in organocobalt corrins.[78,83-85] Indeed, AdoCbl^{+} [25] and both diastereomers of $CF_3CH_2Cbi^{+}$ [38] are known to be pentacoordinate in the vapor phase from mass spectral observations.

In conclusion, the base-on effect, at least in sterically hindered alkylcobalamins such as neopentyl- and benzyl-Cbl, is found to be a steric, rather than an electronic, effect. The higher reactivity of the base-on species is due to a substantial entropic stabilization of the base-off species, probably due to a conformational change of the corrin, reducing the steric interactions between the bulky organic group and the acetamide side chains. However, since sterically undemanding ligands produce R(L)Cbi^{+} complexes that are as reactive as the base-on species, the steric bulk of the axial ligand itself is not important in the base-on effect. Thus, the mechanochemical trigger mechanism of AdoCbl activation does not receive support from the existence of the base-on effect in benzyl- and neopentyl-Cbl. However, it remains possible that steric compression of the axial Co-N bond could play a role in enzymatic activation of AdoCbl if such compression were capable of causing an upward bending of the corrin ring intensifying the steric congestion between the acetamide side chains and the organic ligand. Indeed, the apparent flexibility of the corrin ring permits the persistance of such an hypothesis. Further enlightenment in this area requires the development of an experimental system in which probes of the axial Co-N bond length, the corrin ring conformation, and the mobility of the acetamide side chains can be monitored in complexes of cobalamins with proteins. Attempts to use NMR probes for these purposes are currently in progress.

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Heteronuclear NMR Studies of Cobalamins. 12. Further Studies of Dicyanocobamides and the Complete Proton, Carbon, and Amide Nitrogen NMR Assignments of Dicyanocobalamin[1,2]

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From a combination of ^{31}P-^{1}H chemical shift correlated, homonuclear *J*-correlated, absorption-mode hypercomplex NOE, hypercomplex homonuclear Hartmann-Hahn, ^{1}H-detected heteronuclear, and ^{1}H-detected multiple-bond heteronuclear multiple-quantum coherence NMR spectroscopies, the complete ^{1}H and ^{13}C NMR assignments of dicyanocobalamin in D_2O have been made. From these proton assignments in conjunction with the NOESY map, the ^{1}H spectrum of dicyanocobalamin in DMSO-d_6 could be nearly completely assigned from a NOESY experiment in this solvent. In DMSO-d_6, the amide proton resonances are visible, and these could be unambiguously assigned from observation of numerous NOE's to side-chain methylene and corrin ring protons. Along with our previous determination of the amide ^{15}N chemical shifts and amide proton-nitrogen connectivities from ^{1}H-detected ^{1}H,^{15}N multiple-quantum coherence spectroscopy these assignments permitted, for the first time, the complete assignment of the amide ^{15}N resonances. Comparisons of the ^{13}C resonances of the nucleotides of dicyanocobalamin, the dicyano derivatives of the cobalamin b, d, and e monocarboxylate analogues, and the dicyano derivative of the C13 epimer of cobalamin among each other and with the free base of the detached nucleotide are consistent with the persistance of the previously postulated tuck-in species of base-off dicyanocobalamin, in which the benzimidazole nitrogen B3 is hydrogen bonded to a side-chain amide, in each of the above cobalamin analogues. These comparisons eliminate the b, d, and e amides as possible hydrogen-bond donors in the tuck-in species. Methylation of the benzimidazole B3 nitrogen was shown to prevent formation of the tuck-in species in the dicyano derivative of the trimethylbenzimidazolyl analogue by comparison of its ^{13}C spectrum to that of the detached, N-methylated nucleotide methyl ester. Taken together with previous ^{15}N NMR results, numerous NOE's observed between the benzimidazole B2, B4, and B7 protons and protons on the corrin side chains, ring and ring methyl groups strongly suggest that the g side-chain amide is the hydrogen-bond donor in the tuck-in species.

Introduction

In the last decade the use of modern NMR techniques and high-field spectrometers has given rise to complete ^{1}H and ^{13}C NMR assignments for a number of vitamin B_{12} derivatives including heptamethyl dicyanocobyrinate,[3,4] the base-on[5] and base-off[6] species of 5'-deoxyadenosylcobalamin, 5'-deoxyadenosylcobinamide,[7] and the b and e monocarboxylic acid derivatives of cyanocobalamin.[8] While such data have been used for biosynthetic studies,[3] for an analysis of the conformational consequences of the base-on/base-off reaction of AdoCbl,[6] and for positive identification of CNCbl analogues,[8] their potential use as probes of important conformational effects in the corrin

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ring of cobalamins has barely been exploited. The corrin ring is well-known to be significantly flexible,[9] and such flexibility is widely regarded as being crucial to the enzymatic functioning of the coenzyme form.[9-15] NMR probes of corrin ring conformations seem likely to provide key information on conformational effects in cobalamins and ultimately on the mechanism of enzymatic activation of AdoCbl.

Tantalizing evidence of the conformational sensitivity of such NMR data is already on hand. For instance, comparison of the ^{13}C spectrum of AdoCbi[7] to that of base-off AdoCbl[6] shows that, in addition to anticipated differences in the f side chain and isopropanolamine moiety, several side-chain methylenes and ring carbons have chemical shift differences of 1.0 ppm or more. Similar effects are seen in a comparison of the (incompletely assigned) ^{13}C spectra of CH_3CH_2Cbi and base-off CH_3CH_2Cbl.[16] While such chemical shift differences in the methylene groups of the b, d, and e side chains may well be due to effects of the pendent, protonated nucleotide in the base-off cobalamins, the corrin ring chemical shift differences suggest a subtle effect of the pendent, but uncoordinated, nucleotide on the corrin ring conformation. Indeed, small but finite ^{13}C chemical shift differences between alkylcobinamides and base-off alkylcobalamins enriched in ^{13}C in the α-carbon of the organic ligand have previously been noted[17] and attributed to such conformational effects.

This situation is completely different in the dicyanocobalt corrins. In a previous study[18] in which tentatively assigned ^{13}C spectra of $(CN)_2Cbl$ and $(CN)_2Cbi$ were presented, the spectra were found to be remarkably similar except for anticipated differences in the f side chains and the isopropanolamine moiety and a few peripheral side chains. None of the ring carbons differed in chemical shift by more than 0.15 ppm, and all but two differed by less than 0.1 ppm. This suggests that the corrin ring in these dicyanocobalt corrins is conformationally rigid[19] and that dicyanocobamides can consequently serve as benchmark compounds for studies of corrin ring conformational effects. Consequently, an absolute assignment of the NMR spectra of such compounds is highly desirable.

Dicyanocobalamin is also that ideal species in which to study the so-called "tuck-in" species of base-off, but benzimidazole-deprotonated, cobalamins in which the B3 nitrogen of the axial nucleotide is hydrogen bonded to a side-chain amide. In the neutral species of most RCbl's the affinity of the free base nucleotide for the cobalt atom reduces the tuck-in species to a minor contributor, but it is the major species of base-off $(CN)_2Cbl$. The existence of this species and the hydrogen-bonded nature of the interaction are now well established from NMR spectroscopic[1,18] and thermodynamic studies.[17] However, recent ^{15}N NMR studies,[1] while confirming the existence and nature of the species, have clouded the issue of the identity of the side-chain amide hydrogen-bond donor, originally tentatively assigned as the e side chain.[18] In this work the seven amide ^{15}N resonances of $(CN)_2Cbl$ were observed by polarization transfer (DEPT) and double-quantum (HMQC) methodologies. As previously shown by Di Feo et al.[20] for CNCbl, the b, d, e, and f amide ^{15}N resonances were readily assignable by observation of the b, d, and e monocarboxylate derivatives[8,21,22] and by phase inversion of the f resonance in DEPT spectra and observation of its proton multiplicity in the HMQC maps. Comparison of the ^{15}N spectra of $(CN)_2Cbl$ and $(CN)_2Cbi$ showed that both the e ^{15}N resonances and that of one of the acetamide side chains had chemical shift differences consistent with hydrogen-bond formation in $(CN)_2Cbl$.

We new reoprt the complete ^1H and ^{13}C assignments of $(CN)_2Cbl$. Coupled with our previous observation of the amide $^1H-^{15}N$ connectivities in $(CN)_2Cbl$,[1] homonuclear NOE experiments have now permitted complete assignment of the amide ^{15}N spectrum of $(CN)_2Cbl$ and, by analogy, of CNCbl. Together with ^{13}C NMR observations of the dicyano derivatives of the b, d, and e monocarboxylic acids, the C13 epimer of CNCbl, and the 3,5,6-trimethylbenzimidazole derivative, the assignments of the hydrogen-bond donor in the tuck-in species can now be made with some confidence. In addition, the complete assignments of the amide ^{15}N spectrum of CNCbl now permits the development of strategies for the observation of the ^{15}N NMR resonances and selective observation of the amide proton resonances of protein complexes of CNCbl enriched in ^{15}N. Development of these NMR probes of the details of the interactions of cobalamins with proteins is currently in progress.

Experimental Section

CNCbl was purchased from Sigma. The b, d, and e monocarboxylic acid derivatives of CNCbl were synthesized and purified as recently described.[22] The C13 epimer of CNCbl, CN-13-epiCbl,[2] was obtained by treatment of CNCbl with anhydrous trifluoroacetic acid as described by Bonnett et al.[23,24] and purified by HPLC on a 10 × 250 mm Beckman Ultrashpere C-8 column using the solvent system previously described.[25] The *N*-methyl derivative of CNCbl, $CNMe_3BzmCba$,[2] was obtained by methylation of $(CN)_2Cbl$ with dimethyl sulfate[26] and was also purified by semiprepartive HPLC. N-methylation of α-ribazole 3′-phospate[27] was carried out identically. The major product (of three) was purified by semipreparative HPLC and proved to be the methyl ester of *N*-methyl-α-ribazole 3′-phosphate (vide infra).

^{31}P and ^{13}C NMR spectra of *N*-Me-α-ribazole 3′-P methyl ester[2] were obtained on a Nicolet NT 200 wide-bore NMR spectrometer, as previously described.[27] One-dimensional ^{13}C NMR spectra of CNCbl, the b, d, and e monocarboxylic acid derviatives of CNCbl, CN-13-epiCbl, $CNMe_3BzmCba$, and the dicyano derivatives of all of these were obtained on a Bruker MSL 300 NMR spectrometer. Samples generally consisted of ca. 25 mg of cobamide in 2.25 mL of water, or 0.3 M aqueous KCN, locked to D_2O in a concentric insert (Wilmad). TSP in the insert provided a chemical shift reference.

Two-dimensional NMR experiments were performed on a GE GN 500, a Bruker AM 500, or a Bruker AM 360 NMR spectrometer. For experiments in D_2O, exchangeable protons were deuterated by dissolving CNCbl in 99.8% D_2O and evaporating to dryness three times. Sufficient CNCbl was then dissolved in 0.3 M KCN in "100%" D_2O (Aldrich) to provide a final concentration of $(CN)_2Cbl$ of 50–90 mM. The $^{31}P-^1H$ chemical shift correlation experiment was performed by using a 1024 × 512 data matrix with 16 scans per t_1 value and a 3.0-s delay between scans. Data were collected over 1450- and 3520-Hz sweep widths in the ^{31}P and ^1H dimensions, repsectively, on the GN 500 spectrometer. For the homonuclear *J*-correlated experiment (COSY) a 1024 × 512 data matrix was used with 4 scans per t_1 value and a 2.1-s delay between scans. The data were collected over a 4250-Hz sweep width in both dimensions on the GN 500 spectrometer. The absorption-mode hypercomplex NOE (NOESY) data were collected into a 1024 × 512 data matrix with 32 scans per t_1 value and a 1.0-s delay between scans. The mixing time was

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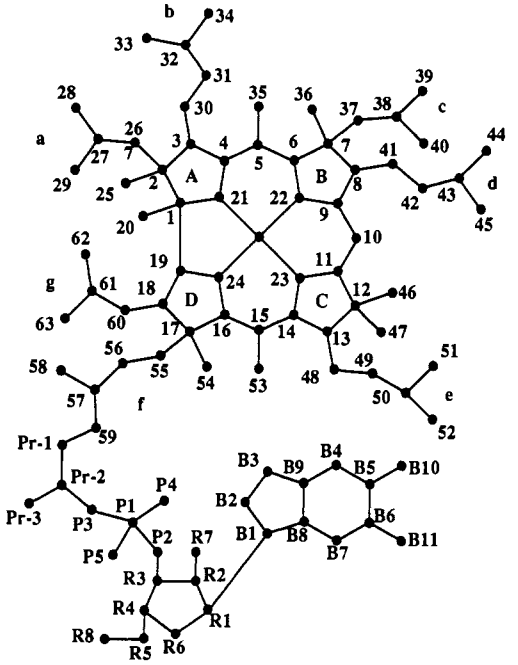

Figure 1. Structure and standard numbering system of cobalamin.

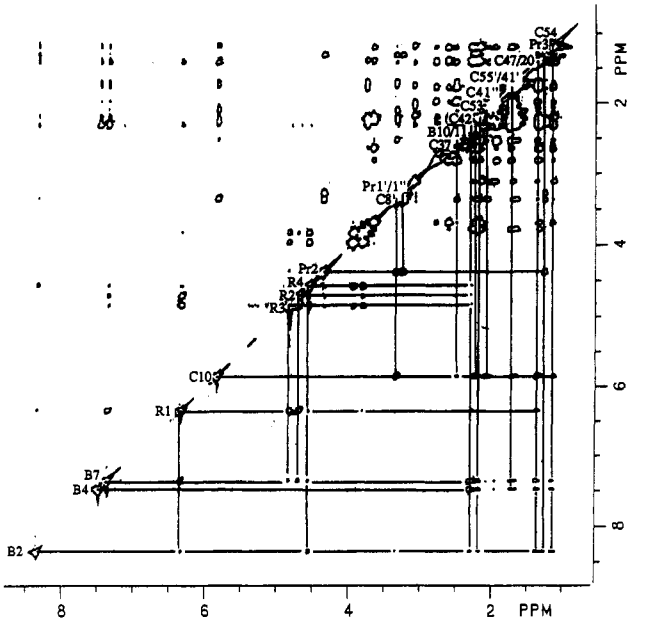

Figure 2. NOESY spectrum of $(CN)_2Cbl$ in D_2O. Through-space connectivities among the ribose and benzimidazole protons are shown, along with others.

500 ms. Data were collected over a 3200-Hz sweep width in both dimensions on the GN 500 spectrometer. The hypercomplex homonuclear Hartmann–Hahn experiment (HOHAHA)[28-31] was carried out by using a 1024 × 512 data matrix with 8 scans per t_1 value, a 1.8-s delay between scans, and 2.5-ms trim pulses. The data were collected over a 4400-Hz sweep width on the GN 500 spectrometer. The ^{1}H-detected heteronuclear multiple-quantum coherence experiment[31,32] (HMQC) was performed as described by Pagano et al.[7] on the GN 500 spectrometer. The ^{1}H-detected multiple-bond heteronuclear multiple-quantum coherence experiment[33] (HMBC) was performed on the Bruker AM 500 spectrometer by using an inverse detection probe. Data were collected into a 4096 × 512 data matrix with 128 scans per t_1 value and a 1.5-s delay between scans. The data were collected over a sweep widths of 4600 (^{1}H) and 26 000 Hz (^{13}C). The values of Δ_1 and Δ_2 were 3.4 and 55 ms, respectively. For the NOESY experiment on $(CN)_2Cbl$ in DMSO-d_6, 50 mg of CNCbl (not exchanged with D_2O) was dissolved in 0.5 mL of 0.17 M NaCN in anhydrous DMSO-d_6 to provide a sample of ca. 75 mM $(CN)_2Cbl$. The experiment was performed on a Bruker AM 360 spectrometer by using a 4096 × 512 data matrix, 32 scans per t_1 value, and a 2.0-s delay between scans. The t_1 increment was 167 μs and the total mixing time was 600 ms.

Results and Discussion

CNCbl and the Monocarboxylic Acids. All of the CNCbl derivatives prepared in this study were characterized by ^{13}C NMR spectrosopy. While this work was in progress, a reassignment[8] of the ^{13}C NMR spectrum of CNCbl[34] appeared. This reassignment was based on the complete ^{1}H and ^{13}C assignments of the conjugate bases of the b and e monocarboxylic acid derivatives of CNCbl by rigorous 2D methods. The ^{13}C spectrum of the insufficiently soluble CNCbl was then assigned by analogy. However, inspection of the influence of hydrolysis of the d side-chain amide on the ^{13}C spectrum of CNCbl strongly suggests that four of the reassignments of the CNCbl spectrum made by Pagano and Marzilli[8] are most likely incorrect. Thus, the group of four methylene carbon resonances between 34.1 and 35.1 ppm (downfield from TSP) were assigned C56, C60, C42, and C55, respectively (proceeding from high to low field) by Pagano and Marzilli (Figure 1). However, in the ^{13}C spectrum of CNCbl-d-COO$^-$ the first, third and fourth resonances remain at precisely the same positions as those of CNCbl (to within 0.04 ppm), while the second one is shifted downfield by 2.2 ppm. Clearly, this resonance must be that of C42, and so the correct order of assignment is C56, C42, C60, and C55. A similar interchange of assignments appears to have been made with the two resonances between 45 and 46 ppm. Pagano and Marzilli assigned the upfield one to C37 and the downfield one to C26. However, in the ^{13}C spectrum of CNCbl-d-COO$^-$, the upfield resonance occurs at the same position as that in CNCbl (to within 0.02 ppm) but the downfield resonance occurs at 0.52 ppm lower field. Given the proximity of the C37 methylene to the d side-chain carboxylate (Figure 1), it seems likely that the upfield member of this pair of resonances is C26 and the downfield one is C37.

NMR Assignments of Dicyanocobalamin and Dicyanocobinamide. We have followed the overall strategy of others in assigning the ^{13}C NMR resonances of $(CN)_2Cbl$ by correlating them to assigned protons in the molecule.[5-8] The proton resonances, for the most part, were assigned by using a combination of COSY, HOHAHA and phase-sensitive NOESY spectroscopy.[28-31] A good starting point for the assignment of proton resonances was to identify the ribose R3H and Pr2H resonances by using conventional phosphorous-detected heteronuclear correlation spectroscopy. It was then possible to assign all of the resonances of the ribose ring protons and the Pr1H and Pr3H resonances from their observed direct and relayed connectivities to the R3H and Pr2H resonances in the COSY and HOHAHA spectra. In the downfield portion of the NOESY spectrum the R1H, R2H, R3H, and R4H resonances show through-space connectivities with B7H at 7.4 ppm (Figure 2). The B7 proton resonance shows an intense crosspeak with a methyl group resonance at about 2.3 ppm and less intense crosspeaks with other upfield resonances. The most intense crosspeak can be assigned to the B11 resonance on the basis of the close proximity of this methyl group to B7. The B4H resonance at about 7.5 ppm can similarily be assigned on the basis of its connectivity with a resonance nearly identical in chemical shift with the B11 methyl resonance. This latter resonance must belong to the B10 methyl protons. The proton resonance at about 8.4 ppm can be assigned to B2H on the basis of its NOE connectivity with the R1 and R4 ribose proton resonances. The only remaining unassigned resonance in the downfield region of the proton spectrum (5.8 ppm) can be assigned to C10H. The connectivities of this resonance with the resonances of other protons in the corrin ring system and their connectivities, in turn, with others allows for a nearly complete assignment of the proton spectrum.

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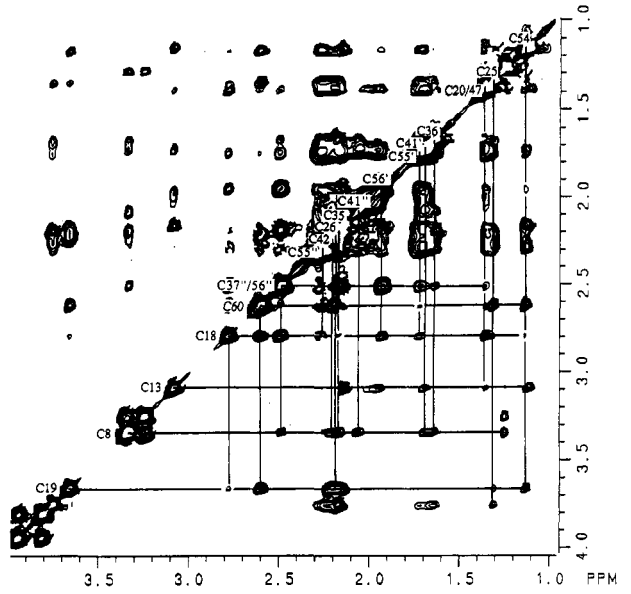

Figure 3. Expansion of the upfield region of the NOESY spectrum of $(CN)_2Cbl$ in D_2O. Through-space connectivities of C8H to the C35 and C36 methyl protons and the B-ring side-chain methylene protons are shown.

In some cases it was not possible to make assignments on the basis of the NOESY spectrum in the absence of data contained in HOHAHA and COSY spectra. For example, the resonance at 3.35 ppm, which overlaps the PR1′,1″H resonances, can be assigned to C8H via its connectivity to C10H in the NOESY spectrum. The C8H resonance has NOESY crosspeaks with two methyl group resonances at 1.66 and 2.20 ppm (C35 and C36), and methylene proton resonances at 1.71, 2.10, 2.25, and 2.50 ppm (Figure 3). The methylene proton resonances at 1.71, 2.10 and 2.25 ppm also appears as crosspeaks in the HOHAHA spectrum, while the COSY spectrum shows C8H connectivities with resonances at 1.71 and 2.10 ppm. Because the COSY spectrum is least likely to show relayed connectivities, these latter resonances can be assigned to C41H″ and C41H′. The remaining crosspeak in the HOHAHA spectrum must then arise from relayed connectivity and can be assigned to C24H's. By elimination, the remaining methylene proton resonance at 2.50 ppm can then be assigned to at least one of the C37H protons.

There are a few proton resonances that could only be assigned via their connectivities to carbons assigned from the HMQC and HMBC spectra (below). For example, the methylene proton resonance at 2.50 ppm previously assigned to one or more of the C37 protons also shows a crosspeak in the NOESY spectrum with C60H (2.62 ppm). With reference to the structure, the nearest methylene groups to C60 are the protons of C55 or C56. Integration of the resonance at 2.50 ppm shows it to be a two-proton resonance. Therefore, half of the intensity of this resonance can be assigned to C37H″ and the remaining intensity to one of the protons of C55 or C56. A definitive assignment of this peak is only possible by noting that the methyl proton resonance of C54 correlates with a carbon resonance at 35.6 ppm in the HMBC spectrum, while the 2.50 ppm proton resonance correlates with a carbon resonance at 34.5 ppm in the HMQC spectrum. Since crosspeaks in HMBC spectra typically arise from two- and three-bond carbon–proton couplings, the proton resonance at 2.50 ppm must arise from C37H″ and C56H″. Once carbon resonances C56 and C37 are assigned, the remaining proton resonances C37H′ and C56H′ could be assigned from NOESY and HMBC spectra. The C55 proton resonances could then be assigned via their connectivities with C56H″, C18H, and C60H in the NOESY spectrum.

A second example of how all available 2D data were used in making assignments arose in the case of making a one-to-one assignment of proton methyl resonances C46H and C47H. On the basis of the chemical shift data of previous studies, there is a tendency to assign the upfield methyl resonance (1.14 ppm) to the C46 protons.[5-8] However, the NOESY data alone make no distinction between the two. If present, connectivity is seen to both of the two methyls. In the HMBC spectrum, however, a crosspeak is present only between the higher field proton resonance and the C13 carbon resonance. Summers et al.[5] were first to assign this methyl group resonance to the C46H's on the basis of the larger expected proton–carbon coupling between groups having a cis orientation across the C12/C13 bond. In keeping with this, we have assigned the upfield proton resonance to the C46H resonance.

Even in the majority of cases where proton resonances could be assigned on the basis of 2D homonuclear correlation experiments alone, the HMBC and HMQC proton–carbon heteronuclear correlation experiments provided data that served to verify the original proton assignments. All of the correlations seen in the 2D spectra from which the carbon and proton resonance assignments were made are summarized in Table I.

Once all of the proton resonances had been assigned, the carbon resonances could be assigned in a straightforward manner by using connectivity data provided by proton-detected carbon–proton correlation HMQC and HMBC 2D experiments.[31-33] Perhaps the most challenging aspect of this was the assignment of the nonprotonated carbon resonances in the carbonyl region of the ^{13}C NMR spectra. The relevant region of the HMBC spectrum used in making these assignments is shown in Figure 4. The C6, C9, and C11 resonances show proton connectivities to four different proton resonances: C6 with the proton resonances of C8H, C37H″, C35H's, and C36H's; C9 with the proton resonances of C10H, C8H, C41H″, and C41H′; C11 with the proton resonances of C10H, C13H, C47H's, and C46H's. The multiple connectivities observed for each of the carbon resonances provided verification of the carbon resonance assignments as well as some of the original proton assignments. This proved to be of particular value in checking the assignments of some of the methylene proton resonances, which strongly overlap with other methyl and methylene proton resonances. For example, in the region of the proton spectrum between about 1.60 and 1.85 ppm proton homonuclear correlation experiments suggested that there was an overlap of methylene proton resonances of C41H, C48H, and C30H. Indeed Figure 4 shows that C9 (at about 174.8 ppm), which was previously assigned primarily by its connectivity to C10H, shows a crosspeak in this region of the proton spectrum at 1.70 ppm arising from its connectivity with one of the C41 protons. This same region of the proton spectrum also contains five additional connectivities between about 179 and 182 ppm on the carbon axis, with three of the crosspeaks at the same proton chemical shift as the C9 contour. One would expect one of these crosspeaks to arise from C43, since this carbon is also within three bonds of C41. Two crosspeaks, one at the same carbon chemical shift as C9, are also apparent in the HMBC spectrum at a proton chemical shift of 2.08 ppm. The second crosspeak of the two has the same carbon chemical shift as one of the lower field crosspeaks at 1.70 ppm. Since 2.08 ppm is in a region of the spectrum where one of the C41 proton resonances is indicated from NOESY data, one may simultaneously assign the two C41H resonances and the C43 carbonyl resonance. In a similar fashion the carbon resonances of C32, C4 and C50 were assigned. These assignments of the C32, C43, and C50 carbonyl resonances were also consistent with the differences between the downfield region of the ^{13}C spectrum of $(CN)_2Cbl$ and those of the dicyano derivatives of the b, d, and e monocarboxylates.

Perhaps the most difficult of all the carbonyl resonances to assign were those of C57 and C38. Both of these resonances occur near 178.0 ppm with less than a 0.2 ppm difference in their chemical shift. The problem is complicated by the fact that the C37H″ and C56H″ resonances overlap at 2.50 ppm. Thus, it is not immediately obvious if the contour in the HMBC spectrum near 178.0 ppm and at 2.50 ppm in the proton dimension arises from the coupling of C56H″ to C57 or from the coupling of C37H″ to C38. Connectivities observed in the HMQC spectrum allowed the assignment of the two C37H resonances at 2.50 and

Table I. Correlation for NMR Connectivities of $(CN)_2$Cbl Observed by Homonuclear *J*-Correlation (COSY), Absorption-Mode NOE (NOESY), Homonuclear Hartmann–Hahn (HOHAHA), and Heteronuclear Multiple-Bond Correlation (HMBC) Methodologies[a]

^{1}H signal	D_2O				DMSO-d_6
	COSY	NOESY	HOHAHA	HMBC	NOESY
C46		C10, C13, C47, C49		C11, C12, C13, C47	C13, C47, C49′, C49″
C54		C18, C19, C53, C55′, C55″, C56′, C56″, C60, (B2), B4, (B7)		C16, C17, C18, C55	Pr2, C13, C18, C49′, C55′, C55″, C56, g′, g″
Pr3		Pr1′, Pr1″, Pr2, B2		Pr1, Pr2	B2, Pr1,[c] Pr2, f, g′, g″
C25		C3, C26, C30′, C30″, C60, B2, (B4), (B7)		C1, C2, C3, C26	C19, C20, a′, a″, g′, g″
C20		C18, C56″, C60, R1, (B2), B4, B7		C1, C19	C18, C19, C25, C60, a′, a″, g′, g″
C47		C10, C13, C46, C48′, C48″, C49		C11, C12, C13, C46	C8, C13, C46, C49′, e″
C36		C8, C35, C37′		C6, C7, C8, C37	C8, C35, C37′, C41, c′, c″, d′, d″
C30′	C3, C30″	C3, C25, C30″, C31	C3, C31	C2, C3, C31, C32	*b*
C48′	C13, C48″	C13, C47, C48″, C49	C13	C12, C13, C50	*c*
C41′	C8, C41″	C8, C10, C41″	C8	C7, C8, C9, C42, C43	C8, C36, c′, c″
C30″	C3, C30′	C3, C25, C30′, C31	C3, C31	C2, C3, C31, C32	*b*
C56′	C55′, C55″, C56″	C18, C54, C55′, C55″, C56″, (B2), (B4), (B7)	C55′, C55″, C56″	C57	C60, f, g′
C55′	C55″, C56′	C18, C55″, C56′, C56″, C60, (B2), (B4), (B7)	C55″, C56″		C19, C54, f
C48″	C13	C13, C47, C48″	C13	C12, C50	C13
C41″	C8, C41′	C8, C10, C41′	C8	C7, C8, C9, C42, C32	C8, C36, c′, c″
C53		C13, C54, (B2), B4, B7		C14, C15, C16	C13, C49′, C54, C55′, C56
C26′		*c*			C3, a′, a″, g′
C37′	C37″	C36, C37″	C37″	C6, C7, C8, C36, C38	C35, c′, c″
C35		C3, C8, C36		C4, C5, C6, C7, C8, C36	C31, C36, C37′, b′, b″, c′, c″
C49′		C13, C46, C47, C48′	C13	C50	C13, C46, C47, C49″, e′, e″
C49″		*c*			C46, C47, C49′, e′, e″
C42	C41′, C41″	C8, C10	C8	C43	d′, d″
C31		C3, B2[d]	C3	C32	C3, C35, a′, a″, b′, b″
C26″		C3, C19, C25			C3, a′, a″, g′, g″
C55″	C55′, C56′, C56″	C18, C55′, C56′, C56″, C60, B2[d]	C56′		C54, C55′, C56″, C60, f
B11	B7	R1, B7	B7	B4, B5, B6, B7	B7
B10	B4	B4	B4	B4, B5, B6, B7	B4
C56″	C55′, C55″, C56′	C18, C54, C55′, C55″, C56′, C60, (B7)	C55′, C55″, C56′		C54, C55′, C55″, C56′, C60, g′, g″, f
C37″	C37′	C8, C10, C36, C37′	C37′	C6, C7, C8, C36, C38	C36, c′, c″
C60[c]	C18	C18, C19, C25, C54, C55′, C55″, C56″	C18, C19	C17, C18, C19, C61	C25, C53, C55′, C55″, C56′, C56″, f, g′, g″
C18	C19, C60	C19, C20, C54, C55′, C55″, C56′, C56″, C60	C19, C60	C1, C17, C19, C54, C60, C61	R4, C19, C20, C54, a′, a″, g′, g″
C13	C48′, C48″, C53, C49	C46, C47, C48′, C48″	C48′, C48″, C49	C11, C12, C14, C46, C48, C49	C46, C47, C48,[c] C49′, C49″, C53, C54, e′, e″
Pr1′	Pr2	Pr2, Pr3	Pr2, Pr3	Pr2, Pr3, C57	Pr2, f
Pr1″	Pr2	Pr2, Pr3, C20	Pr2, Pr3	Pr2, Pr3, C57	*c*
C8	C41′, C42″	C35, C36, C37″, C41′, C41″, C42′	C41′, C41″, C42	C6, C7, C9, C10, C37, C41, C42	C36, C37′, C41′, C47, c′, c″
C19	C18	C18, C25, C26,[c] C54, C60[c]	C18, C60	C1, C2, C16, C18, C20, C60[c]	C18, C20, C25, C55′, C55″, a′, a″
C3	C30′, C30″	C25, C26[c] C30′, C30″, C31, C35	C30′, C30″, C31	C1, C2, C4, C26	C8, C20, C26′, C31, C30, C31
R5′	R4	R3, R4, R5″	R1, R2, R3, R4	R3, R4	*b*
R5″	R4	R2, R3, R4, R5′	R1, R2, R3, R4	R3, R4	*b*
Pr2	Pr1′, Pr1″	Pr1′, Pr1″, Pr3	Pr1′, Pr1″, Pr3		Pr1,[c] Pr3, B2, C54
R4	R3, R5′, R5″	R1, R2, R3, R5′, R5″, B2, B7	R1, R2, R3, R5′, R5″	R3	R2, R3, C18
R2	R1, R3	R1, R2, R3, R4, R5″, B7	R1, R3, R4, R5′, R5″	R1, R4	R1, R4, B2, B7, C53
R3	R2	R1, R2, R4, R5′, R5″	R1, R2, R4, R5′, R5″	R1, R5	R1, R4, Pr3, B2, C53
C10		C8, C37″, C41′, C41″, C42, C46, C47		C8, C9, C11, C12	C8, C46, C47, d′, d″
R1	R2	R2, R3, R4, B7, B11, C20	R2, R3, R4, R5′, R5″	R2, R3, R4, B2, B8	R2, R3, B2, B7
B7	B11	B11, R1, R2, R3, C20, (C25), C53, (C54), C56′	B11	B5, B9, B11	R1, R2, B11
B4	B10	B10, C20, (C25), C53, C54, (C55′), (C56′)	B10	B6, B8, B10	B10
B2		R1, R4, Pr3, (C20), C25, (C53), (C54), (C55′), (C56′)		B8, B9	Pr2, R1, R2, R3

[a] Double prime denotes the downfield signal of diastereotopic methylene protons or the syn proton of an amide syn, anti proton pair, while single prime denotes the upfield signal of such pairs. The letters a, b, c, etc. refer to the amide protons of the a, b, c, etc. side chains. Atoms listed in parentheses show weak crosspeaks, only visible at deep contour levels. [b] Signal not assigned in DMSO. [c] Diastereomeric protons not resolved. [d] Crosspeak to B2H is either from C55H′ or C31H's.

2.16 ppm and the two C56H resonances at 2.50 and 1.95 ppm. Close inspection of the HMBC data revealed that the crosspeak seen at 2.50 ppm had exactly the same carbon chemical shift as a similar correlation seen at 2.16 ppm. On this basis the crosspeak at 178.0 and 2.50 ppm was assigned to C38. Crosspeaks slightly upfield of the C38 resonance are observed at frequencies previously assigned to the two Pr1H resonances and the C56H′ resonance (1.95 ppm). On this basis, the C57 carbonyl resonance has been assigned to the most upfield of the two resonances near 178.0 ppm.

The two remaining carbon resonances near 178.5 ppm must be due to the carbonyl carbons C27 and C61. The only proton responses at this carbon chemical shift on the HMBC map are

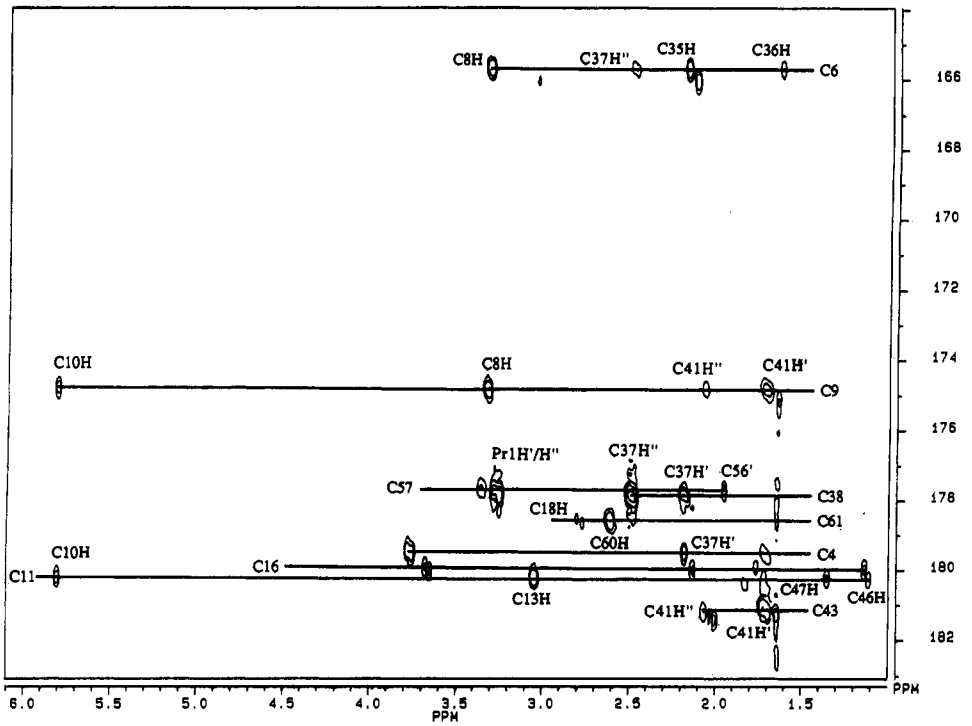

Figure 4. Portion of the ^{1}H-detected, ^{1}H,^{13}C-correlated HMBC spectrum of $(CN)_2$Cbl in D_2O, showing the carbonyl region in the ^{13}C dimension. Different portions of the spectrum have been plotted with different minimum contour levels as indicated within each box. This method of display permits observation of the weaker peaks without the noise associated with the t_1 ridges that accompany the strong peaks and run parallel to the ^{13}C axis.

assignable to C18H (2.8 ppm) and the C60H's (2.6 ppm). Careful alignment of a 1D carbon spectrum plotted on the same scale as the carbon axis of the HMBC map permits assignment of C61 to the more downfield of the two carbon resonances near 178.5 ppm. The remaining carbon resonances near this chemical shift is then assigned to C27 by default. The final assignments of the carbon and proton resonances of $(CN)_2$Cbl in D_2O are given in Table II.

The amide protons of cobalamins are not observable in D_2O due to exchange. However, these protons are readily observed in DMSO-d_6 and the 13 amide proton resonances of $(CN)_2$Cbl have recently been correlated with the amide ^{15}N resonances by ^{1}H-detected ^{1}H,^{15}N heteronuclear multiple-quantum coherence spectroscopy.[1] Armed with the complete assignments of the ^{1}H spectrum of $(CN)_2$Cbl in D_2O (Table II) and the NOESY map in D_2O, nearly complete ^{1}H assignments for $(CN)_2$Cbl in DMSO-d_6 were readily made from the NOESY correlations in DMSO-d_6 (Table I). These assignments are given in Table II. Given the previous one-bond correlations of the amide proton resonances with the amide ^{15}N resonances, the amide ^{15}N spectrum could also be completely assigned. Thus, the pair of amide protons (7.08 and 7.85 ppm) previously correlated with the unassigned acetamide ^{15}N resonance at 115.88 ppm[1] showed NOE cross peaks to C18H, C19H, C20H's, C25H's, C26H', C26H'', and C31H's and could be assigned to the a side-chain amide. This pattern of NOE's was clearly distinguishable from that for the pair of amide protons (7.32, 7.87 ppm) previously correlated with the unassigned acetamide ^{15}N resonance at 111.23 ppm,[1] which showed crosspeaks to Pr3H's, C18H, C20H's, C26H', C26H'', C56H', C56H'', and the C60H's, and could reliably be assigned to the g side-chain amide. While far fewer NOE crosspeaks were observed for the propionamide amide protons than for the acetamides, all of the NOE's observed for the propionamide amide protons were consistent with the previous assignments of the b, d, and e amide nitrogen (and proton) resonances via observation of the monocarboxylate derivatives.[1] Thus, the d amide protons (6.70, 7.11 ppm) had crosspeaks with C10H, C36H's, and C24H's, while the e amide protons (6.92, 7.14 ppm) had NOE's to C13H, C49H', and C49H''. Similarly, the NOE's for the previously assigned f amide proton (8.31 ppm) were consistent with its assignment (C55H', C55H'', C56H', C56H'', C60H's, Pr1H', Pr1H'', and the Pr3H's). Thus, the assignment of the amide ^{15}N spectrum of $(CN)_2$Cbl (and, by analogy, that of $(CN)_2$Cbi[1]) is now complete (Table II); the resonances appear in the order (from high field to low) d, b, e, g, f, c, a. Since the downfield region of the ^{15}N spectrum containing the previously unassigned g, c, and a acetamide resonances is very much less crowded than the upfield, propionamide region (the g, c, and a ^{15}N resonances are separated by 2–3 ppm each) and given the relatively small chemical shift changes in these resonances accompanying the transformation of CNCbl to $(CN)_2$Cbl,[1] it seems reasonable to conclude that the same order of chemical shift assignments is correct for the amide ^{15}N resonances of CNCbl. We also note with interest the recent observation of the ^{15}N resonances of the pyrrole nitrogens of CNCbl enriched in ^{15}N in those nitrogens by fermentation of ^{15}N-labeled 5-aminolevulinic acid in *Propionibacterium shermanii*.[36] Surprisingly, these resonances were spread over 48 ppm. We anticipate the possibility of assigning these resonances in ^{15}N-enriched $(CN)_2$Cbl using the ^{1}H assignments in Table II and heteronuclear ^{1}H–^{15}N NOE spectroscopy. Coupled with the current work, and previous observation and assignment of the ^{15}N resonances of both benzimidazole nitrogens[1] and both the α and β cyanide ligands,[19] this would represent the complete assignment of the ^{15}N spectrum of $(CN)_2$Cbl (15 resonances).

The complete ^{13}C assignments of $(CN)_2$Cbl (Table II) correct a number of misassignments previously made[18] by analogy to other assigned spectra. They also permit reliable assignment of the ^{13}C spectrum of $(CN)_2$Cbi, since, as pointed out above, with the exception of the f side chain, the two carbon spectra are nearly identical. In addition to the chemical shift differences of the three isopropylamine carbons, the C55 methylene in the f side chain undergoes a 0.4 ppm upfield shift when the nucleotide is removed from $(CN)_2$Cbl. This resonance was previously misassigned to the C49 methylene in the e side chain. The only other significant difference (>0.2 ppm) between the ^{13}C spectra of $(CN)_2$Cbl and $(CN)_2$Cbi occurs in the downfield region and is most likely as-

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(36) Kurumaya, K.; Okazaki, T.; Kajiwara, M. *Chem. Pharm. Bull.* **1990**, *38*, 1058–1061.

Table II. Final Assignments of the ^1H and ^{13}C Resonances of $(CN)_2Cbl$ in D_2O, the ^1H and ^{15}N Resonances of $(CN)_2Cbl$ in DMSO-d_6, and the ^{13}C Resonances of $(CN)_2Cbi$ in D_2O

	$(CN)_2Cbl/D_2O$		$(CN)_2Cbl/$ DMSO-d_6		$(CN)_2Cbi/D_2O$		$(CN)_2Cbl/D_2O$		$(CN)_2Cbl/$ DMSO-d_6		$(CN)_2Cbi/D_2O$
atom[a]	δ_{13C}[b] ppm	δ_{1H}[b] ppm	δ_{1H}[b] ppm	δ_{15N}[c] ppm	δ_{13C}[b] ppm	atom[a]	δ_{13C}[b] ppm	δ_{1H}[b] ppm	δ_{1H}[b] ppm	δ_{15N}[c] ppm	δ_{13C}[b] ppm
C53	17.35	2.16	2.26		17.42	C1	85.66				85.75
C35	17.83	2.20	2.22		17.84	R4	85.66	4.57	4.17		
C25	19.02	1.34	1.40		19.15	R1	88.38	6.33	6.30		
C54	19.91	1.16	1.20		20.05	C10	93.71	5.82	5.69		93.65
C47	21.19	1.38	1.37		21.22	C15	105.77				105.79
C36	21.42	1.66	1.57		21.41	C5	107.73				107.79
Pr3	21.42	1.28	1.18		22.23	B7	113.63	7.36	7.44		
B10	22.08	2.31	2.35			B4	121.59	7.46	7.45		
B11	22.60	2.30	2.37			B8	134.15				
C20	24.25	1.38	1.34		24.35	B5	135.07				
C30	27.39	1.71, 1.78	*d*		27.54	B6	135.92				
C48	28.32	1.70, 1.95	1.86[e]		28.40	B9	142.96				
C41	29.34	1.71, 2.06	1.87, 1.90		29.29	B2	145.22	8.35	8.37		
C46	33.06	1.14	1.14		33.08	C6	165.65				165.66
C49	34.44	2.24	1.71, 2.07		34.24	C14	165.96				166.12
C56	34.51	1.95, 2.50	1.82, 2.50		34.57	C9	174.66				174.74
C42	34.75	2.23	2.10		34.65	C57	177.74				177.85
C60	35.05	2.61[f]	2.86[f]		35.18	C38	177.96				177.97
C55	35.60	1.72, 2.30	1.82, 2.27		35.18	C27	178.55				178.65
C31	37.62	2.30	2.50		37.66	C61	178.63				178.65
C18	41.54	2.80	3.59		41.68	C4	179.33				179.51
C26	44.88	2.20[f]	2.04, 2.18		44.96	C16	179.92				180.08
C37	46.41	2.16, 2.50	2.55[e]		46.45	C11	180.16				180.23
Pr1	47.55	3.27, 3.33	3.22[e]		48.97	C32	180.43				180.83
C2	48.79				48.97	C43	181.21				181.26
C12	49.45				49.48	C50	181.34				181.49
C7	51.88				51.81	*a*			7.08, 7.85	115.88	
C13	55.87	3.05	3.13		55.90	*b*			6.85, 7.46	107.89	
C8	57.91	3.33	3.71		57.90	*c*			7.00, 7.60	113.57	
C3	59.02	3.75	2.57		58.99	*d*			6.70, 7.11	107.83	
C17	61.43				61.44	*e*			6.92, 7.14	108.57	
R5	63.46	3.80, 3.93	*g*			*f*			8.31	112.65	
R2	73.78	4.70	4.55			*g*			7.32, 7.87	111.23	
Pr2	74.98	4.35	4.28		69.02	β-CN[h]	141.5				141.6
R3	76.64	4.85	4.65			α-CN[i]	140.2				140.6
C19	77.62	3.67	4.44		77.72						

[a] Figure 1. The letters a, b, c, etc. refer to the a, b, c, etc. side-chain amide nitrogens and protons. [b] ^{13}C and ^1H shifts are relative to internal TSP. [c] Reference 1. ^{15}N shifts were measured relative to external CH_3NO_2 but are reported relative to $NH_3(l)$ ($\delta_{CH_3NO_2}$ = 380.23[35]). [d] Resonance not observed or not assignable. [e] Only one proton was assignable. [f] Diastereotopic protons not resolved. [g] Resonance obscured by water resonance. [h] $\delta_{15N}((CN)_2Cbl)$ = 277.6 ppm, $\delta_{15N}((CN)_2Cbi)$ = 277.4 ppm.[19] [i] $\delta_{15N}((CN)_2Cbl)$ = 284.4 ppm, $\delta_{15N}((CN)_2Cbi)$ = 283.3 ppm.[19]

signable to C32 (the b side-chain carbonyl). This resonance was previously misassigned to the e side-chain carbonyl, C50. Thus, the corrected assignments do not suggest the involvement of the e side in the hydrogen-bonded interaction of the tuck-in species, as previously, tentatively concluded, although there is a slight difference (0.22 ppm) in the ^{15}N chemical shift of the e amide nitrogen between $(CN)_2Cbl$ and $(CN)_2Cbi$.[1] While the 0.4 ppm downfield shift of the b side-chain carbonyl resonance in $(CN)_2Cbi$ relative to $(CN)_2Cbl$ might suggest involvement of the b side-chain amide in the tuck-in interaction, there is no difference in the b amide ^{15}N chemical shift between the two species.[1]

Comparison of the ^{13}C chemical shifts of the corrin ring carbons of $(CN)_2$-Cbl and $(CN)_2Cbi$ show that none differ by more than 0.2 ppm, only five differ by more than 0.1 ppm, and the average absolute deviation is 0.08 ± 0.06 ppm. This is strikingly different from the comparison of base-off AdoCbl[6] and AdoCbi[7] in which the average deviation of the ring carbon chemical shifts is 0.28 ppm, eight of the ring carbons deviate by 0.3 ppm or more, and two deviate by 1.0 ppm. It is also constructive to compare the NMR spectra of base-off AdoCbl[6] with those of $(CN)_2Cbl$ (Table II), the only two examples of base-off Cbl's for which NMR assignments are known with certainty. While the largest ^{13}C chemical shift differences occur at C5, C10, and C15 ($\Delta\delta = \delta_{AdoCbl} - \delta_{(CN)_2Cbl}$ = 3.5, 6.7, and 4.0 ppm, respectively), there are differences of 1.0 ppm or greater (up to 2.5 ppm) at C1, C2, C3, C7, C11, C20, C26, C30, C46, C47, and C53. Many of these deviations are unlikely to be due to electronic effects of the axial ligands and suggest a significant difference in corrin ring conformation. In the ^1H spectra, the largest deviations (1.0–1.2 ppm) are at C10 and C19, and these are most likely due to axial ligand differences. However, significant shifts (0.3–0.6 ppm) in the proton resonances at C3, C8, C13, C26, C30, C47, and C53 again suggest ring conformation effects.

$(CN)_2Me_3BzmCba$ and *N*-Me-α-ribazole 3′-P Methyl Ester. One of the hallmarks of the tuck-in species is the effect that the interaction of the nucleotide with the remainder of the molecule has on the ^{13}C chemical shifts of the benzimidazole moiety. Thus, a comparison of the ^{13}C resonances of the benzimidazole moiety of base-off, but benzimidazole-deprotonated, $(CN)_2Cbl$ and $NCCH_2(CN)Cbl$ with those of the detached, free-base nucleotide (α-ribazole 3′-phosphate dianion) showed substantial differences in the carbon chemical shifts (as large as 1.8 ppm).[18,37] In any $(CN)_2Cbl$ analogues in which structural alterations prevented the hydrogen-bonded interaction responsible for the stability of the tuck-in species,[17] the ^{13}C chemical shifts of the pendent benzimidazole moiety and the free nucleotide would be expected to be identical, assuming there was no interaction whatever between the pendent nucleotide and the remainder of the structure. That this is indeed the case for $(CN)_2Me_3BzmCba$, in which hydrogen bonding to the benzimidazole nitrogen B3 is prevented by its methylation, is shown by the ^{13}C spectrum of the nucleotide of $(CN)_2Me_3BzmCba$ (Table III). While the chemical shifts are quite similar to those of the B3-protonated zwitterion of α-ribazole 3′-P,[18] a more exact comparison was obtained by methylation of

(37) In the comparison drawn in ref 18, structure 10, there is a sign error in the chemical shift difference shown for B8. It should read 1.839, not −1.839.

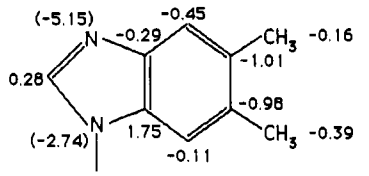
Table III. Comparison of the ^{13}C NMR Spectrum of *N*-Methyl-α-ribazole 3′-Phosphate Methyl Ester to That of the Nucleotide of $(CN)_2Me_3BzmCba$[a]

	$\delta_{^{13}C}$, ppm (J, Hz)[b]	
carbon	*N*-Methyl-α-ribazole 3′-phosphate methyl ester[c]	$(CN)_2Me_3BzmCba$
B2	143.80	*d*
B4	115.64	115.75
B5	140.33	140.18
B6	140.20	140.18
B7	115.22	115.30
B8	131.72	131.69
B9	133.22	133.14
B10	22.35	22.15
B11	22.39	22.68
R1	90.03	89.98
R2	74.20 (4.0)	74.26 (4.0)
R3	77.09 (5.3)	77.25 (5.6)
R4	88.35 (4.2)	88.33 (4.0)
R5	63.84	63.70
N-CH_3	35.90	35.96
O-CH_3	55.73 (6.0)	

[a] In H_2O, locked to D_2O in a concentric insert. Chemical shifts are downfield from internal TSP. [b] J_{CP}. [c] The ^{31}P resonance (1.86 ppm downfield from external 85% H_3PO_4) was a quintet with $^3J_{HP}$ = 10 Hz. [d] Not observed.

α-ribazole 3′-P with dimethyl sulfate by using the procedure of Friedrich and Bernhauer[26] for the B3 methylation of CNCbl. High-performance liquid chromatograms showed that three methylated products were obtained. On purification and NMR characterization, the major product proved to be the methyl ester of *N*-methyl-α-ribazole 3′-phosphate. Thus, in addition to the *N*-methyl resonance at 35.9 ppm in the ^{13}C spectrum, a doublet at 55.7 ppm ($^2J_{CP}$ = 6.0 Hz) was assigned to O–CH_3. ^{31}P NMR demonstrated that it was a phosphate oxygen that had been methylated, as the undecoupled ^{13}P resonance was a nearly symmetrical quintet with an average line spacing of 10 Hz, suggesting that the three-bond H–P couplings are nearly the same for the methyl and R3 protons. The ^{13}C spectrum of *N*-methyl-α-ribazole 3′-phosphate methyl ester is compared to that of the nucleotide of $(CN)_2Me_3BzmCba$ in Table III. With the inexplicable exception of the two ring methyl groups (B10 and B11, $\Delta\delta = \delta_{nuc} - \delta_{Cba}$ = 0.20 and −0.29 ppm, respectively) the spectra are nearly identical. Thus, N-methylation of the nucleotide of $(CN)_2Cbl$ does, indeed, prevent formation of the tuck-in species, and the dangling, N-methylated nucleotide has little or no interaction with the remainder of the molecule.

Dicyano Derivatives of the b, d, and e Monocarboxylates and 13-EpiCbl. We have also attempted to block formation of the tuck-in hydrogen bond by alteration of the b, d, and e side chains of $(CN)_2Cbl$. Table IV shows a comparison of the ^{13}C chemical shifts of the nucleotide loop carbons of $(CN)_2Cbl$ to those of the dicyano derivatives of the cobalamin b, d, and e monocarboxylates and of 13-epicobalamin, in which epimerization at C13 places the e side chain in the abnormal, upward configuration. Agreement of the chemical shifts of individual carbon resonances across the series of compounds is remarkably good, despite some small, but probably significant, deviations in the nucleotide of $(CN)_2Cbl$-b-COO^- (vide infra). This strongly suggests that the conformation of the nucleotide in the base-off dicyanocobamides is the same for all of these derivatives. That this conformation is distinctly different from that of a dangling, noninteracting nucleotide is demonstrated in **1**, in which the difference in chemical shift be-



1

tween the free-base nucleotide (α-ribazole 3′-phosphate dianion)[18] and the average chemical shift of the five dicyanocobamides in Table IV is displayed. Also shown (in parentheses) are the differences in ^{15}N chemical shift of B1 and B3 between the free-base nucleotide and $(CN)_2Cbl$.[1] The only significant differences between the comparison shown in **1** and that previously drawn between α-ribazole 3′-P dianion and $(CN)_2Cbl$[18] result from the interchanges of the assignments of B5 and B6, and B10 and B11.[37] There are substantial chemical shift differences at B4, B5, B6, B8, and both of the nucleotide nitrogens (B1 and B3). These results show conclusively that the tuck-in species persists in all of the derivatives, and they eliminate the propionamides from consideration as hydrogen-bond donors in this species.

Close inspection of the nucleotide ^{13}C chemical shifts in Table IV shows that there is amazing agreement in chemical shift at virtually every carbon atom among $(CN)_2Cbl$, its d and e monocarboxylate analogues, and the 13-epi analogue. Only at R4 does the standard deviation of these chemical shifts exceed 0.07 ppm, and only at 3 (of 17) carbon does the standard deviation exceed 0.05 ppm (B7, R4, and R5). Comparison of the ^{13}C resonances of the nucleotide loop of $(CN)_2Cbl$-b-COO^- to the average values of those of the other four derivatives (Table IV)

Table IV. ^{13}C Chemical Shifts of the Nucleotide Loops of the Dicyano Derivatives of Cobalamin, the b, d, and e Monocarboxylates of Cobalamin, and 13-Epicobalamin[a]

	$\delta_{^{13}C}$, ppm					
carbon	$(CN)_2Cbl$	$(CN)_2Cbl$-d-COO^-	$(CN)_2Cbl$-e-COO^-	$(CN)_2$-13-epiCbl	av ± std dev[b]	$(CN)_2Cbl$-b-COO- (dev)[c]
B2	145.22	*d*	145.14	145.18	145.18 ± 0.04	*d*
B4	121.59	121.57	121.58	121.60	121.59 ± 0.01	121.45 (0.14)
B5	135.07	135.10	135.11	135.13	135.10 ± 0.03	135.10 (0.00)
B6	135.92	136.01	135.98	135.99	135.98 ± 0.04	136.03 (−0.05)
B7	113.63	113.63	113.61	113.75	113.66 ± 0.06	113.81 (−0.15)
B8	134.15	134.16	134.19	134.23	134.18 ± 0.04	134.37 (−0.19)
B9	142.96	142.95	142.93	142.98	142.96 ± 0.02	142.98 (−0.02)
B10	22.08	22.08	22.09	22.06	22.08 ± 0.01	22.11 (−0.03)
B11	22.60	22.58	22.58	22.58	22.59 ± 0.01	22.64 (−0.05)
R1	88.38	88.36	88.35	88.31	88.35 ± 0.03	88.41 (−0.06)
R2	73.78	73.72	73.69	73.76	73.74 ± 0.04	74.04 (−0.30)
R3	76.64	76.69	76.70	76.77	76.70 ± 0.05	77.08 (−0.38)
R4	85.66	85.61	85.50	85.85	85.66 ± 0.15	85.68 (−0.02)
R5	63.46	63.53	63.56	63.63	63.54 ± 0.07	63.86 (−0.32)
Pr1	47.55	47.54	47.56	47.61	47.57 ± 0.03	47.40 (0.17)
Pr2	74.98	74.94	75.01	74.90	74.96 ± 0.05	74.77 (0.19)
Pr3	21.42	21.39	21.46	21.40	21.42 ± 0.03	21.30 (0.12)

[a] In H_2O locked to D_2O in a concentric insert. Chemical shifts are downfield from internal TSP. [b] Average and standard deviation of the chemical shifts for $(CN)_2Cbl$, $(CN)_2Cbl$-d-COO^-, $(CN)_2Cbl$-e-COO^-, and $(CN)_2$-13-epiCbl. [c] Signed difference between the average chemical shift for $(CN)_2Cbl$, $(CN)_2Cbl$-d-COO^-, $(CN)_2Cbl$-e-COO^-, and $(CN)_2$-13-epiCbl and the chemical shift for $(CN)_2Cbl$-b-COO^-. [d] Not observed.

shows a number of statistically significant differences. Chemical shift differences at B4, B7, B8, Pr1, and Pr2, although statistically significant (at least 90% confidence limits), are quite small. More impressive are the chemical shift deviations (0.3 ppm or greater) at R2, R3, and R5, all of which are significant at the 98% confidence limit or greater. These deviations suggest that, while the b side-chain amide is clearly not the hydrogen-bond donor in the tuck-in species, the nucleotide is deployed in this species in such a way as to be in close proximity to the b side-chain carbonyl. This would explain the upfield shift (0.4 ppm) of the b carbonyl carbon (C32) in $(CN)_2$Cbl relative to $(CN)_2$Cbi (Table II). Additional evidence for the deployment of the nucleotide under the western half of cobalamin in the tuck-in species is obtained from a number of conformationally significant NOE's seen in the NOESY spectra of $(CN)_2$Cbl, as discussed below.

Hydrogen-Bond Donor in the Tuck-in Species. The data described above (Table IV) clearly eliminate the propionamides as hydrogen-bond donors in the tuck-in species. While at first glance it might seem impossible to form a hydrogen bond between the benzimidazole nitrogen, B3, and any of the acetamide protons, consideration of models shows that the nucleotide loop has sufficient conformational flexibility to allow B3 to hydrogen bond to the a, c, or g acetamides. Our recent ^{15}N NMR study demonstrated a significant change in ^{15}N chemical shift of only one of the three acetamide nitrogens (the highest field acetamide resonance, near 111 ppm)[1] when the nucleotide is removed from $(CN)_2$Cbl. As this resonance has now been assigned to the g side-chain amide nitrogen, this amide becomes the prime candidate for the donor in the tuck-in species.

A number of conformationally significant NOE's seen in the NOESY spectrum of $(CN)_2$Cbl (Table I) seem to confirm this. Thus, the B2H shows a strong crosspeak with C25H's and with a resonance that cannot be definitely assigned to either C55H″ or C31H's, B4H shows strong NOE's to C20H's and C54H's, and B7H and the R1H both show strong NOE's to C20H's. Weaker NOE's (i.e., only visible in NOESY maps printed at deeper contour levels) are seen between B2H and C20H's, C54H's, C55H′, and C56H′, between B4H and C25H's, C55H′, and C56H′, and between B7H and C25H's, C54H's, C55H′, and C56H′. Use of models shows that these NOE's are consistent with a g-NH-B3 hydrogen-bonded species in which the ribose lies below and just outside the D ring and the benzimidazole moiety is exo to the sugar, pointing away from the D ring, roughly perpendicular to the corrin plane and making an acute angle with the A–D ring junction. However, this conformation would require a strong interaction between the B2H and the C18H, which is not seen at any contour level. If the ribose is shifted closer to the A ring and the nucleotide is in an endo conformation, with the benzimidazole roughly parallel to the A–D ring junction, the requirement of a strong B2H–C18H interaction is lost, and all of the NOE's described above are accommodated. An endo conformation in the nucleotide would also be consistent with the NOE's observed between B7H and R1-, R2-, and R3H's and between B2H and R1H and R4H (Table I). This conformation also places the ribose close to the b side-chain carbonyl and would explain the deviations in the ^{13}C chemical shifts of R2, R3, and R5 in $(CN)_2$Cbl-b-COO^- described above.

The NOESY maps also show NOE's between the C53 methyl hydrogens and B7H, B4H, and B2H, although the latter is weak. These interactions, if they are not somehow relayed, are not explained by the conformation described above. However, simple rotation of the g side chain about the C18–C60 bond permits the nucleotide to flip into a conformation in which the benzimidazole moiety is nearly parallel to and just above the corrin plane, lying quite close to the C53 and C54 methyls. The partial population of such a conformation would easily explain the interactions seen between the C53 methyl protons and the B2, B4, and B7 protons.

Thus, taken together with the previous ^{15}N NMR results,[1] the current data strongly suggest that the tuck-in species results from a hydrogen-bonded interaction between the benzimidazole B3 nitrogen and one of the g amide protons, most likely the upfield (anti[38,39]) proton, which shows a 0.27 ppm downfield shift in $(CN)_2$Cbl relative to $(CN)_2$Cbi and a 25% decrease in its chemical shift thermal gradient.[1,40,41] The NOESY spectrum of $(CN)_2$Cbl, thus far studied at a single mixing time, probably contains sufficient NOE information to accurately determine the three-dimensional conformation of the tuck-in species.

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