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Heteronuclear Nuclear Magnetic Resonance Studies of Cobalt Corrinoids. 15. The Structure of Glutathionylcobalamin: A ^1H and ^{13}C Two-Dimensional Nuclear Magnetic Resonance Study at 600 MHz[†]

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ABSTRACT: Glutathionylcobalamin (GSCbl), the complex formed between glutathione (GSH, γ-glutamylcysteinylglycine) and aquacobalamin (H_2OCbl), has been implicated as an intermediate in the pathway for the formation of the cobalamin coenzymes. In chemical model studies, GSCbl has been shown to be a substrate for methylcobalamin formation in the presence of *S*-adenosylmethionine and a thiol reductant. Although GSCbl was first described in 1964, the structure of this compound, particularly the site of GSH coordination, has been unknown. GSCbl was prepared by reacting GSH (5-fold molar excess) with H_2OCbl in 0.1 M sodium phosphate (pH 6.5) and was purified by gel-permeation chromatography on a Bio-Gel P2 polyacrylamide column. By use of a combination of homonuclear [homonuclear *J*-correlated spectroscopy (COSY), homonuclear Hartmann–Hahn spectroscopy (HOHAHA), and absorption-mode nuclear Overhauser effect spectroscopy (NOESY)] and inverse detected heteronuclear [^1H-detected heteronuclear multiple-quantum coherence (HMQC) and ^1H-detected multiple-bond heteronuclear multiple-quantum coherence (HMBC) spectroscopies] two-dimensional NMR methods at 600 MHz, the complete ^1H and ^{13}C NMR spectra of GSCbl have now been assigned. Comparison of the ^1H and ^{13}C NMR chemical shifts of the GS moiety of GSCbl to those of free GSH and GS^- shows that by far the largest differences occur at the cysteine α and β positions. This result strongly suggests that GSH is coordinated to the cobalt atom in GSCbl via the cysteine sulfur atom.

Glutathionylcobalamin (GSCbl,[1] Figure 1), the complex between glutathione (GSH, γ-glutamylcysteinylglycine) and aquacobalamin (H_2OCbl) was first described in 1964 by Wagner and Bernhauer (1964). Subsequent work has shown that in the presence of thiols, GSCbl is converted to methylcobalamin by treatment with CH_3I or *S*-adenosylmethionine (Jacobsen & Green, 1985, Pezacka et al. 1988) and that GSCbl is a substrate for cobalamin reductase (Pezacka et al., 1990a) and serves as a precursor for 5′–deoxyadenosylcobalamin (AdoCbl, coenzyme B_{12}) using a rabbit spleen extract (Pezacka et al. 1990b). As evidence has also been obtained that GSCbl is a naturally occurring form of vitamin B_{12} in mammalian cells (Jacobsen et al., 1987; Pezacka et al., 1990b; Jacobsen & Green, 1986), GSCbl may well be the natural precursor of the cobalamin coenzymes.

Despite the probable importance of GSCbl, the structure of this vitamin B_{12} derivative, particularly the site of GSH coordination to the metal atom, has never been determined.

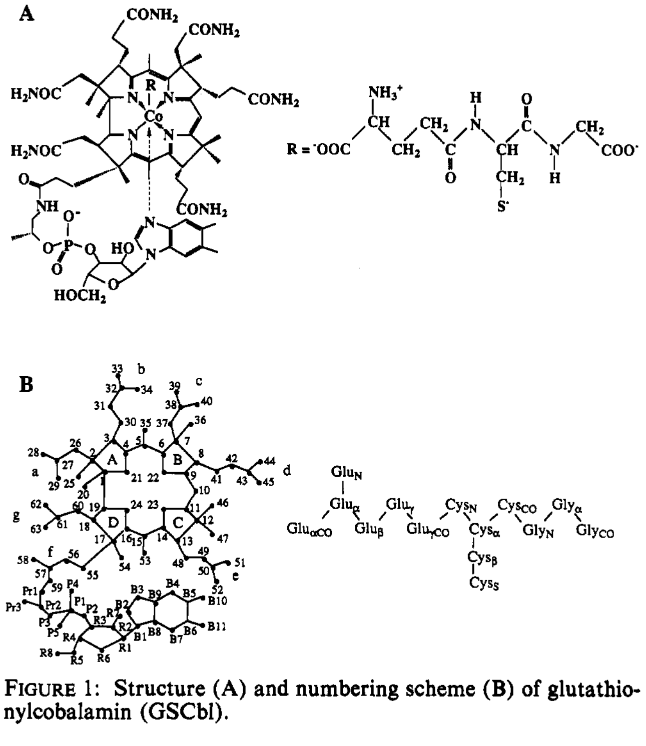

FIGURE 1: Structure (A) and numbering scheme (B) of glutathionylcobalamin (GSCbl).

While Wagner and Bernhauer (1964) assumed that GSH was coordinated to cobalamin via the cysteine sulfur, no evidence of any kind was presented for this structure. We have consequently attempted to use NMR spectroscopy to determine the site of GSH coordination in GSCbl.

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[1] Abbreviations: GSH, glutathione (γ-glutamylcysteinylglycine); GSCbl, glutathionylcobalamin; H_2OCbl, aquacobalamin; AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); CNCbl, cyanocobalamin (vitamin B_{12}); AdePrCbl, adeninylpropylcobalamin; CNCbl–*b*–COO⁻, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamic acid *a,c,d,e,g*-pentaamide (cyanocobalamin *b* monocarboxylic acid); CNCbl–*d*–COO⁻, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamic acid *a,b,c,e,g*-pentaamide (cyanocobalamin *d* monocarboxylic acid); CNCbl–*e*–COO⁻, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamic acid *a,b,c,e,g*-pentaamide (cyanocobalamin *e* monocarboxylic acid); TSP, (trimethylsilyl)propionate.

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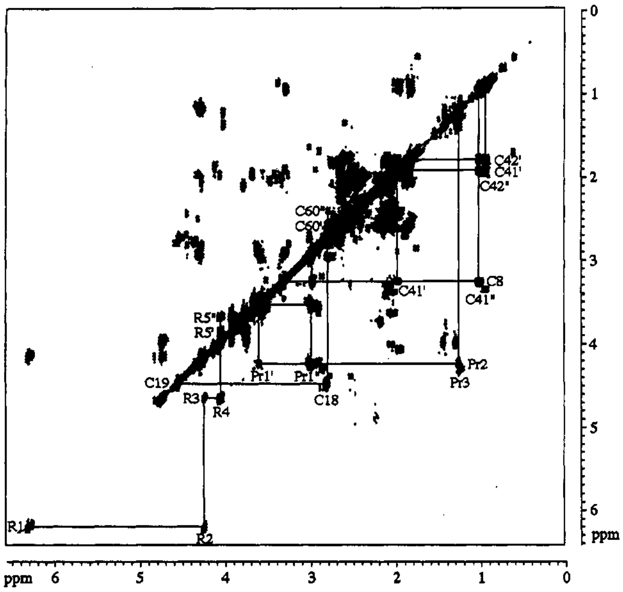

FIGURE 2: Double quantum filtered phase sensitive *J*-correlated (COSY) spectrum of GSCbl showing spin–spin connectivities in the ribose, Pr1–Pr3, C8–C41–C42, and C18–C19–C60 spin systems.

By use of modern two-dimensional NMR methodologies, including homonuclear *J*-correlated spectroscopy (COSY; Aue et al., 1976; Bax & Freeman, 1981), homonuclear Hartmann–Hahn spectroscopy (HOHAHA; Braunschweiler & Ernst, 1983; Davis & Bax, 1985a,b; Bax & Davis, 1985), and absorption-mode nuclear Overhauser effect spectroscopy (NOESY, Jeener et al., 1979; Macara & Ernst, 1980), along with the inverse-detected heteronuclear methodologies heteronuclear multiple-quantum coherence (HMQC; Müller, 1979; Bax & Subramanian, 1986) and multiple-bond heteronuclear multiple-quantum coherence (HMBC; Bax & Summers, 1986; Summers et al., 1986), the ^{1}H and ^{13}C NMR spectra of a number of cobalt corrinoids have been completely assigned, including AdoCbl (Summers et al., 1986) and its base-off form (Bax et al., 1987) and axial nucleotide-free (cobinamide) analog (Pagano et al., 1989), dicyanocobalamin (Brown et al., 1991), the *b* and *e* monocarboxylic acid derivatives of cyanocobalamin (CNCbl, Pagano & Marzilli, 1989), analogs of AdoCbl in which the 5′-deoxyadenosyl moiety is in the "lower" axial ligand position (Brown & Zou, 1992), and the AdoCbl analog adeninylpropylcobalamin (Pagano et al., 1991). Despite the enhanced dispersion engendered by the use of two-dimensional methodologies, both the ^{1}H and portions of the ^{13}C NMR spectra of cobalt corrinoids are sufficiently crowded to make the absolute assignment of these spectra significantly challenging. For instance, AdoCbl displays 72 ^{13}C resonances and 54 ^{1}H resonances, with 46 of the latter falling between 0.5 and 4.7 ppm (Bax et al., 1987). In the ^{13}C spectra of cobalt corrinoids, a crowded methylene region (with six resonances frequently falling within <1.0 ppm) and a downfield region (ca. 177–181 ppm) containing 10 resonances attributable to carbonyl carbons and corrin methines offer substantial challenges. This situation is exacerbated in GSCbl due to the four additional methylene groups and four additional carbonyl carbons of the GSH moiety. We have consequently collected two-dimensional NMR data on GSCbl at 600 MHz which has permitted unambiguous assignment of the ^{1}H and ^{13}C NMR spectra as described below.

EXPERIMENTAL PROCEDURES

GSCbl was prepared as follows. H_2OCbl (500 mg, 0.362 mmol) was dissolved in 10.0 mL of 50 mM sodium acetate, pH 5.0, and 556 mg (1.81 mmol) of GSH was added under dim red illumination. The reaction mixture was stirred for 60 min at room temperature. GSCbl was separated from excess GSH on a 2.5 × 100-cm column of Bio-Gel P2 (200–400 mesh) which had been equilibrated with 50 mM sodium acetate (pH 5.0) and was eluted at a flow rate of 1.0 mL/min. The leading dark band of GSCbl was collected (45 mL) and lyophilized. Subsequent fractions containing excess GSH, as determined by testing with Ellman's reagent (Ellman, 1959), were discarded. The purity of the resulting GSCbl was greater than 98% as determined by HPLC (Jacobsen et al., 1986). To avoid buffer salts in the lyophilized product, GSCbl could also be prepared in HPLC-grade water and purified on a Bio-Gel P2 column that had been equilibrated with water.

A one-dimensional ^{13}C NMR spectrum of GSH was obtained on a Nicolet NT 200 NMR spectrometer operating at 50.311 MHz using a 40 mM D_2O solution (in 25 mM potassium phosphate buffer, pD 6.5) containing TSP as an internal chemical shift reference. A one-dimensional ^{13}C NMR spectrum of GSCbl was obtained on a 12 mM sample (in the same solvent) on a Bruker MSL 300 NMR spectrometer operating at 75.470 MHz. A one-dimensional ^{1}H NMR spectrum of GSH and a one-dimensional ^{13}C NMR spectrum of GS$^-$ (pD 11.5 in 50 mM phosphate buffer) were obtained on a GE QE300 NMR spectrometer. GSH ^{1}H and ^{13}C NMR spectra were assigned according to the literature (Fujiwara et al., 1977, Hucherby et al, 1985). For two-dimensional NMR experiments, a sample of GSCbl was dissolved in 99.8% D_2O and evaporated to dryness four times to replace exchangeable protons with deuterons. The final dried solid was dissolved in "100%" D_2O to provide a 46 mM sample containing TSP as an internal reference.

The double-quantum-filtered phase-sensitive COSY data were collected at 600.141 MHz into a 2048 × 512 data matrix with 16 scans per t_1 value preceded by 32 dummy scans. The sweep width was 6024 Hz in both dimensions. A 1.0-s presaturation pulse was used for solvent suppression. The data were zero filled to a 2048 × 2048 matrix and apodized with a $\pi/3$ shifted sine function. The HOHAHA data were collected and processed in a similar fashion using a 80-ms mixing time. For the NOESY experiment at 600.141 MHz, data were collected into a 2048 × 512 data matrix using 32 scans per t_1 increment preceded by 32 dummy scans and a 1.2-s presaturation pulse. The sweep width was 6024 Hz in both dimensions. The $F1$ data were truncated to 1024 data points and apodized with –5-Hz Gaussian line broadening, and the F_2 dimension was zero-filled to 1024 and similarly apodized. The mixing time was 200 ms. The HMQC experiment was performed at 600.141 and 150.920 MHz with sweep widths of 6024 and 33 202 Hz, respectively. Data were collected into a 2048 × 512 data matrix using 16 scans per 75-μs t_1 increment after 32 dummy scans. A 1.0-s presaturation pulse was employed. The $F2$ dimension was truncated to 1024 data points and processed with a $\pi/2$ shifted Q-sine function. The $F1$ data were zero-filled to 1024 and processed with a $\pi/2$ shifted sine function. The HMBC data were collected similarly, except that 64 scans were acquired per t_1 increment of 150 μs. These data were processed with a $\pi/2$ shifted sine function in both dimensions.

RESULTS

The assignments of the ^{1}H and 13C NMR spectra of GSCbl were made following the general strategies previously used for cobalt corrinoid NMR assignment (Summers et al., 1986, Bax et al., 1987; Pagano et al., 1989; Brown et al., 1991). Of

Table I: Correlation Table for NMR Connectivities of Glutathionylcobalamin (GSCbl) Observed by Homonuclear *J*-Correlation (COSY), Nuclear Overhauser (NOESY), Homonuclear Hartmann–Hahn (HOHAHA), and Heteronuclear Multiple-Bond Correlation (HMBC) Methodologies[a]

^{1}H signal	COSY	HOHAHA	NOESY	HMBC
C53			C13, C18, C47, C56′, C56″, B2	C14, C15, C16
C35			C3, C25, C36, C37″, B4	C4, C5, C6
C54			C10, C13, C48′, C48″	C16, C17, C18, C56
C25			C3, C18, C19, C20, C30′, C30″	C1, C2, C4, C20
C36			C8, C35, C37′, C37″	C7, C8, C9, C37
Pr3	Pr2	Pr1′, Pr1″, Pr2	Pr1′, Pr1″, Pr2, C53, C55′	Pr1, Pr2
B11			B7	B7
B10			C36, B4	B4, B5, B6, B9
C20			C18, C19, C25, C26″, C30′, C30″, B2, B4	C1, C2, C19
C47			C10, C13, C466, C48′, C48″	C11, C12, C13
C41″	C8, C41′, C42′, C42″	C8, C41′, C42′, C42″	C8, C37″, C41′, C42′, B4	C7, C8, C43
C41′	C8, C41″, C42′, C42″	C8, C41″, C42″, C42″	C8, C37′, C41″, C42″, C42″	C8, C42, C43
C30″ [b]	C3, C31	C3, C31	C3, C20, C31, B4	C2, C32
C30′ [b]	C3, C31	C3, C31	C3, C20, C31, B4	C31, C32
C48″ [b]	C13, C49	C13, C49	C13, C49	C12, C13, C49, C50
C48′ [b]	C13, C49	C13, C49	C13, C49, C53	C12, C13, C14, C49
Glu$_{\beta}$″ [b]	Glu$_{\alpha}$, Glu$_{\gamma}$	Glu$_{\alpha}$, Glu$_{\gamma}$	Glu$_{\alpha}$, Glu$_{\gamma}$	Glu$_{\alpha}$, Glu$_{\gamma}$, Glu$_{\alpha\mathrm{CO}}$, Glu$_{\gamma\mathrm{CO}}$
Glu$_{\beta}$′ [b]	Glu$_{\alpha}$, Glu$_{\gamma}$	Glu$_{\alpha}$, Glu$_{\gamma}$	Glu$_{\alpha}$, Glu$_{\gamma}$	Glu$_{\alpha}$, Glu$_{\gamma}$, Glu$_{\alpha\mathrm{CO}}$, Glu$_{\gamma\mathrm{CO}}$
Cys$_{\beta}$″ [b]	Cys$_{\alpha}$, Cys$_{\beta}$′	Cys$_{\alpha}$, Cys$_{\beta}$′	Cys$_{\alpha}$	Cys$_{\alpha}$, Cys$_{\mathrm{CO}}$
Cys$_{\beta}$′	Cys$_{\alpha}$, Cys$_{\beta}$″	Cys$_{\alpha}$, Cys$_{\beta}$″	Cys$_{\alpha}$	Cys$_{\alpha}$, Cys$_{\mathrm{CO}}$
Glu$_{\gamma}$	Glu$_{\beta}$′, Glu$_{\beta}$″	Glu$_{\alpha}$, Glu$_{\beta}$′, Glu$_{\beta}$″	Glu$_{\alpha}$, C19	Glu$_{\alpha}$, Glu$_{\beta}$, Glu$_{\gamma\mathrm{CO}}$
C55″ [b]	C56′, C56″	C56′, C56″	Pr2	C16, C56, C57
C55′ [b]	C56′, C56″	C56′, C56″	Pr1″, Pr2, C56′, C56″	C16, C17, C56, C57
C42″	C41′, C41″, C42′	C8, C41′, C41″, C42′	C10, C36, C41′, C41″, C42′, B4	C7, C8, C41
C42′	C41′, C41″, C42″	C8, C41′, C41″, C42″	C8, C10, C41′, C41″, C42″, B4, R1	C7, C8, C41, C43
C46			C10, C13, C47, C53	C11, C12, C47
C60″	C18	C18	C18, C26′, C54, C60′	C19
C60′	C18	C18, C19	C18, C26′, C54, C60′	C17
C56″ [b]	C55′, C55″	C55′, C55″	C53, C54, C55′, R4, B2	C55, C57
C56′ [b]	C55′, C55″	C55′, C55″	C53, C55′, R4, B2	C55, C57
C49	C48′, C48″	C13, C48′, C48″	C13, C47, C48′, C48″	C13, C48, C50
C31	C30′, C30″	C3, C30′, C30″	C3, C30′, C30″	C3
C18	C19, C60′, C60″	C19	C19, C20, C25, C53, C54, C55″, C56′, C60′	C17, C19, C60, C61
C26″			C3, C25, C26′	C2, C3, C27
C26′			C3, C25, C26″	C2, C25, C27
C37″			C8, C37′	C6, C7, C8, C36, C38
C37′			C8, C36, C37″	C6, C7, C8, C38
Gly$_{\alpha}$				Gly$_{\mathrm{CO}}$, Cys$_{\mathrm{CO}}$
Pr1″	Pr1′, Pr2	Pr1′, Pr2, Pr3	Pr1′, Pr2, Pr3	Pr2, Pr3, C57
Pr1′	Pr1″, Pr2	Pr1″, Pr2, Pr3	Pr1″, Pr2, Pr3	C57
C13	C48′, C48″	C48′, C48″, C49	C46, C47, C48′, C48″, C49, C53	C11, C12, C14, C46, C48, C49
Cys$_{\alpha}$	Cys$_{\beta}$′, Cys$_{\beta}$″	Cys$_{\beta}$′, Cys$_{\beta}$″	Cys$_{\beta}$′, Cys$_{\beta}$″	Cys$_{\beta}$, Cys$_{\mathrm{CO}}$, Glu$_{\gamma\mathrm{CO}}$
Glu$_{\alpha}$	Glu$_{\beta}$′, Glu$_{\beta}$″	Glu$_{\beta}$′, Glu$_{\beta}$″, Glu$_{\gamma}$	C54, C56′, Glu$_{\beta}$′, Glu$_{\beta}$″, Glu$_{\gamma}$	Glu$_{\beta}$, Glu$_{\gamma}$, Glu$_{\alpha\mathrm{CO}}$
C3	C30′, C30″	C30′, C30″, C31′, C31″	C25, C26′, C26″, C30′, C30″, C31, C35	C1, C2, C4, C31, C26
C8	C41′, C41″	C41′, C41″, C42′, C42″	C10, C37′, C37″, C41′, C41″, C42′, C42″	C6, C7, C9, C37, C41
R5″	R4, R5′	R2, R3, R4, R5′	R3, R4, R5′	
R5′	R4, R5″	R2, R3, R4, R5″	R4, R5″	R3, R4
R2	R1, R3	R1, R3, R4, R5′, R5″	R1, R3, B7	R3
Pr2	Pr1′, Pr1″, Pr3	Pr1′, Pr1″, Pr3	C54, C55′, C55″, Pr1′, Pr1″, Pr3	Pr1, Pr3
R3	R2, R4	R1, R2, R4, R5′, R5″	R1, R2, R5″	R5
C19	C18	C18, C60′	C18, C20, C25, C55′, C60′, C60″	C1, C16, C18, C20
R4	R3, R5′, R5″	R1, R2, R3, R5′, R5″	C56′, C56″, B2, R3, R5′, R5″	R2
R1	R2	R2, R3, R4	R2, R4, B2, B7	B2
C10			C8, C42′, C42″, C46, C47	C8, C9, C11, C12
B7		B11	R1, R2, B11	B5, B9
B4			C20, C53, C56′, C56″	B6, B8
B2			C20, C53, C56′, C56″, R1, R4	B8, B9

[a] Prime and double prime indicate downfield and upfield signals, respectively, of diastereotopic methylene groups. [b] The cross peak between the geminal methylene protons is not resolved from the diagonal.

the five resonances in the downfield region (>6.0 ppm) of the ^{1}H spectrum of GSCbl, only one (at 6.33 ppm) is a doublet. This resonance can be confidently assigned to the ribose R1 proton (Figure 1). In the COSY spectrum (Figure 2), in which cross peaks occur only between the resonances of protons which are spin-coupled to each other, this resonance can then be traced through the ribose spin system to the R2, R3, and R4 proton resonances and then to the two resonances of the diastereotopic R5 protons. Similarly, in the upfield region of the ^{1}H spectrum only a single signal with an integral of three protons occurs as a doublet (1.28 ppm). This signal can be assigned to the Pr3 methyl resonance (Figure 1) as this is the only methyl group in the molecule attached to a protonated carbon. The COSY spectrum then permits assignment of the Pr2 and diastereotopic Pr1 protons from the observed Pr3H$_3$–Pr2H and Pr2H–Pr1H′ and –Pr1H″ connectivities.[2] Cross peaks within a number of other spin systems are observable in the COSY spectrum, but absolute assignment of these spin systems cannot be made in the absence of information from other spectra. The spin–spin connectivities observable in the COSY spectrum are summarized in Table I.

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[2] Prime and double prime notations indicate the downfield and upfield signals, respectively, of diastereotopic methylene protons.

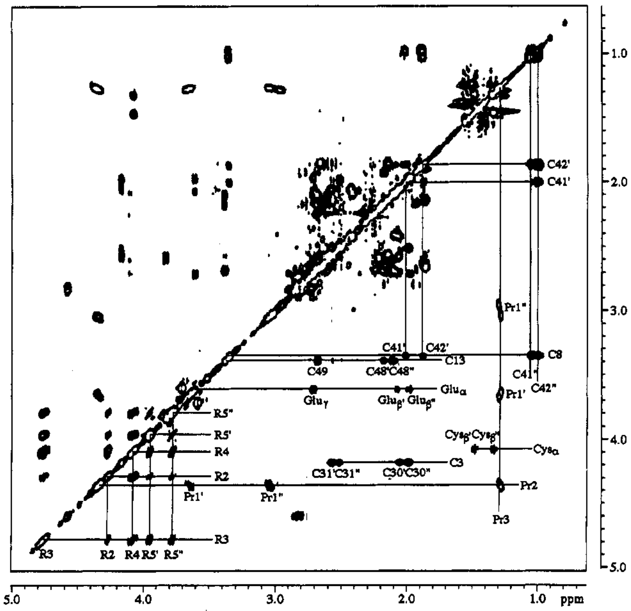


FIGURE 3: Upfield portion of the homonuclear Hartmann–Hahn (HOHAHA) spectrum of GSCbl showing relayed spin–spin connectivities in the ribose, Pr1–Pr3, C3–C30–C31, C8–C41–C42, C13–C48–C49, Cys, and Glu spin systems.

The HOHAHA spectrum (Figure 3) permits direct observation of extended spin systems, since cross peaks can arise from relayed, as well as direct, spin–spin couplings. Thus, a single proton may show cross peaks to all of the protons in the same spin system as seen in Figure 3 for the ribose and Pr spin systems. Because of these relayed connectivities, complete spin systems which are difficult to observe in the COSY spectrum because of crowding can often be readily seen in the HOHAHA spectrum. For example, in the C13–C48–C49 spin system, the C48H–C49H cross peaks are lost in a heavily overlapped region of the COSY spectrum, but this spin system is readily identifiable in the HOHAHA spectrum (Figure 3). The C3–C30–C31 spin system shows similar clarification in the HOHAHA spectrum relative to the COSY spectrum. The Cys and Glu spin systems of the GSH moiety of GSCbl are also clearly evident in the HOHAHA spectrum.

The NOESY spectrum of GSCbl (Figure 4) contains a wealth of information and is crucial in assigning protons which are not spin-coupled to other protons, as well as in confirming assignments tentatively made from spin–spin interactions observed in the COSY and HOHAHA spectra. In a NOESY spectrum, cross peaks arise from through-space nuclear Overhauser effects and can occur between protons located within 4–5 Å of one another. Thus, the assignment of the C8–C41–C42 spin system from the COSY and HOHAHA spectra is confirmed by observation of cross peaks between a proton resonating at 6.12 ppm, assignable to the C10 proton (vide infra), and the C8 and C42 protons. The C10 proton also shows cross peaks to methyl signals which also show cross peaks to the C13 resonance and other signals and are assignable to the C46 and C47 methyl resonances. These methyl groups are distinguishable since only the more downfield one showed cross peaks to the C48 methylene protons on the downward projecting *e* side chain and could hence be assigned to the downward-projecting C47 methyl (Figure 1). In a similar fashion, the most upfield three-proton singlet (0.43 ppm) was assigned to the C20 methyl group from its NOESY cross peaks with signals assignable to the C25 methyl, the C26 and C30 methylenes, the C18 proton, and the C55H′ proton. In the final analysis, very few NOESY cross peaks were observed between protons on the GSH moiety and those on the Cbl itself. The GSH Glu and Cys protons could be assigned by default from the appearance of their spin systems in the COSY and HOHAHA spectra (i.e., the Cys$_\alpha$ proton is coupled only to the Cys$_\beta$ methylene protons in the HOHAHA spectrum while the Glu$_\alpha$ proton shows relayed connectivities to the Glu$_\beta$ and Glu$_\gamma$ methylene protons), and the assignments were confirmable by the observation of NOESY cross peaks among the GSH protons (Table I).

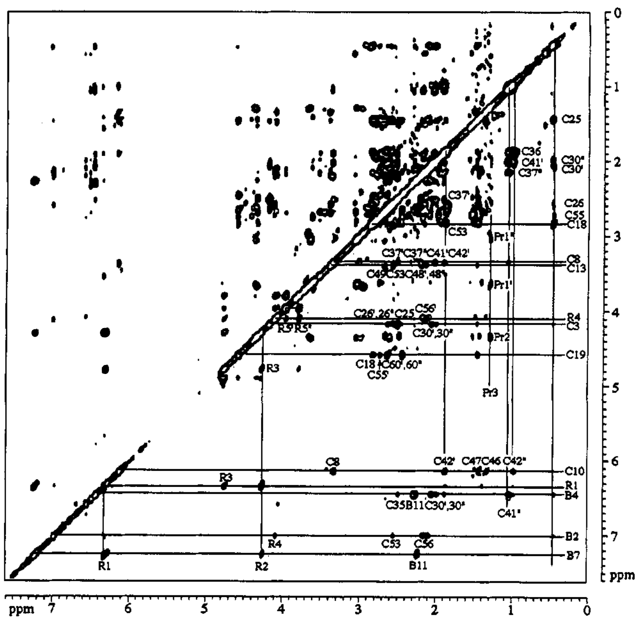


FIGURE 4: Absorption-mode nuclear Overhauser effect (NOESY) spectrum of GSCbl, showing numerous through-space connectivities.

Once the proton spectrum of GSCbl had been assigned, the carbon resonances of the protonated carbons could readily be assigned from the HMQC spectrum in which one-bond C–H spin-spin interactions produce cross peaks and the signal from protons not directly coupled to ^{13}C's are suppressed. Nonprotonated carbons were assigned by use of the HMBC spectrum in which C–H two- and three-bond couplings generate cross peaks but one-bond C–H spin-spin interactions are suppressed. With the ^{1}H assignments in hand, assignment of many of the nonprotonated carbons via the HMBC spectrum is trivial. However, because of the extreme crowding of the downfield region of the ^{13}C spectrum, with 15 resonances occurring between 173.5 and 181.5 ppm, assignment of these carbons remains a substantial challenge, even at 600 MHz. These assignments were made by observing 53 ^{13}C subspectra of the HMBC spectrum taken across the locations of the ^{1}H responses with the downfield ^{13}C signals in the two-dimensional maps. By observation of the assigned carbon resonances in each subspectrum, the protons showing cross peaks with the downfield carbon resonances could be positively identified. By integrating the information from all of the subspectra, the downfield ^{13}C resonances could be unambiguously assigned.

As an example of the use of information from multiple two-dimensional experiments to make unambiguous ^{1}H and ^{13}C assignments, the axial nucleotide resonances were assigned as follows. The resonance assigned to the R1 proton had a cross peak in the HMBC spectrum with a carbon resonance at 144.7 ppm (Figure 5C). As the ribose carbons had previously been assigned from their one-bond connectivities to the ribose protons whose chemical shifts were known from the COSY and HOHAHA spectra, the ^{13}C resonance at 144.7 ppm must be assigned to the B2 carbon. The proton attached to this carbon (6.97 ppm, from the HMQC spectrum) had

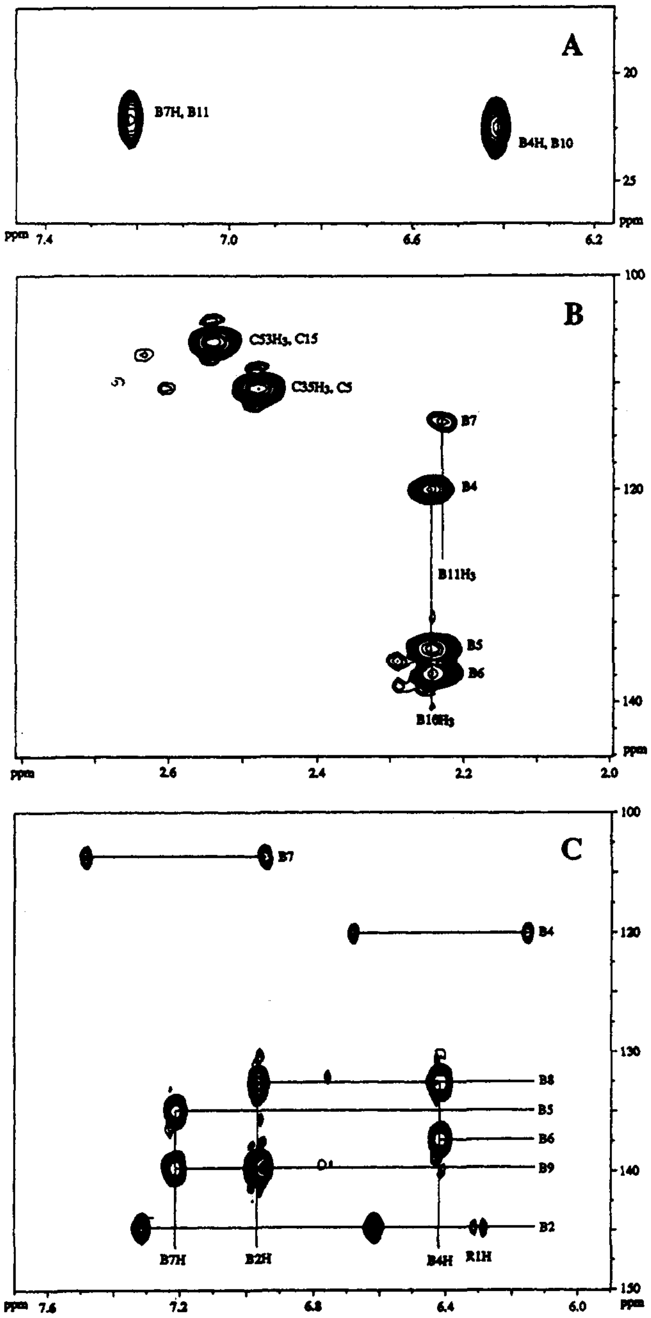
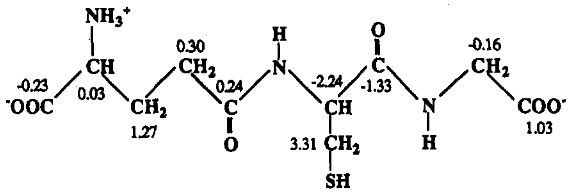


FIGURE 5: Portions of the inverse detected multiple-bond heteronuclear multiple-quantum coherence (HMBC) spectrum of GSCbl showing multiple bond ^1H–^{13}C connectivities for the B10 and B11 methyl protons (A and B) and the R1, B2, B4, and B7 protons (C). Incompletely canceled one-bond ^1H–^{13}C correlations can be seen for B2, B4, and B7 in panel C.

cross peaks with ^{13}C resonances at 132.5 and 139.5 ppm. These must therefore be the B8 and B9 resonances. One of these carbon resonances (132.5 ppm) also had a HMBC cross peak with a proton resonating at 6.42 ppm, while the other (139.5 ppm) has a cross peak with a proton resonance at 7.25 ppm (Figure 5C). These two protons must then be the B4 and B7 protons. In the NOESY spectrum, the proton at 7.25 ppm had cross peaks with the R1 and R2 protons and must therefore be assigned to the B4 proton, while the proton at 6.42 ppm had NOESY cross peaks to $C20H_3$, $C53H_3$, and the C56 methylene protons and must therefore be assigned to the B7 proton (Table I). Summers et al. (1986) have argued that since three-bond H–C couplings in aromatic systems are generally larger than two-bond H–C couplings (Hansen, 1981), the HMBC cross peaks in the benzimidazole nucleotide of cobalamins should represent three-bond H–C correlations. The carbon resonance at 132.5 ppm must then be assigned to B8, and the carbon resonance at 137.2 ppm, which also has a HMBC cross peak with the B4 proton (Figure 5C), must be assigned to B6. The carbon resonance at 139.5 ppm must then be B9. As the B7 proton also has a HMBC cross peak with a carbon resonance at 134.9 ppm, this carbon resonance must be due to B5. As seen in Figure 5A, the B4 and B7 proton resonance assignments permit distinction of the B10 and B11 carbon resonances and hence, from the HMBC spectrum, the proton resonances of these methyl groups. Figure 5B shows that the three-bond correlation rule does not hold for the methyl protons of the axial nucleotide since the B10 methyl protons show correlations to B4, B5, and B6, while the B11 methyl correlates only weakly to B7. With the completion of the assignments of the ^1H resonances of the benzimidazole nucleotide, four of the five downfield resonances in the ^1H spectrum of GSCbl are assigned. The remaining resonance (at 6.12 ppm) can then be assigned to the C10 proton and the C10 ^{13}C resonance assigned to the signal at 97.3 ppm from the HMQC correlation to the C10 proton. The NMR correlations observable in all of the two-dimensional experiments (except the HMQC experiment) are summarized in Table I, and the final assignments of the ^1H and ^{13}C NMR spectra of GSCbl are summarized in Table II.

DISCUSSION

In principle, it should be possible to determine the mode of GSH coordination to cobalamin in GSCbl from the effect of coordination of GSH on the ^1H and ^{13}C chemical shifts of this moiety. Such a comparison of the ^{13}C chemical shifts is shown below as the signed difference in ^{13}C chemical shift between GSCbl and GSH.



$$\Delta\delta_{^{13}C} = \delta_{^{13}C}(\text{GSCbl}) - \delta_{^{13}C}(\text{GSH})$$

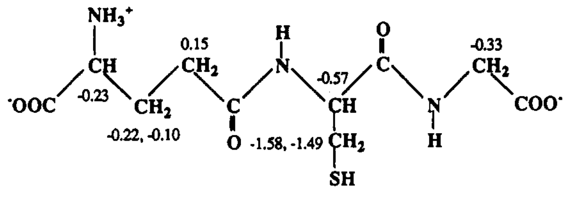
This comparison shows that the largest chemical shift differences by far occur at the Cys_α and Cys_β carbons, suggesting that coordination occurs via the GSH sulfur. However, it is surprising to find that the Cys_β carbon is shifted downfield upon coordination of GSH to cobalamin. Both the known, strongly electron-donating inductive effect of the cobalamin cobalt center (Brown et al., 1985) and the magnetic anisotropy of the cobalt atom dipole ($\Delta\chi = -14.3 \times 10^{29}$ cm^3/molecule for AdoCbl and -3.4×10^{29} cm^3/molecule for CNCbl, Brown & Hakimi, 1986) argue that the Cys_β carbon resonance should be shifted upfield upon coordination, as is the case for the Cys_α carbon resonance. Indeed, a comparison of the ^1H chemical shifts of GSCbl to those of GSH, shown below, shows that both the Cys_α and Cys_β protons are shifted upfield upon coordination and confirms that the largest chemical shift differences occur in the Cys residue of GSH.

Perhaps a more apt comparison would be between the chemical shift difference between GSCbl and GS^- and the chemical shift difference between GSH and GS^-, i.e., a comparison of the effect of GS^- coordination to cobalamin to the effect of GS^- "coordination" to the proton. These

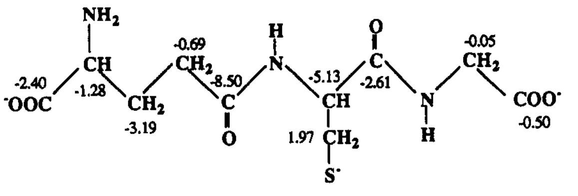
Table II: Final ^1H and ^{13}C NMR Assignments for Glutathionylcobalamin (GSCbl)[a]

atom	^{13}C chemical shift (ppm)	^1H chemical shift (ppm)	^{13}C chemical shift difference[b] (ppm)			atom	^{13}C chemical shift (ppm)	^1H chemical shift (ppm)	^{13}C chemical shift difference[b] (ppm)		
			GSCbl[c]	AdoCbl[d]	AdePrCbl[e]				GSCbl[c]	AdoCbl[d]	AdePrCbl[e]
C53	18.21	2.25	0.5	1.1	0.9	C17	61.16		−0.6	−1.1	−1.2
C35	18.26	2.48	0.3	0.3	0.4	R5	62.93	3.78, 3.93	−0.1	0.4	0.2
C54	19.28	1.43	0.9	1.2	0.9	R2	71.44	4.26	0.0	0.6	0.4
C25	19.28	1.45	−0.1	0.5	0.3	Pr2	75.50	4.34	0.0	0.5	0.3
C36	21.49	1.88	−0.1	0.1	0.0	R3	75.57	4.73	0.0	0.6	0.3
Pr3	21.49	1.28	−0.2	0.1	0.1	C19	76.60	4.57	−0.9	−0.7	−1.0
B11	21.88	2.24	−0.1	0.4	0.4	R4	84.41	4.07	−0.2	0.0	−0.3
B10	22.36	2.26	−0.1	0.0	−0.1	C1	88.48		0.8	0.8	0.1
C20	22.36	0.43	0.6	1.7	1.7	R1	89.47	6.33	−0.4	−0.2	−0.5
C47	23.83	1.43	2.1	2.1	1.6	C10	97.27	6.12	−0.2	0.2	−0.2
C41	28.64	1.04, 2.00	0.1	0.2	−0.1	C15	105.96		−0.7	0.3	−0.1
C30	28.89	1.99, 2.05	0.4	0.7	0.9	C5	110.33		0.3	−1.7	−2.0
C48	29.15	2.09, 2.17	0.6	−0.3	0.2	B7	113.64	7.25	−0.4	−0.5	−0.8
Glu_β	30.22	1.95, 2.07				B4	119.89	6.42	0.9	2.4	2.0
Cys_β	31.66	1.35, 1.46				B8	132.47		0.0	0.8	0.5
Glu_γ	34.25	2.71				B5	134.91		−0.7	−1.1	−1.5
C55	34.25	2.47, 2.65	−0.8	−0.8	−0.2	B6	137.19		−0.5	−0.9	−1.2
C42	34.35	0.98, 1.87	0.1	0.6	0.5	B9	139.54		0.3	1.8	1.4
C46	34.35	1.33	0.5	0.3	0.7	B2	144.66	6.97	0.3	0.3	0.1
C60	34.40	2.43, 2.63	−0.4	0.0	−0.1	C6	165.79		−2.1	−1.3	−2.1
C56	34.93	2.15, 2.19	0.8	0.5	0.2	C14	167.12		−1.5	−1.4	−2.0
C49	37.72	2.10, 2.15	0.5	0.9	0.9	Cys_{CO}	173.65		−1.3		
C31	37.84	2.46	0.3	0.8	0.8	C9	174.87		−1.2	−3.0	−3.8
C18	41.94	2.80	0.4	0.9	1.0	$Glu_{\alpha CO}$	176.51		−0.2		
C26	45.28	2.42, 2.62	−0.3	0.8	0.4	$Glu_{\gamma CO}$	176.67		0.2		
C37	45.75	2.20, 2.49	0.2	−0.3	−0.3	C57	177.67		0.3	0.8	0.3
Gly_α	45.99	3.65				C38	178.32		0.6	0.1	−1.0
Pr1	47.88	3.04, 3.63	−0.1	−0.2	−0.2	C61	178.32		0.0	0.7	0.1
C2	49.43		−0.4	−0.3	−0.4	Gly_{CO}	178.65		1.0		
C12	49.62		−1.1	−1.2	−1.2	C27	178.99		0.5	0.8	−0.2
C7	53.69		−0.3	−0.9	−1.1	C11	179.13		−0.3	−1.8	−3.0
C13	55.67	3.37	−0.6	−0.4	−0.3	C43	179.87		0.1	0.6	0.2
Cys_α	55.94	4.00				C16	180.48		−1.0	−2.8	−4.2
Glu_α	56.98	3.60				C32	180.65		0.2	0.6	0.0
C3	58.55	4.15	−0.3	−0.4	−0.8	C50	180.87		0.1	0.1	0.0
C8	58.89	3.33	0.6	−0.7	−1.1	C4	181.43		−1.2	−3.9	−4.6

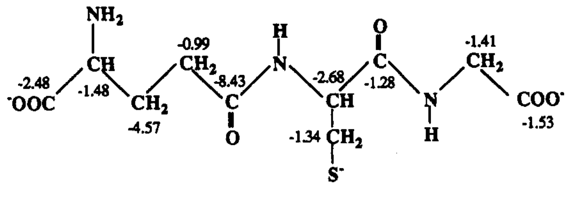
[a] In D_2O, pH 6.5, chemical shifts relative to internal TSP. [b] Difference in ^{13}C chemical shift between the RCbl and CNCbl [Pagano & Marzilli (1989) as corrected by Brown et al. (1991)]. [c] This work. [d] Summers et al. (1986). [e] Pagano et al. (1991).



$\Delta\delta_{^1H} = \delta_{^1H}(GSCbl) - \delta_{^1H}(GSH)$



$\Delta\delta_{^{13}C} = \delta_{^{13}C}(GSCbl) - \delta_{^{13}C}(GS^-)$



$\Delta\delta_{^{13}C} = \delta_{^{13}C}(GSH) - \delta_{^{13}C}(GS^-)$

comparisons are shown below as the signed difference in ^{13}C chemical shift between GSCbl and GS^- and between GSH and GS^-, where the chemical shifts of GSH and GS^- were determined at pD 6.50 and 11.50, respectively.[3] This comparison is complicated by the fact that in addition to complete deprotonation of the Cys -SH at pD 11.30 ($pK_a = 9.1$; Huckerby et al., 1985) the Glu α-NH_3^+ is also completely deprotonated under these conditions ($pK_a = 9.5$; Hucherby et al., 1985). Nonetheless, the comparison shows that the effect of protonation of GS^- and of coordination of GS^- to cobalamin are fundamentally different in terms of the direction in which the Cys_β carbon resonance is displaced. Perhaps the significance of this observation lies in a fundamental difference in the bonding interactions of a thiolate with the proton and with a cobalamin chelated cobalt center, possibly suggesting the importance of metal-to-sulfur back bonding in the GSCbl complex. While the final answer to this question will require further investigation by other techniques, we nonetheless can conclude that GSH coordinates to cobalamin via its cysteine sulfur.

Unfortunately, very few NOE interactions between the GSH moiety and the cobalamin are observed in the NOESY spectrum of GSCbl (Table I), at least at the mixing time employed here (200 ms). The only such interactions detected

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[3] It seems that the assignments of the $Glu_{\alpha CO}$ and $Glu_{\gamma CO}$ in Huckerby et al. (1985) may be reversed since the latter would be expected to undergo a larger chemical shift change than the former when the pH is raised and the α-NH_3^+ ionizes.

were between the Glu$_\gamma$ protons and the C19 proton, between the Glu$_\alpha$ proton and the C54 methyl group, and between the Glu$_\alpha$ proton and one of the C56 methylene protons. These interactions suggest a conformation for the glutathionyl moiety in which the Glu residue lies directly above the D ring–C15 junction (Figure 1).

Finally, the complete NMR assignments of GSCbl permit a valuable NMR comparison among base-on cobalamins, as this is the fourth such species for which unambiguous NMR assignments have been made. The NMR spectra of AdoCbl (coenzyme B$_{12}$), the first cobalamin for which rigorous NMR assignments were made, were assigned by Summers et al. in 1986. More recently, the NMR assignments of an AdoCbl analogue, adeninylpropylcobalamin (AdePrCbl), in which the ribose moiety of the adenosyl group is replaced by a propylene chain, have been reported (Pagano et al., 1991). The complete ^{13}C NMR spectrum of CNCbl (vitamin B$_{12}$), which may be considered as the benchmark compound of the series, was originally assigned by Bratt and Hogenkamp (1984), using classical methods including labeling studies, chemical shift arguments, and observation of the ^{13}C spectra of various analogs. Pagano and Marzilli (1989) subsequently made unambiguous ^{1}H and ^{13}C NMR assignments of the CNCbl analogs in which the *b* or *e* propionamide side chains had been hydrolyzed to carboxylates (CNCbl–*b*–COO$^-$ and CNCbl–*e*–COO$^-$) by two-dimensional NMR spectroscopy. By analogy, these authors then reassigned the ^{13}C spectrum of CNCbl, correcting a number of misassignments previously made by Bratt and Hogenkamp (1984). However, a subsequent NMR study (Brown et al., 1991) of the *d* monocarboxylic acid derivative of CNCbl (i.e., CNCbl–*d*–COO$^-$) showed that several misassignments had been made by Pagano and Marzilli (1989) as well. Consequently, the ^{13}C chemical shifts of CNCbl used in the following comparisons are those of Pagano and Marzilli (1989) as corrected by Brown et al. (1991). These comparisons are shown in Table II as the signed difference in chemical shift of the ^{13}C resonances of GSCbl, AdoCbl, and AdePrCbl and those of CNCbl.

These data permit the observation of changes in equatorial chemical shifts in response to changes in a single axial ligand, i.e., a spectroscopic cis effect. The corrin ring carbons C4, C9, C11, and C16, the most downfield of the ring carbon resonances, show the largest sensitivity to upper axial ligand substitution, their ^{13}C chemical shifts varying by as much as 4.6 ppm across the series. Ring carbons C5, C6, and C14 form the next most sensitive group, whose chemical shifts vary by as much as 2.0 ppm. A third group of ring carbons, C7, C8, C12, C17, C18, and C19, show much less sensitivity to axial ligand substitution, their chemical shifts varying by only 1.2 ppm at most, while a fourth group of ring carbons, C1, C2, C3, C10, C13, and C15, show little or no sensitivity to the axial ligand, their chemical shifts varying by $<$1.0 ppm. It seems unlikely that these effects can be completely attributed to inductive effects of the upper axial ligand since, for instance, the C5 methine carbon falls in the second most sensitive group (with variations as high as 2.0 ppm) while the apparently electronically equivalent C15 methine is insensitive to the axial ligand (variations $\leq$0.7 ppm). This suggests that changes in corrin ring conformation accompanying axial ligand substitution cause changes in ring carbon hybridization.

Chemical shift changes among several methyl carbons across the series of base-on cobalamins also suggest conformational differences among these compounds. Thus, the C20, C47, C53, and C54 methyl groups show ^{13}C chemical shift differences across the series of at least 1.1 ppm and as large as 2.1 ppm. These chemical shift differences cannot be attributed to inductive effects as two of these methyls, C20 and C53, are attached to ring carbons showing the least sensitivity to axial ligand substitution, while the other two methyls are attached to ring carbons showing the next least sensitivity. In addition, only one of these methyl groups, C54, projects upward from the corrin plane, suggesting that the chemical shifts of the other three are unlikely to be significantly affected by direct interactions with the upper axial ligand.

In summary, the complete, unambiguous assignment of the ^{1}H and ^{13}C NMR spectra of GSCbl have permitted the determination of the site of coordination of the glutathionyl moiety by comparison to the NMR spectra of free glutathione. It is thus shown that glutathione coordinates to the cobalamin cobalt atom via the cysteine sulfur. NOE interactions between protons on the glutamate residue of the glutathionyl moiety and cobalamin protons suggest that this residue lies directly above the D ring–C15 junction and that the glutamate α-amino group may be hydrogen bonded to the *f* side-chain carbonyl oxygen. Finally, comparison of the ^{13}C NMR spectra of four base-on cobalamins whose NMR assignments are known with certainty suggests the existence of important differences in corrin ring conformation across the series of compounds.

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