen in the HOHAHA spectra (Figures 2 and S2), to their specific methylene groups from any of the homonuclear two-dimensional experiments used. There is no proton on C17, the carbon to which the f side chain is attached to the corrin ring, and so there is no correlation shown for the C55 methylene protons in the COSY spectrum. However, assignments of the C55 and C56 protons can be made from ^1H–^{13}C correlation experiments (see below).

Assignment of the ^{13}C NMR Spectra. With the methylene proton signals of the *b*-, *d*-, and *e*-propionamide side chains and almost all of the ^1H NMR spectra assigned, it was possible to use the HMBC spectra to identify unambiguously the cyanocobalamin-*b*- and -*e*-monocarboxylic acids. The HMBC experiment shows connectivity between protons and carbons that are two to three bonds away. Therefore, both of the sets of methylene protons in the *b*- and *e*-propionamide side chains should give correlations to the carbonyl carbons of these side chains. In the HMBC spectrum of the b isomer (Figure 4a) the C30 and C31 methylene protons show a correlation to C32, assigning it to the most downfield carbon signal in the ^{13}C NMR spectrum. Likewise, the C48 and C49 methylene protons show a correlation to C50 in the HMBC spectrum of the e isomer (Figure 4b), assigning the most downfield peak in the ^{13}C NMR spectrum to C50. Further evidence for the identity of the monocarboxylic acid isomers comes from the HMBC spectra at pH 2.0 (see below). In addition to assigning the carbonyl carbon of the carboxyl group, most of the other carbon signals were assigned from the HMBC spectra. Connectivity around the corrin ring can be established from the HMBC spectra (Figures 4, 5, S6, and S7), assigning most of the corrin carbons, as described previously (Summers et al., 1986). The ambiguity about the proton assignments of $C46H_3$ and $C47H_3$ mentioned earlier can now be removed from the intensity of the correlations of C13H to C46 and C47 in the HMBC spectra (Figure 5), as has been discussed previously (Summers et al., 1986). C46 and C47 were assigned from correlations with $C47H_3$ and $C46H_3$, respectively, in the HMBC spectra.

Most of the dimethylbenzimidazole carbons were assigned from the HMBC spectra, although their assignments were not as easily made as those of the corrin carbons. R1H does not show connectivity to B8 in the HMBC spectra, making the distinction between B8 and B9 difficult. These two carbons were assigned from correlations with B4H and B7H, respectively, by use of arguments described previously (Summers et al, 1986). This same argument was used to assign B5 and B6 from their correlations with B7H and B4H, respectively. B2 was assigned from a correlation with R1H. B10 and B11 were assigned from correlations with B4H and B7H, respectively. The assignments of B4 and B7 were impossible from the HMBC spectrum (Figure 4b) of the e isomer, since the only protons which would show correlations to B4 and B7 ($B10H_3$ and $B11H_3$, respectively) have overlapping signals in the ^1H NMR spectrum; they can be assigned from HMQC spectra (see below). The signals of the B10 and B11 methyl groups are slightly separated in the HMBC spectrum of the b isomer, making the assignment of B4 and B7 possible.

Most of the ribose carbons were also assigned from the HMBC spectra. Some difficulty was encountered in the assignments because not all of the possible connectivities are seen in the spectra. One proton signal that does show all possible connectivities in the HMBC spectra is R5H′. It shows two connectivities to carbons at ~85 ppm (84.75 and 84.82 ppm in the b and e isomers, respectively) and ~76 ppm (75.69 and 75.85 ppm in the b and e isomers, respectively). The carbon at ~85 ppm also shows connectivity to R2H and, in the spectrum of the e isomer, to R3H, assigning it to R4 since that is the only carbon that would show connectivity to all three protons. In the case of the b isomer, R4 can be assigned from the HMQC spectrum (see below). Since R5H′ can only show connectivity to R4 and R3, the peak at ~76 ppm is assigned to R3. R2H shows one other connectivity, besides the one to R4, to a carbon at ~90 ppm (89.76 and 89.72 ppm in the b and e isomers, respectively). It is possible for R2H to show connectivities with R1, R3, and R4. Since R3 and R4 have already been assigned, the peak at ~90 ppm must be R1. R1H shows only one correlation in the HMBC spectra to a carbon at ~71.5 ppm (71.53 and 71.49 ppm in the b and e isomers, respectively). This carbon must be R2 since all the other carbons to which R1H could show correlations (i.e., R3 and R4) have already been assigned. The remaining ribose carbon, R5, cannot be assigned from the HMBC spectra because it shows no connectivities to any of the ribose protons in these spectra. R5 can, however, be assigned from HMQC spectra (see below).

The remaining carbons which can be assigned from the HMBC spectra are those of the *f*-propionamide side chain and the propanolamine group. C55 can be assigned from a correlation with $C54H_3$ (Figure S6). Pr1H″ shows correlations to two carbons. The carbon at ~75.7 ppm (75.69 and 75.76 ppm in the b and e isomers, respectively) also shows a cor-

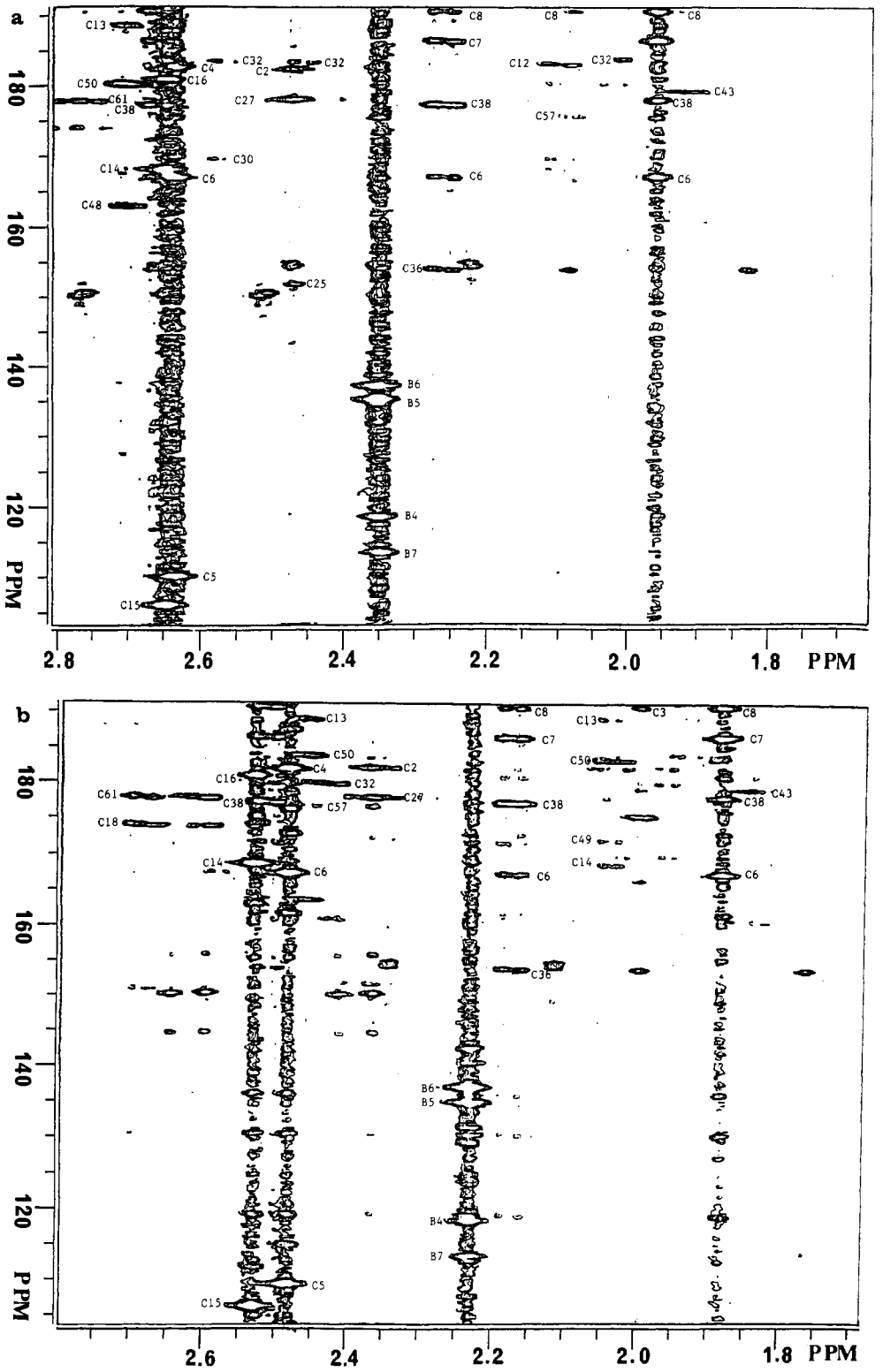


FIGURE 4: Part of the HMBC spectra of (a) the b isomer and (b) the e isomer displaying the multiple-bond $^{1}H-^{13}C$ correlations of the methylene protons to the carbonyl carbons of the side chains.

relation with $Pr3H_3$, assigning it to Pr2. The other peak to which Pr1H″ shows connectivity is in the carbonyl region of the ^{13}C NMR spectrum (176.23 and 177.41 ppm in the b and e isomers, respectively) and is assigned to C57. Pr2H shows no connectivities in the HMBC spectra. Pr3 shows no correlations to any of the propanolamine protons in the HMBC spectra but can be assigned from the HMQC spectra (see below).

The HMQC spectra enable the ^{13}C assignments to be made for the carbons that could not be assigned from the HMBC spectra. This experiment shows correlations between protons and the carbons to which they are attached. The methyl groups, as expected, give the strongest signals in the HMQC spectrum, making the assignments of C20, C25, C35, C53, C54, and Pr3 fairly easy. The HMQC spectrum of the b isomer removes the ambiguity in the assignments of B4 and B7 in the HMBC spectrum. The downfield region of the spectrum shows that B7 is upfield of B4, assigning B7 to the peak at 114.08 ppm and B4 to the peak at 119.17 ppm in the ^{13}C NMR spectrum.

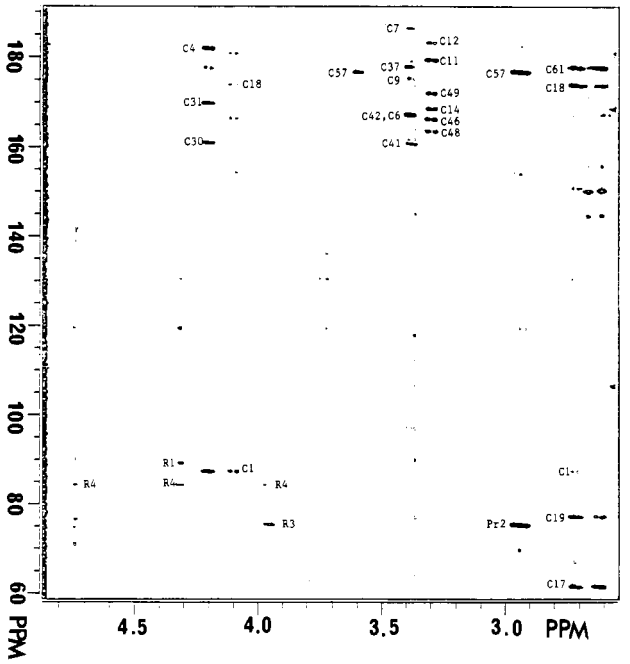


FIGURE 5: Part of the HMBC spectrum of the e isomer showing the methine proton region. There is foldover in the upfield (<59 ppm) region of the ^{13}C spectrum. The most upfield signal in the foldover region is also the most upfield signal in the ^{13}C spectrum.

It is difficult to determine exact ^{13}C chemical shifts from the HMBC and HMQC spectra. In regions where there are many peaks in a short chemical shift range (i.e., between 34 and 35.5 ppm), the relative positions of correlations to these carbons in the HMBC and HMQC spectra were used in conjunction with the one-dimensional ^{13}C spectra to obtain exact shifts. In the HMBC spectra, correlations to C46, C60, C42, and C55 showed that their order from upfield to downfield is C46 < C60 < C42 < C55. The HMQC spectra (Figure S8) show that C56 is upfield of C60 and downfield of C46. The C55 and C56 protons were also assigned from the HMQC spectra. In both isomers, the two resonances in the middle of the four f side chain methylene proton signals (determined from the HOHAHA spectra, see above) were assigned to C55H′ and C55H″. Both of these protons give correlations to C55 in the HMQC spectra (Figure S8b). The most upfield and most downfield of the four f side chain ^1H signals were assigned to C56H″ and C56H′, respectively, since both give correlations to C56, upfield of C60, in the HMQC spectra. The ^1H and ^{13}C NMR assignments of the deprotonated B_{12}-*b*- and -*e*-monocarboxylic acids are now complete and shown in Tables I and II.

NMR Experiments at pH 2.0. Although it is reasonable to assume that the most downfield carbonyl carbon signal in the deprotonated acid belongs to the carboxyl group, conclusive evidence is found in the comparison of the changes in the shifts of all the carbonyl carbons when the acid is protonated. To achieve this objective, three types of experiments were performed on both isomers at pH 2.0. First, a NOESY spectrum of each isomer was used to identify the methine protons, particularly C3H and C13H, by establishing connectivity around the corrin ring as discussed above. Second, the HOHAHA experiment gave the shifts of the b, d, and e side chain methylene protons (see above). No attempt was made to assign the methylene protons to their specific carbons since it was not necessary for determining the shift of the carbonyl carbon of the carboxylic acid. Since both sets of methylene protons in the propionamide side chains are expected to show connectivity to the carbonyl carbons and as long as the protons have been assigned to a specific side chain, more specific assignments are unnecessary. Third, HMBC spectra (Figure

Table I: ^1H and ^{13}C NMR Chemical Shifts and Signal Assignments for Cyanocobalamin-*b*-monocarboxylic Acid at pH 7.3[a]

assignment	δ ^{13}C NMR	δ ^1H NMR	assignment	δ ^{13}C NMR	δ ^1H NMR
C53	17.88	2.58	R5	63.07	3.94, 3.75
C35	18.33	2.57	R2	71.53	4.28
C54	18.58	1.39	Pr2	75.69	4.30
C25	19.57	1.41	R3	75.69	4.73
Pr3	21.71	1.26	C19	77.62	4.10
C36	21.71	1.87	R4	84.75	4.08
C20	21.71	0.48	C1	87.74	
C47	21.95	1.45	R1	89.76	6.37
B11	22.14	2.27	C10	97.59	6.10
B10	22.68	2.27	C15	106.66	
C41	28.61	1.02	C5	110.66	
C30	29.71	1.97	B7	114.08	7.28
C48	30.73	1.98	B4	119.17	6.54
C46	34.04	1.19	B8	132.59	
C56	34.25	2.66, 1.82	B5	135.91	
C60	34.39	2.71	B6	137.84	
C42	34.95	2.02, 1.84	B9	139.39	
C55	35.23	2.51, 2.12	B2	144.43	7.10
C49	37.33	2.64	C6	167.62	
C31	40.59	2.48, 2.41	C14	168.69	
C18	41.76	2.77	C9	176.23	
C37	45.59	2.60, 2.18	C57	177.49	
C26	45.70	2.40	C38	177.83	
Pr1	47.96	3.61, 2.97	C61	178.38	
C2	50.06		C27	178.55	
C12	50.78		C11	179.40	
C7	54.12		C43	179.76	
C13	56.37	3.34	C50	180.82	
C8	58.37	3.44	C16	181.52	
C3	59.51	4.15	C4	183.34	
C17	61.87		C32	183.98	

[a] Shifts relative to internal TSP.

Table II: ^1H and ^{13}C NMR Chemical Shifts and Signal Assignments for Cyanobalamin-*e*-monocarboxylic Acid at pH 7.1[a]

assignment	δ ^{13}C NMR	δ ^1H NMR	assignment	δ ^{13}C NMR	δ ^1H NMR
C53	17.92	2.60	R5	63.23	3.95, 3.75
C35	18.10	2.55	R2	71.49	4.30
C54	18.64	1.39	Pr2	75.76	4.31
C25	19.51	1.41	R3	75.85	4.70
C36	21.74	1.88	C19	77.62	4.09
Pr3	21.78	1.27	R4	84.82	4.11
B11	22.03	2.27	C1	87.80	
C20	22.09	0.47	R1	89.72	6.37
C47	22.09	1.45	C10	97.66	6.10
B10	22.62	2.27	C15	106.95	
C41	28.61	1.02	C5	110.04	
C30	28.74	1.97	B7	114.18	7.29
C48	31.37	2.04, 1.94	B4	119.19	6.52
C46	34.08	1.19	B8	132.63	
C56	34.20	2.66, 1.84	B5	135.68	
C60	34.24	2.69	B6	137.76	
C42	34.92	2.00, 1.82	B9	139.42	
C55	35.12	2.52, 2.16	B2	144.63	7.13
C31	37.64	2.58, 2.50	C6	168.00	
C49	39.83	2.51	C14	169.29	
C18	41.69	2.76	C9	176.03	
C37	45.47	2.58, 2.19	C57	177.41	
C26	45.68	2.41	C38	177.85	
Pr1	47.95	3.61, 2.98	C61	178.41	
C2	49.92		C27	178.41	
C12	50.99		C43	178.54	
C7	54.23		C11	179.74	
C13	56.84	3.33	C32	180.06	
C8	58.47	3.41	C16	180.52	
C3	59.01	4.19	C4	181.60	
C17	61.88		C50	182.60	

[a] Shifts relative to internal TSP.

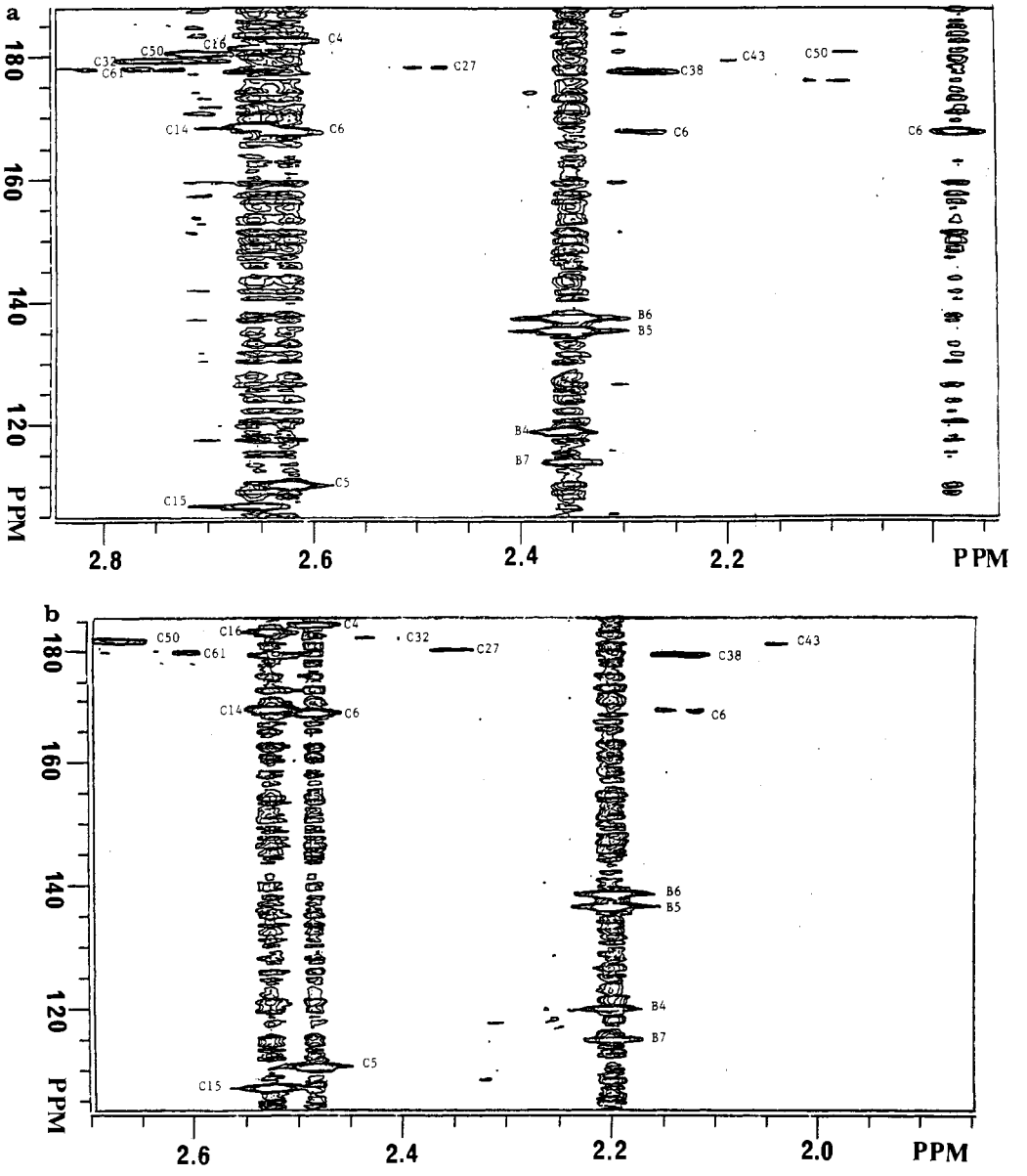


FIGURE 6: Part of the HMBC spectra of (a) the b isomer and (b) the e isomer at pH 2 displaying the multiple-bond $^{1}H-^{13}C$ correlations of the methylene protons to the carbonyl carbons of the side chains.

6) of each isomer showed the positions of the carbonyl carbons (see above). In the HMBC spectrum of the b isomer (Figure 6a) it was found that C32 shifts upfield 4.2 ppm on going from the deprotonated (pH 7.3) to the protonated (pH 2.0) form of the acid, much more than any other carbon in the downfield region. Likewise, in the e isomer (Figure 6b) C50 exhibits an upfield shift of 2.7 ppm on going from the deprotonated (pH 7.1) to the protonated acid, which is also a much larger shift than any other carbon in the downfield region of the spectrum.

Additional evidence for the isomer assignment was found from the change in shifts of the b, d, and e side chain methylene carbons. It is known that the ^{13}C signals of α- and β-carbons of carboxylic acids shift upfield on protonation (Hagen & Roberts, 1969). In the b isomer C31 and C30 show greater upfield shifts in their ^{13}C signals (3.17 and 1.71 ppm, respectively) than any of the other propionamide methylene carbons. Likewise, in the e isomer C49 and C48 show greater upfield shifts in their ^{13}C signals (4.09 and 0.59 ppm, respectively) than any of the other propionamide methylene carbons. These results unambiguously identify the peak II and peak III acids (Anton et al., 1980) as cyanocobalamin-*b*-monocarboxylic acid and cyanocobalamin-*e*-monocarboxylic acid, respectively.

Discussion

Our unambiguous structural assignment of the cyanocobalamin-*b*- and -*e*-monocarboxylic acid isomer through the use of long-range $^{1}H-^{13}C$ connectivity confirms the previous identification of these isomers made with less definitive methods (Anton et al., 1980). The third monocarboxylic acid produced by the mild acid hydrolysis of CNCbl is too insoluble to study by these methods. The third acid must also be a propionic acid, namely, the d isomer, because the *b*-, *d*-, and *e*-propionamide groups are much more readily hydrolyzed than the sterically hindered acetamides (Cason et al., 1953). The only other propionamide group in the molecule is the f side chain. However, such a hydrolysis would cleave the nucleotide loop, but there are peaks present for the benzimidazole and ribose protons of the loop in the 1D ^{1}H NMR spectrum of the third acid isomer (not shown).

Our complete assignment of the ^{13}C spectra of the b and e acid isomers allows verifications or corrections to be made in the previous assignments of the ^{13}C spectrum of vitamin B_{12}

(Bratt & Hogenkamp, 1984; Anton et al., 1982; Scott et al., 1974; Doddrell & Allerhand, 1971). Those carbons which do not show significant differences in chemical shift between the b and e acid isomers should have very similar shifts in CNCbl. This led us to determine that approximately one-fourth of the most recent ^{13}C assignments of CNCbl (Bratt & Hogenkamp, 1984) are incorrect. These incorrect assignments consist mostly of methyl and side-chain methylene carbon resonances. A comparison of earlier assignments with each other (Bratt & Hogenkamp, 1984; Anton et al., 1982; Scott et al., 1974; Doddrell & Allerhand, 1971) and with our results is informative.

The first ^{13}C assignments of vitamin B_{12} involved mostly the nucleotide portion of the molecule and some of the corrin ring carbons (Doddrell & Allerhand, 1971). These assignments were based on model compounds, spectral comparisons within corrinoids, off-resonance single-frequency decoupling, scalar coupling to ^{31}P, partially relaxed spectra, and ^{13}C T_1 values. Incorrect assignments were made by switching the positions of the following pairs of ^{13}C resonances: C2 and C17, B6 and B8, and Pr2 and R2.

More detailed assignments of the corrin ring and methylene carbon resonances were made from CNCbl biosynthesized from ^{13}C-labeled precursors (Scott et al., 1974a). These assignments of the side-chain methylene carbon resonances were later used to identify the CNCbl monocarboxylic acid isomers by ^{13}C NMR (Anton et al., 1980). In the ^{13}C-labeling study (Scott et al., 1974a), four different ^{13}C-labeled precursors were fed to *Propionibacterium shermanii*, and the resulting labeled CNCbl was extracted. The sites of ^{13}C enrichment were determined from a previous study (Scott et al., 1974b) using the same four precursors labeled with ^{14}C and the carbon-by-carbon degradation of the resulting molecules. Assignments made within the groups of labeled carbons were based on structural similarity, ^{13}C–^{13}C coupling, and the γ effect (Dalling & Grant, 1972).

The ^{13}C-labeled precursors used were [2-^{13}C]ALA, [*methyl*-^{13}C]-L-methionine, [8-^{13}C]PBG, and [5-^{13}C]ALA. The precursor [2-^{13}C]ALA gives CNCbl labeled at the C26, C31, C37, C42, C49, C56, and C60 methylene groups and a methyl group, C46. The precursor [*methyl*-^{13}C]-L-methionine labels the C20, C25, C35, C36, C47, C53, and C54 methyl groups. The methylene carbons C30, C41, C48, and C55 of the propionamide groups were labeled by the precursor [8-^{13}C]PBG. [5-^{13}C]ALA labels the corrin ring carbons C4, C5, C9, C10, C14, C15, and C16.

The chemical shift values given by Scott et al. (1974a) were found to be incorrect by one of the authors due to a computer error (Scott, 1979), but the assignments were unchanged. Using the correct chemical shift values (Scott, 1979), we found most of the group or individual assignments of the 26 biosynthetically ^{13}C-enriched carbons of the resonances to be in agreement with our assignments when corrected for bulk magnetic susceptibility (Kalinowski et al., 1988) and the different reference used. The major exception is the misassignment of C60. We found the resonance of C60 at 34.6 ppm to be quite upfield to the reported 37.5 ppm (Scott, 1979). Also, the assignments of C55 and C42 appear to be switched, but these two resonances are separated by only 0.3 ppm. Another discrepancy is in the shift of C14, which is 0.5 ppm and 0.6 ppm upfield of the value we found for the *b*-monocarboxylic acid isomer and the value of Anton et al. (1982) for CNCbl, respectively. The shift of C14 given by Scott (1979) is actually closer to the C6 value found by both Anton et al. (1982) and us. However, since the shifts found for C4, C5, C9, C10, C15, and C16 agree with our assignments, it is impossible to rationalize the ^{13}C–^{13}C coupling patterns observed by Scott et al. (1974a) for these carbons with the assumption that C6 is labeled by [5-^{13}C]ALA instead of C14. Therefore, we suspect that the C14 shift value and not the ^{13}C incorporation site is incorrect. Interpretation of the ^{13}C NMR spectra of biosynthetically ^{13}C-labeled CNCbl's using the γ effect and coupling constants gives only one major error, the misassignment of C60. Since this misassignment does not change the sites of incorporation of the labeled precursors, we have verified all the conclusions made by Scott et al. (1974a).

The shifts of C30, C41, and C48, identified correctly as a group by Scott et al. (1974a), were used to identify the monocarboxylic acid isomers by ^{13}C NMR (Anton et al., 1980). C41 was assigned to the resonance that shifted the most between CNCbl and the two derivatives, CNCbl lactam (X-ray crystal structure known; Hodgkin et al., 1955) and CNCbl lactone in which there is a ring closure between the c side chain and C8. C48 was assigned as the resonance that shifted the most between CNCbl and cyano-13-epicobalamin, where there is an inversion in the configuration of the *e*-propionamide side chain (Stoeckll-Evans et al., 1972). The e acid isomer was then assigned as the acid in which C48 shifted the most with pH and in comparison to CNCbl. C30 and C41 had chemical shifts too similar to be used to identify the other acid isomers.

The shift of C42 in CNCbl, found by comparison with CNCbl lactam and CNCbl lactone, was used to identify the d acid isomer. However, the authors (Anton et al., 1980) stated that the position of this resonance could not be determined exactly because of several overlapping resonances; in fact, we found this assignment and others in the region to be incorrect. Since there are no methylene carbons from the b or e side chains in this region, the downfield shift of one of the resonances in the region when CNCbl is converted to the d acid isomer was sufficient to identify this isomer. C49 was assigned, like C48, from the change in shift in cyano-13-epicobalamin. The b acid isomer was assigned by difference and confirmed by the shifts of C31 in the acid with pH.

The three monocarboxylic acid isomers were identified correctly by Anton et al. (1980) despite the fact that similar methods led to nearly one-fourth of the most recent ^{13}C assignments of CNCbl (Bratt & Hogenkamp, 1984) being incorrect. One group of incorrect assignments includes about half of the methyl carbons (i.e., C25, C35, C46, C47, and C53). Our results also indicate incorrect assignments were made by Anton et al. (1982) of five closely spaced signals in the methylene carbon region (C46, C56, C60, C42, and C55). The assignments of C26 and C37 were also switched. These two carbons were assigned as a group by Scott et al. (1974a), and C37 was assigned on the basis of chemical shift changes in CNCbl lactone and the *d*-monocarboxylic acid. This switch could have been caused by incorrect extrapolation to its position in CNCbl. The remaining incorrect assignments are in some of the nucleotide loop carbons. The assignments of Pr2 and R2 were incorrectly interchanged as were those of B5 and B6. Our assignments of the ^{13}C NMR spectrum of CNCbl are shown in Table III along with the previous assignments of Bratt and Hogenkamp (1984).

The results of any studies before 1980 using the CNCbl monocarboxylic acids or derivatives made from them can now be reinterpreted with the correct structural assignments of the isomers. These studies include determination of the activities of coenzyme B_{12} analogues modified at the propionamide side chains in the enzymes diol dehydrase (Toraya et al., 1979), ribonucleotide reductase (Morley et al., 1968), and glycerol

Table III: ^{13}C NMR Chemical Shifts and Signal Assignments for Vitamin B_{12} (Cyanocobalamin)

assignment[a]	δ[b]	assignment[a]	δ[b]	assignment[a]	δ[b]
C53 (C53)	18.0	C37 (C26)	45.6	B7 (B7)	114.3
C35 (C36)	18.2	C26 (C37)	45.9	B4 (B4)	119.3
C54 (C54)	18.8	Pr1 (Pr1)	48.3	B8 (B8)	132.8
C25 (C35)	19.6	C2 (C2)	50.1	B5 (B6)	135.8
C36 (C25)	21.8	C12 (C12)	50.9	B6 (B5)	137.9
Pr3 (Pr3)	21.9	C7 (C7)	54.2	B9 (B9)	139.5
C47 (C46)	22.1	C13 (C13)	56.5	B2 (B2)	144.7
C20 (C20)	22.1	C8 (C8)	58.5	C6 (C6)	168.1
B11 (B11)	22.2	C3 (C3)	59.2	C14 (C14)	168.8
B10 (B10)	22.8	C17 (C17)	62.0	C9 (C9)	176.4
C41 (C41)	28.8	R5 (R5)	63.3	C57 (C57)	177.5
C30 (C30)	28.8	R2 (Pr2)	71.7	C38 (C38)	177.9
C48 (C48)	30.8	Pr2 (R2)	75.8	C61 (C61)	178.5
C46 (C55)	34.1	R3 (R3)	75.9	C27 (C27)	178.6
C56 (C47)	34.3	C19 (C19)	77.7	C11 (C11)	179.7
C60 (C42)	34.6	R4 (R4)	84.9	C43 (C43)	180.0
C42 (C56)	35.0	C1 (C1)	87.9	C32 (C32)	180.7
C55 (C60)	35.3	R1 (R1)	89.8	C50 (C50)	181.0
C49 (C49)	37.5	C10 (C10)	97.7	C16 (C16)	181.7
C31 (C31)	37.8	C15 (C15)	106.9	C4 (C4)	182.8
C18 (C18)	41.9	C5 (C5)	110.3		

[a] Previous assignments (Bratt & Hogenkamp, 1984) are given in parentheses. [b] Shifts have been referenced to TSP by adding 2.8 ppm to the values given by Bratt and Hogenkamp (1984), in which neat Me_4Si was used as the reference.

dehydratase (Yakusheva et al., 1977). In all of these studies modifications that were thought to be made on the *b*-, *e*-, and *d*-propionamide side chains were actually made on the *d*-, *b*-, and *e*-propionamide side chains, respectively. In the diol dehydrase study (Toraya et al., 1979), conclusions were based on the activities of the three isomers of the monocarboxylic acid, methyl ester, and methylamide derivatives of coenzyme B_{12} (all made from the CNCbl monocarboxylic acid isomers) relative to the activity of unmodified coenzyme B_{12}. A modification of the *d*- (formerly identified as the *b*-) propionamide side chain showed that the carboxylic acid has a greater activity (86% of unmodified coenzyme B_{12}) than the methylamide or methyl ester derivatives (16% and 14%, respectively). It was suggested that steric effects are operative near this side chain in the holoenzyme since the methylamide and methyl ester groups are bulkier than the carboxyl group. On the other hand, a modification in the *b*-propionamide side chain (formerly identified as the e side chain) showed that the methylamide derivative has a greater activity (41% of unmodified coenzyme B_{12}) than the carboxylic acid and methyl ester derivatives (11% and 7%, respectively). It was suggested that hydrogen bonding with the enzyme is more important at this side chain since hydrogen-bond donation from the coenzyme to the apoenzyme is possible with the methylamide, but not with the carboxylic acid and methyl ester groups. Modifications in the e side chain (formerly identified as the d side chain) give an activity of the carboxylic acid (66% of unmodified coenzyme B_{12}) that is greater than that of the methylamide and methyl ester derivatives (43% and 14%, respectively), indicating a slight steric effect at this side chain in the enzyme. However, the fairly large difference in the activities of the methylamide and methyl ester derivatives seems to indicate that hydrogen bonding is also important.

In the ribonucleotide reductase study (Morley et al., 1968) the *b*-monocarboxylic acid (formerly identified as the e isomer) of coenzyme B_{12} and derivatives made from the acid were used. The acid was produced by hydrolysis of coenzyme B_{12} and was identified by comparing its properties to those of the acid synthesized from the corresponding CNCbl monocarboxylic acid isomer. It was found that the monocarboxylic acid is neither active nor inhibitory, indicating a lack of binding to the enzyme. However, the anilide and 2,4-dinitroanilide derivatives have activity (44% and 11%, respectively, of coenzyme B_{12}) despite the bulky aromatic group. The less bulky methylamide and ethylamide derivatives are quite active (82% and 67%, respectively, of coenzyme B_{12}). The absence of activity for the carboxylic acid could be explained by the inability of the ionized carboxyl group to donate a hydrogen bond to the enzyme, indicating the presence of a hydrogen-bond acceptor group at this site in the enzyme. The activity of the bulky aromatic derivatives indicates that steric effects are not very important in the interaction of the b side with the enzyme.

As in the study mentioned above, coenzyme B_{12} analogues modified at only one of the propionamide side chains, namely, the b side chain, were used in the glycerol dehydratase study (Yakusheva et al., 1977). Activities of the carboxylic acid and ethyl ester derivatives were found to be 12% and 65% of the value for the unmodified coenzyme. Affinities of the enzyme for the carboxylic acid and ethyl ester derivatives were factors of 25 and 10, respectively, less than for coenzyme B_{12}. From this information it was concluded that the *b*-propionamide group is important for coenzyme B_{12} binding with the enzyme but not essential for the catalytic properties of glycerol dehydratase.

The CNCbl monocarboxylic acid isomers have also been used in studies of the interaction of CNCbl with intrinsic factor (Lien et al., 1974; Ellenbogen, 1975) and the transport of vitamin B_{12} (Kensley et al., 1978). In the intrinsic factor study (Lien et al., 1974) the binding of CNCbl lactone was compared to the binding of what was thought to be the d acid isomer. However, the reassignment of this acid as the e isomer makes the comparison less meaningful. It was found that the monocarboxylic acid binds to intrinsic factor as well as aquocobalamin while CNCbl lactone binds rather poorly. In a previous study (Hippe et al., 1971) in which the binding of CNCbl lactone was studied, it was concluded that the corrin ring side chains, especially those at the B pyrrole ring, are important in binding CNCbl to intrinsic factor. This conclusion was refuted by the evidence of the strong binding of the monocarboxylic acid. However, since the carboxyl group and the lactone are not in the same region of the corrin as was originally thought, no clear conclusion can be made from the study.

In the study on transport of vitamin B_{12} in *Escherichia coli* (Kensley et al., 1978) it was found that the three monocarboxylic acids and their derivatives gave different dissociation constants for the different isomers of the same derivative as determined by competition with CN[^{3}H]Cbl in binding to the receptor. The major conclusions were reinterpreted by one of the authors (Bradbeer, 1982) in light of the reassignments of the acid isomers by Anton et al. (1980). Affinity for the receptor decreases slightly with *d*- and *e*-monocarboxylic acid isomers. Since the e and d isomers of the amino-*N*-hexylamide derivative are equal and worse cofactors than the corresponding acid, respectively, it was concluded that steric restrictions are more important at the d side chain. The *b*-monocarboxylic acid is a much worse substrate than the other acid isomers. Also, the substitution of the *b*-amide with a methyl or larger group gave dissociation constants much greater than that of CNCbl. From these facts, it was concluded that there are severe steric restrictions at the binding site of the *b*-propionamide side chain and the presence of negative charges in this region is incompatible with binding.

In light of our reinterpretation of the studies before 1980 using cobalamins modified at the propionamide side chains, there appears to be a need for more studies of B_{12} binding sites, particularly in B_{12}-dependent enzymes. Cobalamins modified at the propionamide side chains should still prove useful as probes for the binding site structure. With our unambiguous confirmation of the structural assignments of the three CNCbl monocarboxylic acid isomers, derivatization of the carboxyl group can be made without the need for further 2D NMR or X-ray structural analysis to confirm the site of derivatization.

Acknowledgments

We thank Prof. Harry Hogenkamp for providing us with samples of the monocarboxylic acid isomers.

Supplementary Material Available

Eight figures depicting the HOHAHA spectrum of the b isomer, part of the HOHAHA spectrum of the e isomer showing the connectivities between the methylene protons, the 2D COSY spectra of both isomers, the NOESY spectra of both isomers, the hypercomplex NOE spectrum of the b isomer, part of the HMBC spectrum of the b isomer displaying connectivities of the methyl protons, part of the HMBC spectrum of the e isomer showing the methine proton region, and part of the HMQC spectra of both isomers (13 pages). Ordering information is given on any current masthead page.

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