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Structural Determination of 5′-OH α-Ribofuranoside Modified Cobalamins via ^{13}C and DEPT NMR

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Received January 14, 2003

Abstract: A protocol for the rapid NMR characterization of cobalamin (vitamin B_{12}) analogues with 5′-hydroxy-α-ribofuranoside modification is reported. The structure of cyanocobalamin in DMSO-d_6 has been assigned using COSY, NOESY, HSQC, and HMBC NMR methods. The robust precision of ^{13}C NMR assignments in DMSO-d_6 allows for the rapid structural determination of 5′-hydroxy-α-ribofuranosyl cyanocobalamin derivatives with solely 1-D ^{13}C and DEPT NMR spectra and only 10 mg of derivatized cobalamin. Using this method, the ^{13}C NMR resonances of four cobalamin analogues were determined with the most significant variance of ^{13}C chemical shifts occurring in the α-ribofuranoside ring. In DMSO-d_6, cobalamin concentrations greater than 30 mM can be achieved for an improved signal-to-noise ratio.

A facile NMR analysis protocol has been developed to determine the structure of 5′-hydroxyl-modified cobalamin (vitamin B_{12}) analogues. The ribofuranose 5′-hydroxyl is an especially attractive site for the covalent modification of cobalamin and the attachment of chemotherapeutic drugs or imaging agents for targeted delivery to cancer cells. Unambiguously proving chemical modification of the primary 5′-ribofuranosyl-OH in preference to the less reactive, but still available, secondary 2′-ribofuranosyl-OH has been difficult in the absence of a crystal structure for each analogue. A robust method of structural analysis must be sufficiently sensitive to detect modification at other locations on cobalamin (including the 2′-OH of the α-ribofuranoside) and versatile enough to encompass cobalamin derivatives of vastly different solubility when a hydrophobic chemotherapeutic or fluorescent imaging agent is attached. Herein, we report the synthesis and NMR characterization of four 5′-OH α-ribofuranoside modified cobalamin conjugates. The central feature of the structural analysis protocol is to acquire 1-D ^{13}C and distortionless enhancement by polarization transfer (DEPT) NMR spectra in DMSO-d_6. The inherent complexity of the aliphatic region of the ^1H NMR of cobalamin makes ^{13}C NMR the preferred method to quickly characterize cobalamin analogues due to the sensitivity of ^{13}C chemical shifts resulting from covalent modifications and the unique chemical shift of each carbon resonance. In addition, the choice of DMSO-d_6 as the solvent allows for the accommodation of cobalamin analogues of varying solubility that becomes an issue upon the conjugation of extremely nonpolar chemotherapeutic or fluorescent imaging agents.

Bioconjugate derivatives of cobalamin with cytotoxic, fluorescent, and radiographic imaging agents are being evaluated as vehicles to target the delivery of cancer drugs and diagnostic agents in vivo.[1−6] Cancer cells have an increased requirement for cobalamin relative to non-neoplastic cells, as cobalamin is an essential cofactor for the enzyme methionine synthase. This enzyme is required for the conversion of dUMP to dTMP prior to DNA synthesis. Since cobalamin is a micronutrient, the human body has developed a sophisticated transport pathway to sequester and to maintain cobalamin at appropriate physiological levels.[7,8] In addition, Collins and Hogenkamp have shown that rapidly dividing cells up-regulate cobalamin binding receptors during DNA replication.[3,4] By taking advantage of the body's own machinery to concentrate and to maintain cobalamin levels in vivo, combined with the up-regulation of cobalamin binding receptors in neoplastic cells, cobalamin has the potential to be a site-selective drug delivery vehicle for the treatment, imaging, and diagnosis of cancer.

A number of synthetic handles are available on cobalamin for the attachment of chemotheraputic or tumor imaging agents. The *β*-ligand to cobalt, the *b*, *c*, *d*, and *e* side chain amides, as well as the 5′-OH (and to a lesser extent the 2′-OH) of the α-ribofuranoside, are synthetically plausible locations for the attachment of chemotherapy agents.[2,9,10] However, due to the light sensitivity of the carbon−cobalt bond,[11] and the low synthetic yields of the *b*, *c*, *d*, and *e* side chain acids,[12] the 5′-OH of the α-ribofuranoside is the most attractive handle for the attachment of chemotherapy and imaging agents with high synthetic yields, while maintaining high binding efficiency to transcobalamin II and light stability.[9]

The complete NMR characterization of CNCbl in D_2O has been reported previously.[13−19] However, the low solubility of CNCbl and its analogues in D_2O (100 mg/8

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10.1021/jo0340399 CCC: $25.00
Published on Web 08/09/2003

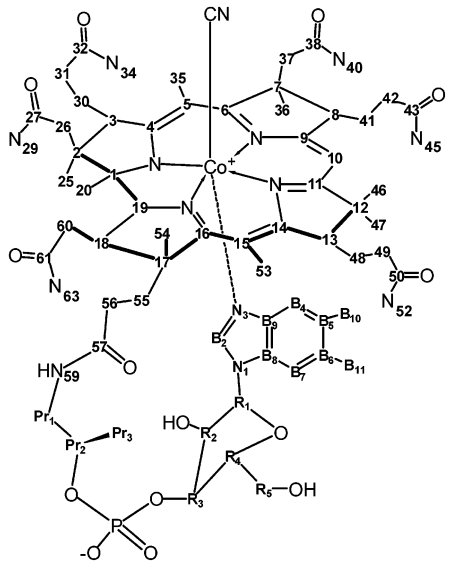

FIGURE 1. Atomic numbering scheme of CNCbl.

mL) and the further decrease in solubility of cobalamin upon conjugation to extremely nonpolar chemotherapeutic agents make the acquisition and analysis of NMR experiments in D_2O lengthy. In the present study, we selected DMSO-d_6 as the NMR solvent to obtain a higher concentration of cobalamin (>400 mg/8 mL), thereby enabling the acquisition of 1-D NMR data in a very short time with a high signal-to-noise ratio. In addition, the choice of DMSO-d_6 not only enables the observation of protons that would otherwise undergo exchange in protic solvents, but also allows for the analysis of pH-sensitive chemotherapeutic agents conjugated to cobalamin.

Only partial NMR characterization of CNCbl in DMSO-d_6 has been reported to date.[13,20] The complete characterization of the parent CNCbl molecule in DMSO-d_6 by 1- and 2-D NMR methods allows for the rapid structural characterization of cobalamin analogues solely by 1-D ^{13}C and DEPT NMR methods. Extensive characterization of subsequent cobalamin analogues by COSY, NOESY, HSQC, and HMBC multidimensional NMR methods is not necessary.

NMR Characterization of CNCbl in DMSO-d_6. The structure of underivatized CNCbl (Figure 1) was determined with COSY, NOESY, HSQC, and HMBC multidimensional NMR experiments in DMSO-d_6. Since the complete structure of cobalamin in D_2O has been published previously, this discussion will focus on areas of interest or controversy that were encountered in assigning the NMR structure of cobalamin in DMSO-d_6.

Characterization of the Corrin Ring. A classic alkene proton shift was observed at 5.9 ppm in the ^1H NMR. From this, HSQC correlations were used to find the C10 carbon of the corrin ring at 93.38 ppm. C10 is the only trisubstituted olefinic carbon in CNCbl. This provides the starting point to characterize the rest of the corrin ring by HSQC, HMBC, DEPT, COSY, and NOESY correlations (see Table 1). The assignment of side-chain amide carbons is less robust because of a lack of multiple correlations to these carbons. However, ^1H and ^{15}N NMR characterization of the amide side chains has been reported for CNCbl in DMSO-d_6.[20]

Characterization of the Propyl Side Chain. The propyl side chain of CNCbl was assigned based upon the unique COSY correlation between the Pr3 methyl and the Pr2 methylene carbons, which is the only such occurrence in CNCbl. From these assignments, Pr1 can be easily assigned based on HMBC, COSY, and NOESY correlations. A COSY correlation is also observed between Pr1 and N59 to clearly identify the amide proton.

Characterization of the Dimethylbenzimidazole. A good location to begin the assignment of the dimethylbenzimidazole moiety is B2. The dimethylbenzimidazole B2 is a phenolic methine carbon with a large downfield shift of 142.34 ppm due to neighboring heteroatoms.

From this assignment, the remaining dimethylbenzimidazole moiety can be easily assigned from HMBC correlations. Although the B10 and B11 methyl groups have similar ^{13}C shifts, they can be differentiated based on the relative strength of their HMBC correlations to the B4 and B7 methine carbons.

Characterization of the α-Ribofuranoside. The remaining five carbons of the α-ribofuranoside are assigned starting from R5, which is the only unassigned methylene in the expected sugar region. Following the assignment of R5, COSY correlations can be used to assign the remainder of the α-ribofuranoside and complete the assignment of cyanocobalamin. NOESY correlations were also observed between R1, R2, and R3, providing additional support for the assignment of the α-ribofuranoside. Additional NOESY correlations between R1 and B7, as well as R4 and B2, provide spatial information about the relative position of the dimethylbenzimidazole and are used to differentiate between the two halves of the pseudo-C_2 symmetric dimethylbenzimidazole.

CNCbl-5′-OH Modification. Activation of the 5′-hydroxyl is preformed according to a modified literature procedure by treatment of CNCbl with 1,1′-dicarbonyldi-(1,2,4-triazole) (CDT).[10] The more nucleophilic primary 5′-hydroxyl reacts with CDT, resulting in electrophilic intermediate **1,** which can be isolated and stored for modification by a variety of nucleophiles (see Scheme 1). Reaction with glycine methyl ester followed by saponification yields **2**, resulting in a convenient carboxylic acid handle. Diamines such as 1,6-diaminohexane and 4,7,10-trioxa-1,13-tridecanediamine also can be used to react with **1** under dilute reaction conditions to yield a nucleophilic amine handle on compounds **3**, **4**, and **5**, respectively.

5′-Modified Cobalamin Characterization. Assignment of 2. A direct comparison of the ^{13}C NMR shifts of **2** with fully characterized CNCbl leads to the rapid assignment of the majority of the resonances. The new resonance at 156.06 ppm is assigned to the newly formed carbamate. The methylene carbon has a resonance at 44.62 ppm, whereas the carboxylic acid has a chemical shift of 171.71 ppm. As expected, the only significant

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TABLE 1. NMR Assignments of CNCbl in DMSO-d_6

assignment	^{13}C shift	DEPT	HSQC	HMBC	COSY	NOESY
C4	179.43			C35		
C16	178.22			C53, C54		
C11	175.02			C10, C13, C46, C47		
C9	173.58			C10		
C50[a]	173.40					
C32	173.14					
C43[a]	172.71					
C27[a]	172.47			C26		
C38[a]	172.34					
C61	171.04			C60		
C57	170.80			C55		
C6	165.20			C35, C36		
C14	164.51			C13, C53		
B2	142.34	CH	7.01	R1		C48, C53, R4
B9	136.27			B2, B7		
B6	132.40			B4, B10		
B5	131.06			B7, B11		
B8	129.57			B2, B4		
B4	116.23	CH	6.45	B10		B10, C36, C41
B7	111.35	CH	7.31	B11		B11, R1, R2
CN	110.04					
C5	105.82			C35		
C15	102.94			C13, C53		
C10	93.37	CH	5.90	C8		C8, C46, C47
R1	85.39	CH	6.27		R2	B7, R2, R3
C1	84.30			C19, C20, C25		
R4	81.41	CH	3.88		R3, R5	B2, R3, R5
C19	74.84	CH	3.93	C20	C18	C26, C54, C60
R3	74.50	CH	4.49		R2, R4	R1, R2, R4
Pr2	70.62	CH	4.10		Pr1, Pr3	Pr1, Pr3
R2	69.08	CH	3.90		R1, R3	B7, R1, R3
R5	61.75	CH_2	3.57	R3	R4	R4
C17	58.57			C54		
C3	54.97	CH	4.67	C25	C30	C26, C35
C8	54.09	CH	3.70	C10, C36, C37	C41	C10, C37, C41
C13	52.87	CH	3.13	C46, C47, C49	C48	C46, C48, C53
C7	50.23			C8, C36		
C12	47.33			C10, C46, C47		
C2	46.53			C20, C25, C30		
Pr1	45.35	CH_2	2.68, 3.57	Pr3	N59, Pr2	C8, Pr2
C37	42.08	CH_2	1.74, 2.44	C36		N45
C26	41.54	CH_2	2.03, 2.16	C25		C3, C19, C30
C18	38.12	CH	2.74	C54	C19, C60	C20, C56
C31	35.06	CH_2	2.22		C30	C25, C30
C49	33.64	CH_2	2.29, 2.39		C48	
C60	31.79	CH_2	2.46		C18	C19, C54, C55
C56	31.65	CH_2	2.46		C55	C18, C54, C55
C46	31.56	CH_3	1.05	C13, C47		C10, C13, C47
C55	31.30	CH_2	1.75	C54	C56	C56, C60
C42	29.85	CH_2	2.01, 2.64		C41	C41
C48	27.03	CH_2	1.62, 1.93		C13, C49	B2, C13, C47
C41	25.80	CH_2	0.92, 1.78		C8, C42	B4, C8, C36, C42
C30	25.46	CH_2	1.66, 1.78		C3, C31	C25, C26, C31, C35
Pr3	20.19	CH_3	1.04		Pr2	Pr2
C47	19.83	CH_3	1.33	C46		C10, C46, C48
B10	19.80	CH_3	2.16	B4		B4
B11	19.72	CH_3	2.16	B7		B7
C20	19.43	CH_3	0.28			C18, C25
C36	18.59	CH_3	1.70	C37		B4, C35, C41
C54	16.49	CH_3	1.22	C18		C19, C53, C56, C60
C25	16.31	CH_3	1.17			C20, C30, C31
C35	15.52	CH_3	2.47			C3, C30, C36
C53	14.88	CH_3	2.43			B2, C13, C54

[a] Assignment is less-rigorous in the absence of chemical derivatization at this position.

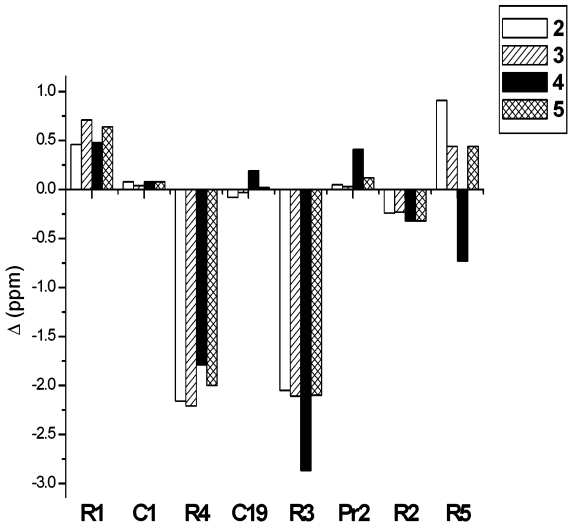
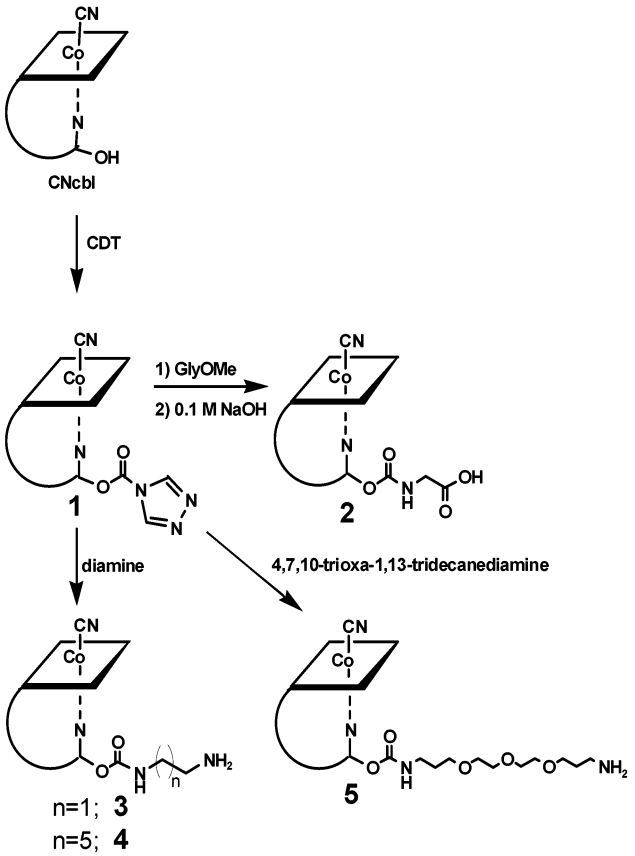


FIGURE 2. ^{13}C NMR chemical shifts of key carbons of cobalamin analogues relative to CNCbl.

SCHEME 1. Synthesis of 5′-Modified Cobalamins



variation in chemical shifts occurred in the sugar region of the spectra. Assignment of the α-ribofuranosyl carbons of cobalamin is accomplished with the help of a DEPT experiment to first identify the R5 methylene at 62.66 ppm. The remaining methine carbons at 85.85, 79.25, 72.45, and 68.84 ppm are assigned as R1, R4, R3, and R2, respectively. A plot of key carbon resonance values, with respect to CNCbl in the α-ribofuranoside region (60 to 90 ppm), demonstrates the large shift in resonances for the majority of the α-ribofuranosyl carbons (1−3 ppm), with virtually no change in the rest of the molecule

(0.02−0.32 ppm). See Figure 2. This provides evidence that only the 5′-OH of the α-ribofuranoside is chemically modified in the synthesis of compound **2**.

Assignment of 3. The fully characterized CNCbl leads to the rapid assignment of the ^{13}C NMR resonances of compound **3**. The newly formed carbamate is assigned to the new quaternary carbon observed at 156.43 ppm, and the two new methylene carbons are assigned to the resonance at 48.00 and 41.35 ppm. Consistent with compound **2**, only the α-ribofuranosyl carbons of compound **3** show a variation greater than 0.7 ppm when compared to CNCbl. After identifying the R5 methylene carbon resonance at 62.19 ppm from the DEPT spectra, the remaining resonances at 86.11, 79.20, 72.39, and 68.85 ppm are assigned to the α-ribofuranosyl methylene carbons R1, R4, R3, and R2, respectively.

Assignment of 4. Similarly, a comparison of the observed ^{13}C NMR chemical shifts of **4** with the CNCbl assignments allows for the complete characterization of analogue **4**. Once again, only small changes in chemical shift values are observed for the majority of the carbons (0.01−0.74 ppm). The new resonance at 156.55 ppm is assigned to the newly formed carbamate. Additional methylene resonances at 39.77, 38.13, 28.11, 26.69, 24.96, and 24.72 ppm constitute the new carbons from the addition of the hexane tether. Again, the only significant variation in chemical shift occurs in the α-ribofuranoside region of the spectra. The DEPT experiment is useful for identifying the R5 methylene resonance at 61.02 ppm. The remaining resonances at 85.87, 79.62, 71.63, and 68.76 ppm are assigned to the α-ribofuranosyl methine carbons R1, R4, R3, and R2, respectively.

Assignment of 5. The ^{13}C NMR shift values of **5** are assigned in a similar manner as compounds **2**−**4**. Comparison of the ^{13}C resonances of **5** to that of CNCbl allows for the complete assignment of ^{13}C NMR resonances of the molecule. As seen before, only small changes in chemical shift values are observed for the majority of the carbons (0.02−0.77 ppm). The new resonance at 156.32 ppm is assigned to the newly formed carbamate. Due to the large number of new resonances in the region of 60−90 ppm, the ^{13}C resonances are assigned by comparison to CNCbl, as well as **2**−**4**. The DEPT experiment is used to identify the new methylene resonances at 69.81, 69.76, 69.48, 69.46, 67.95, 67.81, 41.96, 37.58, 36.95, and 29.65 ppm, and these resonances are assigned to the carbons from the 4,7,10-trioxa-1,13-tridecanediamine tether of **5**. As seen in the previous two 5′-modified analogues, the only significant variations in chemical shifts occur in the α-ribofuranosyl region of the spectra (see Figure 2). The only remaining methylene in the α-ribofuranosyl region at 62.19 ppm is assigned R5. The remaining resonances at 86.03, 79.41, 72.40, and 68.76 ppm are assigned to methine carbons R1, R4, R3, and R2, respectively.

Prediction of 2′ vs 5′ Modification. To address the possibility of 2′-OH activation versus 5′, chemical shift predictions are performed.[21] Using standard substituent effects, the chemical shift changes are calculated as listed in Table 2. Carbons R1, R2, and R4 all have observed chemical shift variations that are consistent with 5′ modification. Although the magnitude of the change observed for R5 is less than predicted, a significant chemical shift alteration is observed, thereby supporting our claim that the 5′-hydroxyl group is the site of modification. Carbon R3, however, has a dramatic change in chemical shift contrary to simple substituent effect predictions. It is thought that the large alteration in chemical shift is caused by a conformational change in the geometry of the phosphate group attached to the R3 position, due to perturbation of the sugar 5′-hydroxyl. This may result in a more dramatic shift to R3 than predicted by simple substituent effects.

TABLE 2. Predicted Chemical Shift Changes of Ribose Region ^{13}C NMR Resonances with Comparison to Analogue 2

	R1	R2	R3	R4	R5
2′ modified	−3.6	2.7	−3.6	0.2	0.0
5′ modified	0.0	0.0	0.2	−3.6	2.7
observed (**2**)	0.48	−0.32	−2.87	−1.79	0.73

Previous to the work reported herein, the complete NMR characterization of cobalamin analogues required extensive multidimensional NMR experiments for each analogue. This required considerable instrument time as well as in excess of 40−50 mg of material, resulting in concentrations of only about 15 mM in D_2O. With the addition of most chemotherapeutic drugs, the solubility decreases further, requiring even longer instrument times if D_2O is used as solvent. By using DMSO-d_6, concentrations of greater than 30−40 mM can be obtained with only 10 mg of material in 250 μL of DMSO-d_6.

Multidimensional NMR experiments were performed on only the parent compound, cyanocobalamin, to provide a reference library for comparison of ^{13}C and DEPT NMR resonances of various cobalamin analogues. Also, the amide protons of cobalamin are observed in the COSY and NOESY experiments of cyanocobalamin because of the ability to observe exchangeable protons in DMSO-d_6, thereby providing additional support for the assignment of the amide side chains. Comparing the ^{13}C and DEPT NMR resonances of compounds **2**−**5** to cyanocobalamin, only the α-ribofuranoside carbon resonances have shifted (1−3 ppm), whereas the remaining carbon resonances shifted only 0.01−0.77 ppm. This facile comparison of ^{13}C and DEPT NMR resonances provides strong evidence that only the 5′-OH of the α-ribofuranoside is modified in compounds **2**−**5,** whereas all other functional groups on cyanocobalamin remain unmodified. This reliable and sensitive technique allows for the complete characterization of cobalamin analogues for use as either chemotherapeutic drug delivery agents or for probing the enzymatic mechanism of reactions that use cobalamin as a cofactor.

Acknowledgment. We thank the National Institute of Health for funding (Grant No. CA73003) to C.B.G. and F.G.W. We also thank Dr. Charles Mayne for NMR technical help.

Supporting Information Available: Proton, ^{13}C, DEPT, COSY, NOESY, HSQC, and HMBC NMR spectra of cyanocobalamin in DMSO-d_6, atomic numbering scheme for compounds **2**−**5**, ^{13}C and DEPT NMR spectra of **2**−**5**, and comparative shifts of **2**−**5** versus cyanocobalamin. Experimental procedures for the synthesis of compounds **2**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0340399

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