Homocoenzyme B_{12} and Bishomocoenzyme B_{12}: Covalent Structural Mimics for Homolyzed, Enzyme-Bound Coenzyme B_{12}

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Dedicated to Professor Heinz Falk on the occasion of his 65th birthday

Abstract: Efficient electrochemical syntheses of "homocoenzyme B_{12}" (**2**, Co_β-(5′-deoxy-5′-adenosyl-methyl)-cob(III)alamin) and "bishomocoenzyme B_{12}" (**3**, Co_β-[2-(5′-deoxy-5′-adenosyl)-ethyl]-cob(III)alamin) are reported here. These syntheses have provided crystalline samples of **2** and **3** in 94 and 77% yield, respectively. In addition, in-depth investigations of the structures of **2** and **3** in solution were carried out and a high-resolution crystal structure of **2** was obtained. The two homologues of coenzyme B_{12} (**2** and **3**) are suggested to function as covalent structural mimics of the hypothetical enzyme-bound "activated" (that is, "stretched" or even homolytically cleaved) states of the B_{12} cofactor. From crude molecular models, the crucial distances from the corrin-bound cobalt center to the C5′ atom of the (homo)adenosine moieties in **2** and **3** were estimated to be about 3.0 and 4.4 Å, respectively. These values are roughly the same as those found in the two "activated" forms of coenzyme B_{12} in the crystal structure of glutamate mutase. Indeed, in the crystal structure of **2**, the cobalt center was observed to be at a distance of 2.99 Å from the C5′ atom of the homoadenosine moiety and the latter was found to be present in the unusual *syn* conformation. In solution, the organometallic moieties of **2** and **3** were shown to be rather flexible and to be considerably more dynamic than the equivalent group in coenzyme B_{12}. The homoadenosine moiety of **2** was indicated to occur in both the *syn* and the *anti* conformations.

Keywords: coenzyme B_{12} · crystal structures · electrochemistry · enzyme inhibitors · radicals

Introduction

Nature has evolved a variety of radical-based strategies for dealing with metabolic problems that require chemistry which is considered "difficult" (see, for example, ref. [1]). Coenzyme B_{12} dependent enzymes catalyze complex and particularly fascinating rearrangements in apparently well-controlled enzymatic radical reactions (see, for example, refs. [2–6]). In these enzymes, coenzyme B_{12} (**1**, 5′-deoxy-5′-adenosyl-cob(III)alamin; see Scheme 1A,) undergoes substrate-induced homolysis of its organometallic bond to produce the catalytically active 5′-deoxyadenosyl radical (and the "spectator" cob(II)alamin). Coenzyme B_{12} is thus a "pre-catalyst" and serves as a "reversibly functioning source" of the 5′-deoxyadenosyl radical.[7] The critical "activation" of the protein-bound coenzyme B_{12} towards rapid homolytic cleavage of its organometallic bond is still a puzzling problem (see, for example, refs. [8–12]). An "upwards conformational deformation" and an increased "folding" of the corrin moiety were considered to be crucial structural factors in this respect.[7] However, the corrinoid homolysis product of coenzyme B_{12}, cob(II)alamin, and the corrinoid moiety of the intact coenzyme have a strikingly similar structure.[13,14] This finding rendered a deformation of the corrin ligand very unlikely as a significant means for the enigmatic enzyme-induced activation towards homolysis of the Co–C bond and suggested the binding and the mutual positioning of the protein-bound homolysis products to be critical.[13] Indeed, some analogues of coenzyme B_{12}, in which flexible and satu-

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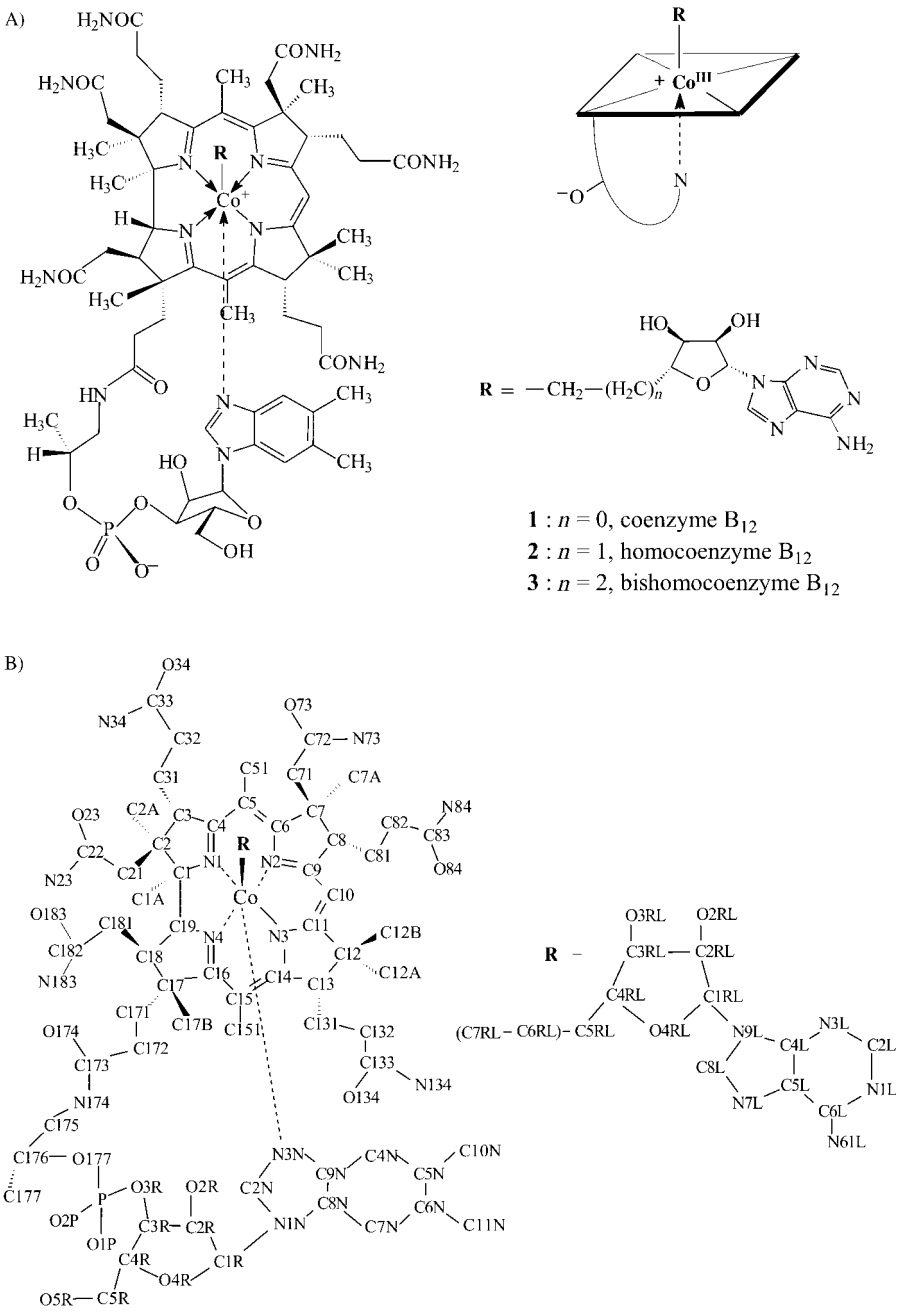


Scheme 1. A) Structural formulae of coenzyme B_{12} (**1**), homocoenzyme B_{12} (**2**), and bishomocoenzyme B_{12} (**3**); B) atom numbering used for coenzyme B_{12} derivatives.[23]

rated long chains connect the corrin and the adenine or adenosine moieties were found to bind well to the apo forms of coenzyme B_{12} dependent enzymes and to function as inhibitors.[15,16] Several such analogues of coenzyme B_{12} were suggested to mimic the transition-state geometry of coenzyme B_{12} dependent reactions.[15,17]

Deeper structural insights into the puzzling B_{12} problems have recently been provided by several atomic-resolution crystal structures of coenzyme B_{12} dependent enzymes.[8,9,18–20] Indeed, in all of the structures, the corrin ligand of the protein-bound corrinoid cofactor, if deformed at all to a significant degree, appeared to be slightly flatter than in the unbound coenzyme. However, X-ray crystal analysis of the coenzyme B_{12} dependent glutamate mutase revealed the activated cofactor to occur in two structures, in which the organometallic bond was apparently (partially) broken: The cobalt center and the adenosyl C5′ atom were at distances of about 3.2 and 4.2 Å, respectively, in these protein-bound, activated forms of coenzyme B_{12}, whose interconversion was accompanied by a pseudorotation of the adenosine ribose unit, (Figure 1).[9,21] The crystal structures of the related coenzyme B_{12} dependent enzymes, diol-dehydratase[22] and ribonucleotide reductase[20] were solved in the presence of the flexible coenzyme B_{12} analogue 5′-(9-adeninyl)-pentyl-cobalamin and showed electron density corresponding to an intact bound organometallic corrinoid. Based on the crystallographic studies with diol-dehydratase and other investigations with various organometallic analogues of coenzyme B_{12} (**1**), the existence of an "adenine-binding pocket" was suggested in diol-dehydratase.[16] The affinity of the "adenine-binding pocket" for the adenine heterocycle would help displace the organometallic ligand from the cobalt center of protein-bound **1**, by stretching the Co–C bond of **1** (to a calculated value of about 3.0 Å) and by activating it towards homolysis.[4]

We have set out to examine homologues of coenzyme B_{12} that might function as covalent structural mimics of the hypothetical enzyme-bound "activated" (that is, "stretched" or even homolytically cleaved) states of the B_{12} cofactor. Here we report, 1) on the synthesis of "homocoenzyme B_{12}" (**2**, Co_{β}-(5′-deoxy-5′-adenosyl-methyl)-cob(III)alamin) and on in-depth investigations of the structure of **2** in solution and in the crystalline state, as well as 2) on the synthesis and on detailed studies of the solution structure of "bishomocoenzyme B_{12}" (**3**, Co_{β}-[2-(5′-deoxy-5′-adenosyl)-ethyl]-cob(III)-alamin), which was also obtained in crystalline form

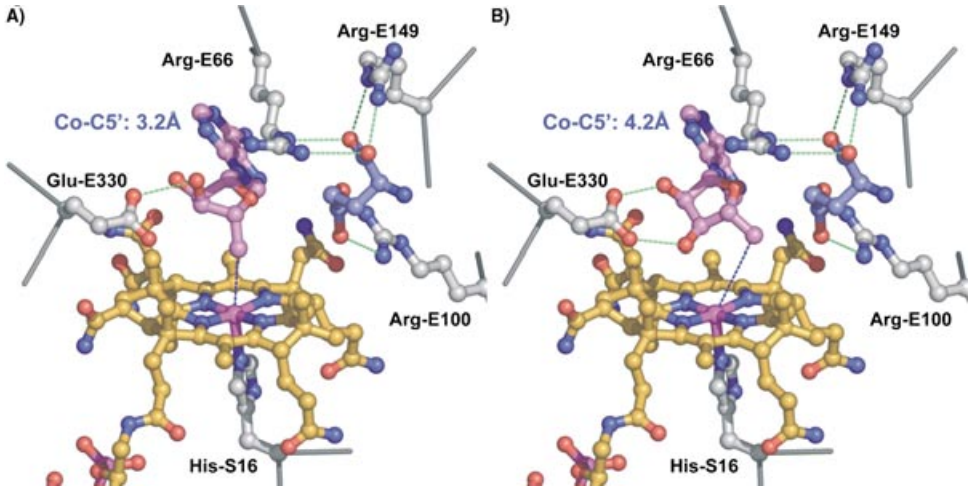


Figure 1. Coenzyme B_{12} bound to glutamate mutase from *Clostridium cochlearium*. The two conformers (A and B) of the adenosyl moiety as observed in the crystal structure (PDB code: 1i9c[9]) are shown in magenta. The substrate glutamate is drawn in blue, the B_{12} core in orange, and selected amino acid residues in grey. Potential hydrogen bonds are indicated by green dashes. Figures 1, 3, and 4 were produced by using the PyMol program (http://www.pymol.org/).

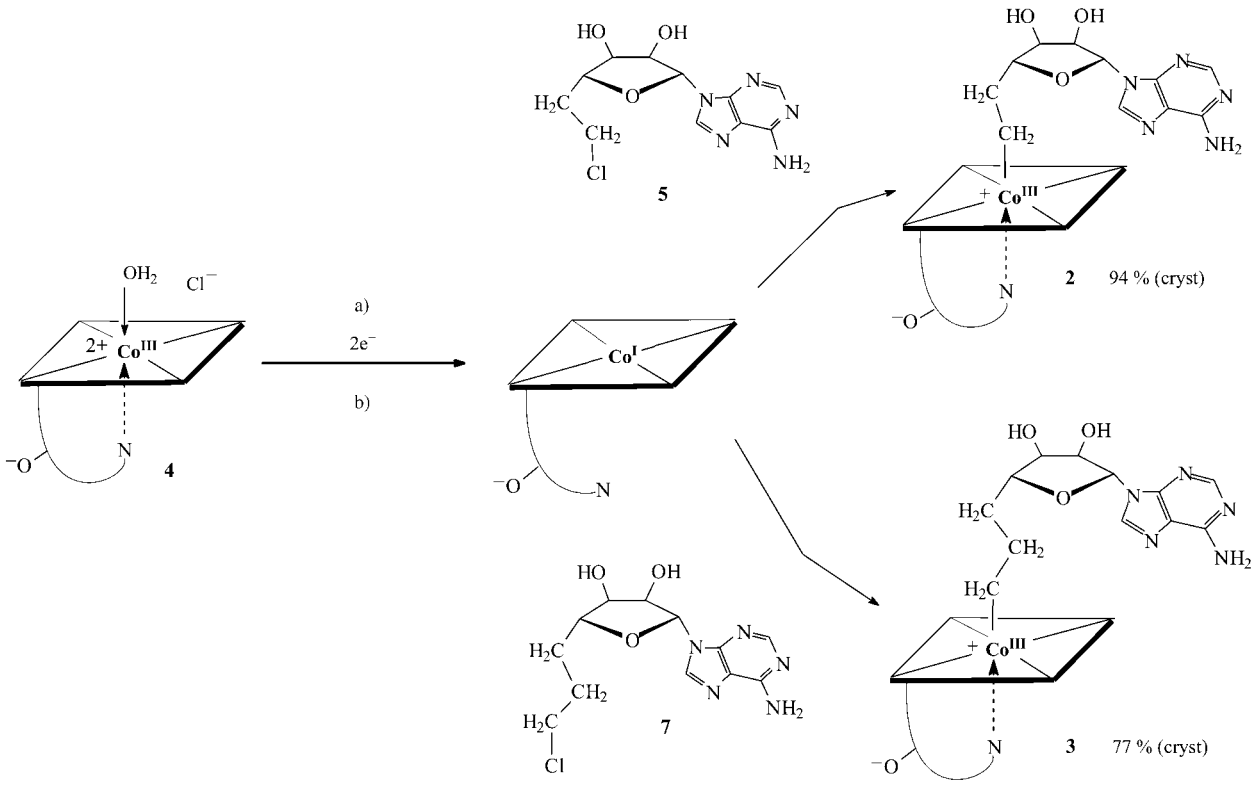
(Scheme 1A). From crude molecular models, the crucial distances from the corrin-bound cobalt center to the C5′ atom (labeled C5RL in Scheme 1B[23]) in **2** and **3** were estimated to be about 3.0 and 4.4 Å, respectively; these values are roughly the same as those found in the two "activated" forms of coenzyme B_{12} (**1**) in glutamate mutase.[9] Bishomocoenzyme B_{12} (**3**) was first prepared by Hogenkamp and coworkers,[24,25] who identified it by UV/visible spectroscopy and paper chromatography only. This coenzyme B_{12} homologue was found at that time to be a competitive inhibitor of adenosylcobalamin-dependent ribonucleotide reductase (with an inhibition constant, K_i(**3**), of 5.5 μmol, relative to an apparent Michaelis constant, K_m(**1**), of 4.7 μmol for coenzyme B_{12} (**1**) itself)[26] and diol-dehydratase (with an K_i(**3**) value of 0.58 μmol, relative to an apparent K_m(**1**) value of 0.80 μmol).[27]

Results

Electrochemical reduction of aquocob(III)alamin chloride (**4**) and cobalt alkylation of the highly nucleophilic cob(I)alamin formed in situ is an established means for the efficient synthesis of pure organometallic B_{12} derivatives.[28] The synthesis of the coenzyme B_{12} homologues **2** and **3** was thus planned to be achieved by in situ alkylation of electrochemically produced cob(I)alamin with proper alkylation agents (Scheme 2). Accordingly, the preparation of 5′-(chloromethyl)-5′-deoxyadenosine (**5**) and of the homologue 5′-(2-chloroethyl)-5′-deoxyadenosine (**7**) were first worked out to provide properly functionalized derivatives of 5′-deoxyadenosine. Adenosine was a convenient starting material for the synthesis of the adenosine derivative **5** (in 6 steps and 5% yield overall) via 5′-homoadenosine (**6**),[29] as well as for the synthesis of the homologue **7** (in 6 steps and 9% yield overall) via 5′-(2-hydroxyethyl)-5′-deoxyadenosine (**8**).[25,29] The substitutive refunctionalizations of the adenosine-homologues **6** and **8** to give the chlorodeoxy-derivatives **5** and **7** were achieved in 74 and 80% yields, respectively, by using thionyl chloride in hexamethylphosphortriamidate.[30]

Crystalline homocoenzyme B_{12} (**2**) was obtained in 94% yield from electrochemical reduction of aquocob(III)alamin chloride (**4**) to cob(I)alamin and in situ alkylation of the latter with 5′-(chloromethyl)-5′-deoxyadenosine (**5**). The molecular formula of the light-sensitive red corrinoid was confirmed by FAB mass spectrometry and the structure was established by using the relevant spectroscopic means. The purity (in terms of B_{12} content) of crystalline **2** was shown to be high (>98%) by analysis of a high-quality 500 MHz ^{1}H NMR spectrum (Figure 2A), which also confirmed the sample of **2** to contain no detectable amount (less than 0.2%) of coenzyme B_{12} (**1**).

Analysis of the structure of **2** in aqueous solution by one-dimensional and two-dimensional (heteronuclear) NMR spectroscopy[31,32] provided firm signal assignments for all relevant hydrogen and carbon nuclei. The data were consistent with the expected insignificant structural differences of the cobalt–corrin moiety in **2** compared to that in coenzyme B_{12} (**1**; Table 1). In contrast, investigations with NOE studies (ROESY spectra) clearly showed the organometallic moiety to be rather flexible in **2** and to exist in various orientations with respect to the corrin ring. Several such features were of particular interest: NOE contacts of both of the two adenine protons H(2L) and H(8L) with H(1RL), H(2RL), and H(3RL) of the ribose ring of the adenosine moiety were not compatible with an exclusive existence of the nucleoside in the "classical" *anti* conformation, but pointed to a significant population of the unusual *syn* conformation[33] (Tables 2 and 3). NOE contacts between the diastereotopic methylene protons $H_{a/b}$(5RL) and $H_{a/b}$(6RL) of the organometallic-bound linker and protons H(71), H(12B), H(17B), and H(19) of the corrin moiety clearly indicated the linker to be oriented predominantly "southeast" (that is, near ring C of the corrin ligand), with a mean torsion angle N4–Co–C6RL–C5RL of roughly 70–80°. While NOE contacts of the adenine proton H(2L) were observed to reporter protons of all segments of the "top" face of the corrin ligand, the major contacts of H(2L), as well as of H(8L), were observed with H(17B). The NOE data thus suggested the adenine moiety to be positioned mainly above ring D. In this respect the orientation of the organometallic



Scheme 2. The synthesis of the coenzyme B_{12} homologues **2** and **3** by in situ alkylation of electrochemically produced cob(I)alamin with alkylation agents **5** and **7**, respectively: a) reduction potential −1.1 V versus 0.1 N CE, CH_3OH, 0.1 M TBAHFP, RT; b) reduction potential −1.1 V versus 0.1 N CE, H_2O, *t*BuOH (1:1), 0.1 M $LiClO_4$, RT.

ligand of **2** clearly differs from that of coenzyme B_{12}, where the adenine unit is situated near ring C.[34–38] The chemical-shift data for relevant protons on the corrin ring also pointed to differing shielding effects of the adenine unit, consistent with the indicated altered conformational preferences: Compared to the signals in the ^{1}H NMR spectrum of **1**,[34] the signals of the methyl protons H(17B) and of H(19) in the spectrum of **2**, were shifted by nearly 0.4 ppm to higher field, those of the methyl protons H(12B) and of H(13) are at lower field (see Table 1). Less pronounced shifts in the same directions were observed in **3** (see below) and in analogues of coenzyme B_{12} with longer connecting chains.[17]

In the NOE spectra of **2** in aqueous solution, correlations between the diastereotopic methylene protons $H_{a/b}$(5RL) and $H_{a/b}$(6RL) of the organometallic-bound linker and protons H(71), H(12B), H(17B), and H(19) at the corrin moiety helped to assign H_a(5RL) as $H_{pro\text{-}S}$(5RL) and H_a(6RL) as $H_{pro\text{-}R}$(6RL) (and the two H_b protons correspondingly). However, the NOE correlations of H(2L) and H(8L) with H(71) and H(21) are inconsistent with a single major orientation of the organometallic moiety with respect to the corrin macrocycle. They indicate that the 6′-deoxy-5′-homoadenosine group of **2** also samples orientations that place the adenine heterocycle more closely to the acetamide side chains extending from the C2 and C7 atoms. In solution, the organometallic moiety of homocoenzyme B_{12} (**2**) is thus considerably more flexible than in coenzyme B_{12} (**1**): The homoadenosine group in **2** is observed in *syn* and *anti* conformations and in a more extended array of orientations with respect to the corrin ligand.

Crystals of homocoenzyme B_{12} (**2**) were grown from aqueous acetone and were studied by X-ray crystal analysis. Synchrotron radiation was used to record the diffraction pattern of a cryo-cooled crystal specimen to a crystallographic resolution of 0.85 Å. Anisotropic refinement of the structure against these diffraction data led to a crystallographic residual of 0.0585 for all reflections, as described in the experimental section, thereby revealing a well-ordered (single) molecular structure for crystalline **2** (Figure 3). Not unexpectedly, the cobalt–corrin moiety of **2** showed structural features similar to those in coenzyme B_{12} (**1**): In **2** the folding angle of the corrin ligand is only slightly larger than in **1** (15.5° in **2**, 13.3° in **1**), the lengths of the axial Co–N3N and Co–C6RL bonds were only slightly shorter than in **1** (2.192 and 2.003 Å in **2**, 2.24 and 2.04 Å in **1**), and the base tilt is similar (9.5° versus 10.1°).[38] Coenzyme B_{12} (**1**) and its homologue **2** differ in an interesting and significant way with respect to the structures of the homologous organometallic ligands (Figure 4A): Whereas the Co–C5RL–C4RL bond angle in **1** has the remarkable and much discussed value of 125.4°, the corresponding Co–C6RL–C5RL angle in **2** is only about 115.0°, closer to the tetrahedral value. In crystalline **2**, the critical C5RL atom (C5′ of the adenosine ribose moiety) is at a distance of 2.99 Å from the cobalt center and

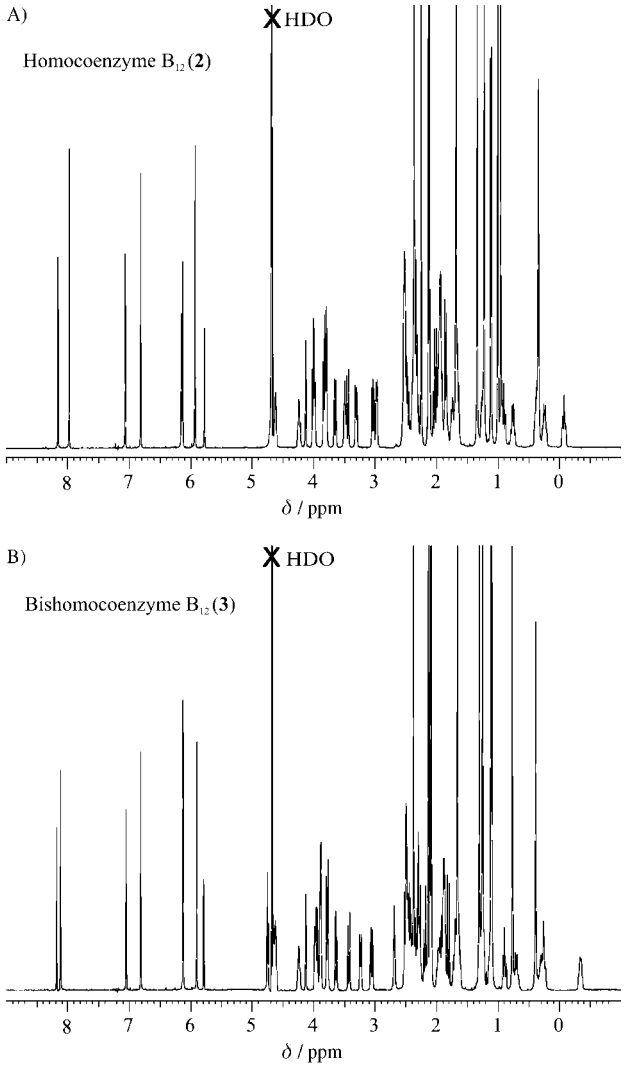


Figure 2. 500 MHz ^{1}H NMR spectra of A) homocoenzyme B_{12} (**2**) and B) bishomocoenzyme B_{12} (**3**) in D_2O (26 °C).

is therefore remarkably close to where it is in the "short" stretched state of bound and "activated" coenzyme B_{12} (**1**) in glutamate mutase[9] (Figure 4B). The organometallic ligand of **2** exhibits a nearly tetrahedral bond angle at the C5RL carbon atom (C6RL–C5RL–C4RL 110.1°) and both torsion angles around the corresponding C–C bonds correspond closely to ideal antiplanar conformations (Co–C6RL–C5RL–C4RL 177.2°; C6RL–C5RL–C4RL–C3RL 175.8°). In the structure of coenzyme B_{12} (**1**) the Co-C5RL–C4RL–C3RL torsion angle equals −169°; this places the ring oxygen atom O4L nearly synclinal to the cobalt center (Co–C5RL–C4RL–O4RL 77°). The observed N4–Co–C6RL–C5RL torsion angle of 107.2° positions the organometallic ribofuranose segment of **2** near ring C and a remarkable (roughly) 80° counterclockwise, when compared to the situation of the same moiety in coenzyme B_{12} (where the N4–Co–C5RL–C4RL angle equals 16°; Figure 4A). In both crystal structures (**1** and **2**) the ribofuranose moieties are present in 3′-*endo* conformations. However, in **2** the ade-

Table 1. List of assigned signals in the 500 MHz ^{1}H and 125 MHz ^{13}C NMR spectra of coenzyme B_{12} (**1**),[a] homocoenzyme B_{12} (**2**),[b] and bishomocoenzyme B_{12} (**3**)[b] (in D_2O).

	1		**2**		**3**	
	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$
C(1A)	0.34	23.5	0.33	23.2	0.37	23.3
C(1)		88.5		87.9		87.8
C(2A)	1.24	19.9	1.21	19.4	1.23	19.5
C(2)		49.5		48.8		48.9
C(21)	2.30	46.2	2.01	45.9	1.78/2.15	45.7
C(3)	3.99	58.5	3.82	58.3	3.92	58.0
C(31)	1.85/1.95	29.2	1.83/1.92	28.8	1.84/1.92	28.8
C(32)	2.38	38.3	2.32/2.37	37.6	2.33–2.42	37.7
C(5)		108.4		108.7		108.1
C(51)	2.34	18.3	2.34	17.9	2.35	18.0
C(7A)	1.59	21.7	1.66	21.3	1.64	21.5
C(7)		53.1		52.7		52.5
C(71)	1.61/2.08	45.3	1.85/2.35	45.2	1.78/2.27	45.1
C(8)	3.18	57.5	3.29	57.3	3.21	57.3
C(81)	0.69/1.64	28.7	0.75/1.73	28.7	0.66/1.70	28.4
C(82)	0.81/1.61	34.8	0.93/1.64	34.3	0.87/1.62	33.9
C(10)	5.81	97.7	5.91	97.4	5.88	97.1
C(12)		49.5		49.1		49.2
C(12A)	1.19	23.9	1.32	22.6	1.28	22.5
C(12B)	0.72	34.2	0.93	34.2	0.74	34.1
C(13)	2.76	55.8	2.95	56.0	2.66	56.0
C(131)	1 .89/2.09	30.3	1.93	30.4	1.84	30.3
C(132)	2.43	38.1	2.51	37.6	2.33–2.42	37.7
C(15)		106.9		106.4		106.3
C(151)	2.33	18.8	2.24	17.9	2.06	17.7
C(17B)	1.23	19.6	0.98	18.8	1.09	19.3
C(17)		60.8		60.3		60.2
C(171)	1.95/2.33	34.4	1.64/2.32	34.3	2.27/2.47	34.4
C(172)	1.71/2.43	34.6	1.91/2.32	34.2	1.89/2.27	34.7
C(18)	2.52	42.5	2.51	42.1	2.48	42.0
C(181)	2.52	34.8	2.45/2.50	34.2	2.46	34.4
C(19)	4.24	76.8	3.80	76.3	3.86	76.5
C(175)	3.03/3.43	47.8	3.01/3.44	47.5	3.04/3.40	47.2
C(176)	4.22	76.0	4.22	75.6	4.22	75.6
C(177)	1.09	21.7	1.10	21.0	1.09	21.2
C(R1)	6.13	89.4	6.13	88.8	6.10	88.9
C(R2)	4.22	72.0	4.11	71.7	4.10	71.6
C(R3)	4.60	76.2	4.61	75.7	4.60	75.5
C(R4)	4.10	84.6	3.97	84.1	3.95	83.9
C(R5)	3.77/3.62	63.4	3.63/3.79	63.1	3.61/3.75	62.8
C(2N)	6.83	144.7	6.80	144.5	6.79	144.2
C(4N)	6.13	121.4	6.11	120.8	6.10	121.7
C(5N)		134.5		136.7		136.2
C(10N)	2.08	22.5	2.09	21.8	2.09	22.1
C(6N)		136.8		134.5		133.9
C(11N)	2.08	22.3	2.09	21.8	2.09	21.8
C(7N)	7.04	113.5	7.07	113.3	7.03	113.0
C(8N)		133.3		133.3		133.0
C(9N)		141.0		141.3		140.8
C(1RL)	5.46	91.0	5.75	92.0	5.76	98.6
C(2RL)	4.42	75.6	4.65	75.6	4.73	75.2
C(3RL)	3.63	76.6	3.98	76.3	3.86	76.8
C(4RL)	2.53	88.6	3.48	85.7	3.75	86.2
C(5RL)	0.44/1.40	27.3	−0.09/0.22	38.2	1.09/1.25	35.1
C(6RL)			0.36/1.24	24.6	−0.38/0.22	29.2
C(7RL)					0.23/1.25	30.0
C(2L)	8.08	156.0	8.13	156.1	8.15	155.6
C(4L)		151.8		151.6		151.9
C(5L)		121.8		121.7		121.1
C(6L)		158.7		158.6		158.4
C(8L)	7.89	143.8	7.95	143.1	8.10	143.0

[a] Adapted from ref. [34]. [b] Results from this work.

Table 2. Relevant NOE contacts[a] between the corrin ligand and homoadenosine moiety of **2**.

	H(2L)	H(8L)	H(1RL)	H(4RL)	H_a(5RL)	H_b(5RL)	H_a(6RL)	H_b(6RL)
H(21)	3	2					nd[b]	nd[b]
H(71B)	3	3		6				
H(71A)	2			5	12		nd[b]	
H(8)	2				1		1	
H(10)	2				6	2	4	nd[b]
H(12B)					24	15	8	nd[b]
H(13)	6	3		4	2	1	1	nd[b]
H(151)	8	4			4	2		
H(17B)	27	21	5		6	24	nd[b]	nd[b]
H(181)	11	2						nd[b]
H(19)	2			nd[b]	1	24	2	34

[a] Given in % of NOE intensity of cross-peak H_a/H_b(C6RL). [b] nd = not detected due to signal overlap.

Table 3. Selected NOE contacts[a] within the homoadenosine moiety of **2**.

	H(1RL)	H(2RL)	H(3RL)	H(4RL)	H_a(5RL)	H_b(5RL)	H_a(6RL)	H_b(6RL)
H(2L)	3	3	3					
H(8L)	33	91	69	3	2	5	2	2

[a] Given in % of NOE intensity of cross-peak H_a/H_b(C6RL).

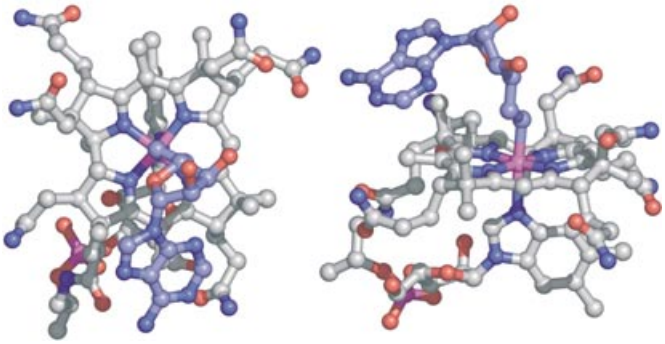


Figure 3. Crystal structure of homocoenzyme B_{12} (**2**) in two perpendicular views. The B_{12} core is shown in grey and the organometallic ligand in light blue. Hydrogen atoms have been omitted for clarity.

nine unit is in a unique *syn* conformation (O4RL–C1RL–N9L–C4L 66.2°)[33] and is turned by about 180° relative to its *anti* conformation in **1** (O4RL–C1RL–N9L–C4L −109°). This unusual conformational feature of **2** in the crystal is clearly not merely a consequence of crystal packing as it had already been revealed by NMR spectroscopy as a characteristic of the (dynamic) structure in solution.

Crystalline bishomocoenzyme B_{12} (**3**) was obtained in 77% yield from alkylation of electrochemically produced cob(I)alamin with 5′-(2-chloroethyl)-5′-deoxyadenosine (**7**). In fact, a first synthesis of **3** was described by Hogenkamp,[24] who prepared coenzyme B_{12} (**1**) and several organometallic analogues in apparently good yield (70–85%) by use of cob(I)alamin, generated in situ by sodium borohydride reduction of aquocob(III)alamin (**4**). Alkylation with 5′-(2-chloroethyl)-5′-deoxyadenosine (**7**) produced in situ gave **3**, identified by UV/visible spectroscopy and paper chromatography. A more thorough analysis and structural characterization was apparently not carried out at that time. In the present work, the structure of bishomocoenzyme B_{12} (**3**) was established by the relevant set of spectroscopic data: The molecular formula of the light-sensitive red corrinoid was confirmed by FAB mass spectrometry again. Crystalline **3** contained no detectable amount (less than 0.2%) of coenzyme B_{12} (**1**) and was highly pure in terms of its B_{12} content (>98%), as revealed by careful analysis of a ^{1}H NMR spectrum (Figure 2B).

Analysis of the structure of **3** in aqueous solution by one-dimensional and two-dimensional (heteronuclear) NMR spectroscopy again provided firm signal assignments for most relevant hydrogen and carbon nuclei and was consistent with the expected insignificant structural differences of the cobalt–corrin moiety of **3** in comparison with the analogous moiety in coenzyme B_{12} (**1**; Table 1). Investigations with NOE studies (ROESY spectra) showed the organometallic ligand to be rather flexible in **3**, but the accumulated spectral data suggested it to exist in a rather narrow range of orientations with respect to the corrin ring (Tables 4 and 5): NOE contacts between the proton H(2L) and the adenine ring are seen for H(1RL) but not for H(2RL) and H(3RL) of the ribose ring of the adenosine moiety. These findings are compatible with the *anti* conformation of the nucleoside of **3** and exclude significant populations of a *syn* conformation. However, hydrogen atoms at all positions of the extended ribose unit exhibit NOE contacts with H(8L), a fact indicating otherwise considerable flexibility within the organometallic ligand. NOE contacts between the adenine unit and the corrin ligand were generally weaker than those observed in the homologue **2**. The more intensive NOE cross-peaks correlated H(2L) and H(8L) with H(12B), as well as H(8L) with H(71); these results suggest the adenine moiety to be positioned mainly above ring C, with similar orientations to those in coenzyme B_{12} (**1**).[34] NOE contacts were again observed between various protons at the β-face of the corrin ligand and the diastereotopic methylene protons H(5RL), H(6RL), and H(7RL) of the organometallic linker (which could not individually be assigned due to signal overlap). These data, as well as the chemical-shift data available for all relevant protons at the corrin ring all pointed to rather similar (but weaker) shielding effects of the adenine unit in **3** to those in **1** (Table 1). The data are all consistent with a predominant "south to south-east" orientation of the linker (near rings C and D of the corrin ligand). In bishomocoenzyme B_{12} (**3**) the organometallic moiety thus exists predominantly in an *anti* conformation of the adenine ring and appears to be bound in a rather flexible way but mainly populating a rather narrow range of orientations ("south to south-east") with respect to the corrin ligand.

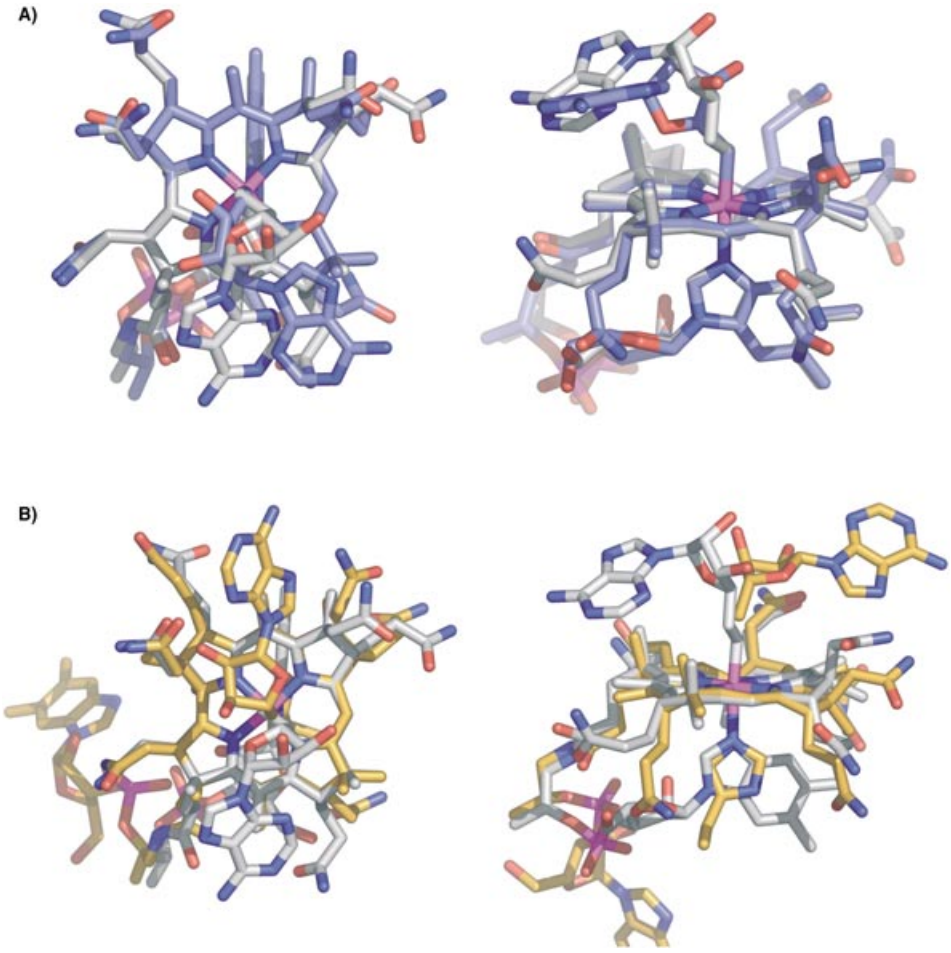


Figure 4. Superposition of the crystal structures of homocoenzyme B_{12} (**2**) and coenzyme B_{12} (**1**) in its free form[38,62] (A) and bound to glutamate mutase[9] (B). **2** is shown in grey, whereas **1** is drawn in blue (free) and orange (protein-bound). In the case of the protein-bound cofactor, only conformation A (see Figure 1A) is shown. The central cobalt and the four corrin nitrogen atoms were used for superimposing the structures.

Table 4. Relevant NOE contacts[a] between the corrin ligand and bishomoadenosine moiety of **3**.

	H(2L)	H(8L)	H(1RL)	H(4RL)	H_a(5RL)	H_a(6RL)	H_b(6RL) H_a(7RL)	H_b(7RL)
H(21)				nd[b]	nd[b]		12	nd[b]
H(71)		3	2		nd[b]	7	12	nd[b]
H(10)						1	6	nd[b]
H(12B)	5	8	2	2		17	14	nd[b]
H(13)						2	2	nd[b]
H(17B)				3		28	3	nd[b]
H(181)							3	17
H(19)					17	2	9	12

[a] Given in % of NOE intensity of cross-peak $H_a/H_b(C_{7RL})$. [b] nd = not detected due to signal overlap.

Discussion

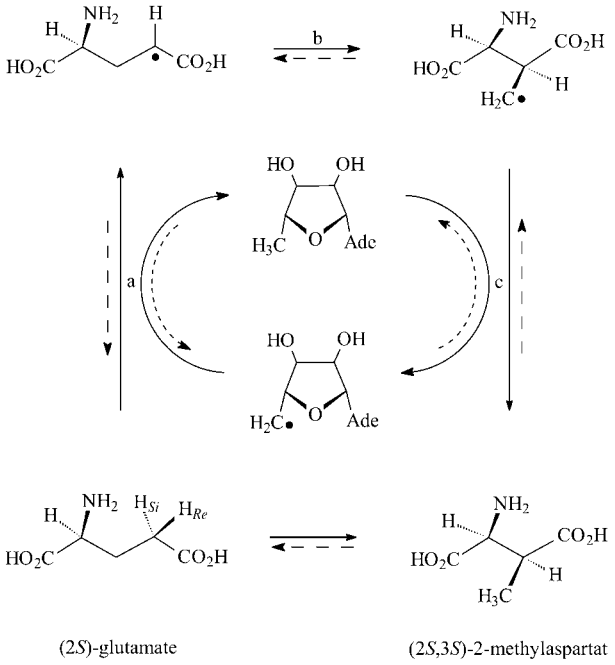
Human, animal, and microbial metabolism depends upon coenzyme B_{12} dependent enzymatic reactions to achieve transformations whose chemistry is considered "difficult"[1] through mechanistically intriguing sequences of apparently well-controlled radical steps.[2,4–6,21] As outlined (for example, for glutamate mutase;[9,39–42] Scheme 3), the rearrangement reaction catalyzed by these enzymes proceeds through (a minimum of) five steps involving radicals and radicaloid species. In most of these enzymes, substrate loading triggers the cascade of radical steps by inducing the bound organometallic cofactor, coenzyme B_{12} (**1**) to undergo homolysis of its Co–C bond and to produce a 5′-deoxyadenosyl radical. In an intriguing and highly controlled manner, this radical abstracts the H_{Si} atom from the substrate (step a), to produce a substrate radical in suitably activated form for rearrangement to a product radical (step b). The product radical then reabstracts a hydrogen atom from the 5′-methyl group of 5′-deoxyadenosine to give the product and to furnish the 5′-deoxyadenosyl radical (step c). The latter radical is generated in the vicinity of the corrinoid homolysis fragment cob(II)alamin and may recombine with the latter to regenerate the organometallic cofactor **1**. The high reactivity of carbon-centered radicals and the frequent lack of selectivity in reactions with such radicals would be expected to require tight binding of the reacting species by the protein matrix, in order to control the enzymatic radical steps. Selectivity in radical reactions would thus be achieved by exact geometric support for the main reaction channel, as well as by steric inhibition of competing reactions, along the lines of the concept of "negative catalysis".[43,44]

Coenzyme B_{12} dependent enzyme reactions thus must provide the structural and dynamical prerequisites for the completely controlled operation of a complex and exquisite machinery. The extent to which such radical machineries tolerate structural changes of the protein, the substrate, or the cofactor is of considerable interest. By studying single-point mutations (for examples, see refs. [45–51]) or by using substrate analogues[6,39,52,53] the questions on the former two aspects have

Table 5. Selected NOE contacts[a] within the bishomoadenosine moiety of **3**.

	H(1RL)	H(2RL)	H(3RL)	H(4RL)	H_a(5RL)	H_b(5RL)	H_a(6RL)	H_b(6RL)
H(2L)	3							
H(8L)	32	35	15	8	12	2		5

[a] Given in % of NOE intensity of cross-peak H_a/H_b(C7RL).



(2*S*)-glutamate (2*S*,3*S*)-2-methylaspartat

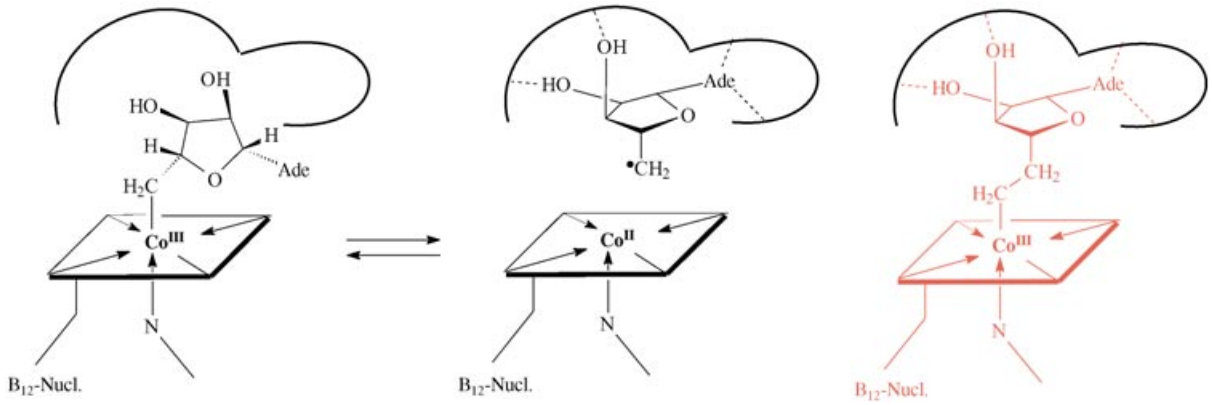
Scheme 3. Suggested mechanism (in three steps: a, b, c) of the enzyme-catalyzed isomerization reaction of (2*S*)-glutamate and (2*S*,3*S*)-2-methylaspartate, a reversible carbon-skeleton rearrangement, catalyzed by the coenzyme B_{12} dependent enzyme glutamate mutase.[41,42]

been addressed. Likewise, H/D-isotope effects were investigated to probe the mechanisms[54–57] and the transition states of hydrogen-atom transfer reactions.[4,44,50,58]

The present work is intended to provide close homologues of coenzyme B_{12} as an entry to test the structural tolerance with respect to the geometric properties of the adenosyl group of the radical-generating B_{12} cofactors. Changing the hydrogen-bonding interaction capacity of the adenine heterocycle by the use of corresponding analogues of **1** had significant effects on their binding and cofactor activities, for example, in studies with diol-dehydratase.[4,16] However, when the adenosine ribose unit of **1** was substituted by oligomethylene chains of suitable lengths, analogues of **1** were obtained with the properties of inhibitors in a variety of coenzyme B_{12} dependent enzymes.[20,22,26,27,59] In a related fashion, intermediate- and long-chain homologues of **1** were prepared, in which oxa-oligomethylene chains $(O(CH_2)_n$, with $n=3$–7) were inserted, formally, between cobalt and C5RL (of the adenosine moiety) of **1**, and these homologues were investigated as inhibitors of coenzyme B_{12} dependent enzymes.[15,17] At that time, these homologues of **1** were conceived as transition-state mimics for adenosylcobamide-dependent reactions with a long hypothetical distance (of about 10 Å) between cobalt and C5RL in the activated form of protein-bound **1**.[17]

The mentioned recent crystallographic work on glutamate mutase[21] and on methylmalonyl-CoA-mutase[8] supported two major modes of deformation of the bound corrinoid cofactor, in which the adenosine function was displaced from its known position in unstrained coenzyme B_{12} by reorientation and by stretching the Co–C bond. Specifically, in the activated, protein-bound forms of coenzyme **1** in glutamate mutase C5RL was found to be at distances of about 3.2 and 4.2 Å from the cobalt center[9,21] (Figure 1). Similarly, in diol-dehydratase, the evidence pointed to the existence of an "adenine-binding pocket", which apparently helped to stretch the Co–C bond of the bound cofactor to a distance of about 3.0 Å.[4,11] The synthetic work reported here thus concerned the preparation of close homologues of **1**, in which such distances between the C5RL and cobalt atoms would be available in an intact covalent mimic. Indeed, "homocoenzyme B_{12}" (**2**) and "bishomocoenzyme B_{12}" (**3**) appeared to meet these criteria very closely. Such homologues of coenzyme B_{12} thus could already have a geometric resemblance to the hypothetical strained enzyme-bound forms of the coenzyme (see Scheme 4). In keeping with the idea that a mechanochemical effect would be a major contributor to the activation of the cofactor, homocoenzyme B_{12} (**2**) and similar homologues of coenzyme B_{12} would be much less prone to homolysis of their Co–C bond and would bind better to the apoproteins. A first basic question, to be addressed with such homologues, therefore, could concern the enigmatic activation of the enzyme-bound cofactor towards homolysis of its Co–C bond. When bishomocoenzyme B_{12} (**3**) was used earlier in enzyme assays with ribonucleotide reductase and with diol-dehydratase, it was found—as expected—to be a good competitive inhibitor.[26,27]

The preparation of natural and unnatural organometallic B_{12} derivatives, in line with the organometallic nature of coenzyme B_{12} (**1**),[60] has attracted considerable interest.[12,28,61,62] Most of the standard procedures developed were based on the cobalt alkylation of the highly nucleophilic Co^{I} corrins with organic alkylating agents[63] and rely, therefore, both on methods for the efficient reduction of Co^{III} corrins to cob(I)alamin and similar Co^{I} corrins (and their in situ alkylation) and on the low tendency of alkyl Co^{III} corrins to be reduced themselves under these conditions.[28,64] In cases where alkyl Co^{III} corrins are themselves susceptible to reduction, sufficiently reactive alkylating agents can alternatively be used to alkylate Co^{II} corrins.[65] In the present work, homocoenzyme B_{12} (**2**) and bishomocoenzyme B_{12} (**3**) were prepared in pure crystalline form, free of coenzyme B_{12} (**1**). Cobalt alkylation of the highly nucleophilic cob(I)alamin, which was produced by controlled potential electrolysis of aquocob(III)alamin chloride (**4**),[28]



Scheme 4. The protein part of coenzyme B_{12} dependent enzymes is suggested to assist the Co–C bond homolysis by providing stabilization to "activated" (homolyzed) coenzyme B_{12} (center), but not to intact, unstrained coenzyme B_{12} (**1**, left). The structure of enzyme-bound "activated" coenzyme B_{12} may be mimicked by intact homocoenzyme B_{12} (**2**), which would thus be bound with less strain (right). Nucl. = nucleotide moiety.

provided the organometallic coenzyme B_{12} analogues **2** and **3** in 94 and 77% yield, respectively, after crystallization from aqueous acetone.

Modern means of spectroscopic analysis allow the reliable structural characterization in solution of B_{12} derivatives[31,32] and the analogues were examined to highlight the advances possible through the use of new NMR spectroscopic[34] and mass spectrometric methods.[66] However, in earlier times, the basis for the identification and characterization frequently was of a more qualitative nature (such as UV/visible spectra),[60,61,67,68] supplemented, in a few cases, with analysis of the crystal structure.[36] In the present work, the structures of **2** and of **3** in aqueous solution were analyzed in detail, by heteronuclear NMR spectroscopy and mass spectrometric methods in particular. These studies first of all confirmed the homologous nature of (the organometallic ligands of) **1**–**3**. At the same time, they revealed characteristic differences in the orientation, conformations, and (deduced) flexibility of the organometallic group. In the crystalline state, structural studies with coenzyme B_{12} (**1**)[35–38] suggest the organometallic 5′-deoxyadenosyl group to be held in the position of minimal steric interactions and the adenine heterocycle to be positioned rather tightly above ring C. Steric impediment of such an arrangement, for example, by an inverted configuration at C13 and β orientation of the propionamide side chain, as in neocoenzyme B_{12}, eliminated such a minimum and was found to result in an increased flexibility of the organometallic ligand.[69] When "rationalizing" the structure of vitamin B_{12}[70] it is thus tempting to interpret the presence of the methyl group C(12B) (which is the result of a remarkable decarboxylation of an acetic acid side chain in the course of the B_{12} biosynthesis[71,72]) as a "deletion" from the ligand structure of natural corrinoids to reduce steric strain near ring C in coenzyme B_{12} (**1**).

In **2** and **3**, one or two methylene units are inserted, formally, between the cobalt center and the adenosine moiety, to give the two closest homologues of coenzyme B_{12}. An interesting consequence of this "stretching" of the organometallic function, first of all, is a considerably enhanced flexibility, as similarly observed, for example, in 9-(3-propyl)-adeninylcobalamin.[59] A mobile and apparently rather unstrained (homo)adenosine function is observable in the (solution) structure of homocoenzyme B_{12} (**2**), in which the organometallic adenosine group exists in both the *anti* and *syn* conformations. NOE contacts of the adenine moiety with β side chains from all four sectors of the corrin ligand indicate a dynamic organometallic moiety and the absence of a pronounced conformational minimum.

X-ray analysis of crystals of **2** provided a high-resolution crystal structure (Figure 3). It revealed the organometallic (homo)adenosine group of **2** to be present in a *syn* conformation and the adenine heterocycle in an unencumbered and possibly rather floppy "tuck-in" position near the junction between rings C and D. Aside from these conformational features, the organometallic ligand appeared to have the characteristics of a rather unstrained group, with close to standard values for bond lengths, bond angles, and torsion angles. As expected, the critical C5RL atom was observed to be at a distance of about 3.0 Å from the cobalt center, that is, at a similar distance to that found for C5RL in "activated" coenzyme B_{12} in the crystals of glutamate mutase (Figure 4B).[9] The crystal structure thus supported the notion that homocoenzyme B_{12} could be a promising structural mimic of protein-bound and activated coenzyme B_{12}.

The crystals obtained for bishomocoenzyme B_{12} (**3**) have not, so far, been suitable for X-ray analysis. However, extensive heteronuclear NMR spectroscopy in aqueous solution revealed **3** also to be a rather unstrained and flexible "stretched" homologue of coenzyme B_{12} (**1**), in which the organometallic ligand was actually oriented mainly in the same direction as in **1**. From a structural model of **3**, with an antiperiplanar arrangement of the organometallic chain Co–C7RL–C6RL–C5RL, C5RL was calculated to be at a distance of about 4.4 Å from the cobalt center, that is, at a similar distance as is found in one form of homolyzed coenzyme B_{12} in the crystals of glutamate mutase.[9]

Homocoenzyme B_{12} (**2**) and bishomocoenzyme B_{12} (**3**) are two homologues of coenzyme B_{12} (**1**), in which the adeno-

sine function is attached in a "stretched" and conformationally flexible way: The formal insertion of one or two methylene units into the Co–C bond of **1** positioned the C5RL atom at a distance from the cobalt center in the intact organometallic analogues **2** and **3** where the radical center in the 5′-deoxyadenosyl radical of enzyme-bound "activated" (homolyzed) **1** has been observed. Secondly, the observed flexible nature of the organometallic group in **2** and in **3** would also be helpful for the adaptation of their structure to the holoenzyme. Two structural features are thus present in **2** and **3** that are expected to help adapt their structures to the protein surface of the unstrained enzyme (Scheme 4). The intact homologues **2** and **3** would thus be likely to have an increased binding affinity to the apoproteins of coenzyme B_{12} dependent enzymes and, in this way, to act as (competitive) inhibitors of such enzymes by displacing the "real" B_{12} cofactor from the active site. This behavior has been observed for **3** in earlier studies with ribonucleotide reductase and with diol-dehydratase.[26,27] On the other hand, the expected ability of the two homologues of coenzyme B_{12} to bind more effectively to the protein part of coenzyme B_{12} dependent enzymes would also render them suitable as mimics of coenzyme B_{12} with a lesser sensitivity to be activated towards homolysis and thus with a higher stability in the enzymes, a property useful for crystallographic purposes. Experiments examining the possible roles of **2** and **3** as cocatalysts or inhibitors and their ability to stabilize the protein structure in a variety of coenzyme B_{12} dependent enzymes thus appear of particular interest: So far, studies with the coenzyme B_{12} dependent enzymes glutamate mutase[73] and with diol-dehydratase[74] have been carried out, and these studies will be reported in due course.

Experimental Section

General: Chemicals and solvents: Aquocob(III)alamin chloride (**4**, vitamin B_{12a}; pyrogen-free Fr. Ph. BP; 10.7% loss on drying; <2% cyanocobalamin) was obtained from Roussel Uclaf. Tetra-*n*-butylammonium hexafluorophosphate (TBAHFP; Fluka, Neu-Ulm, Germany; puriss.) was recrystallized once from dichloromethane/diethyl ether and once from methanol/diethyl ether. All other chemicals were of the highest available purity from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany), or Sigma (Deisendorf, Germany). Thionyl chloride was distilled before use, CH_3OH (Fluka; puriss.) was distilled from magnesium, and dichloromethane was filtered over basic aluminum oxide (Fluka; for chromatography; activity grade 1). Chromatography materials were purchased from Merck (Darmstadt, Germany) and Macherey–Nagel (Düren, Germany). XAD-4 and aluminum oxide were purchased from Sigma (Deisendorf, Germany).

Spectral measurements: UV/Vis spectra (λ_{max} [nm], $\log\varepsilon$ [$dm^3 mol^{-1} cm^{-1}$]): in H_2O, Hitachi U-3000 apparatus. CD spectra ($\lambda_{max/min}$ [nm], $\Delta\varepsilon$ [$dm^3 mol^{-1} cm^{-1}$]): in H_2O, Jasco-J715 spectropolarimeter. FTIR spectra ($\tilde{\nu}$ [cm^{-1}]): Mattson 3000 Galaxy Series instrument. NMR: Varian 500 UNITY Plus spectrometer, equipped with a pulse-field gradient unit, 5 mm indirect-detection probe, and 5 mm broadband direct-detection probes; apparent coupling constants J [Hz]. 1D ^1H NMR and ^{13}C NMR experiments: in [D_6]DMSO, δ([D_6]DMSO) = 2.49 ppm; in D_2O, δ(HDO) = 4.68 ppm. For 2D NMR homonuclear experiments spin-locked NOE (ROESY)[75,76] and total correlation spectroscopy (TOCSY)[77–79] was applied. For heteronuclear experiments, pulsed field gradient enhanced hetero-single-quantum coherence experiments (PFG-HSQC) were used for indirect detection of the low-γ carbon nuclei,[80,81] while pulsed field gradient enhanced ^1H-detected multiple-bond heteronuclear multiple-quantum coherence experiments (PFG-HMBC) were done with the standard gradient-enhanced HMQC pulse sequence[80] with an additional low-pass filter as in the original HMBC experiment.[82] FAB MS spectra (m/z (rel. int.)): Finnigan MAT95 spectrometer, with nitrobenzyl alcohol (NOBA) matrix and Cs^+ bombardment.

Electrosynthetic experiments: All preparative electrochemical experiments were carried out in a glove box (Mecaplex GB-80) containing less than 10 ppm of oxygen. The work up of the reactions was done outside of the glove box in a dark room under dim light. Electrolysis: PAR model 170, two-compartment electrolysis cell, Hg-pool working electrode, Pt-counter electrode, 0.1 N calomel reference electrode (0.1 N CE). (See ref. [28] for a general discussion of these methods.)

Syntheses and analyses

Preparation of 5′-(chloromethyl)-5′-deoxyadenosine (5): With protection from air, hexamethylphosphortriamidate (HMPT; 3 mL) was added to a dry 10 mL 2-necked round-bottom flask, which was flushed with argon and cooled to 0 °C (external ice bath). Then, freshly distilled thionyl chloride (300 μL, 4.12 mmol, 491 mg) was added and the yellow solution was stirred for 20 min. After addition of dry 5′-homoadenosine (**6**; 290 mg, 1.03 mmol), the resulting mixture was stirred at 0 °C for 6 h, whereupon a homogeneous solution was formed. The reaction mixture was poured on to ice, 25% aqueous NH_3 (1 mL) was added, and the solvents were removed by using a rotatory evaporator and water-aspirator vacuum. Monitoring by TLC (silica gel 60; CH_2Cl_2/CH_3OH 5:1) indicated complete conversion. The remaining solvents were removed under high vacuum and the solid residue was suspended in CH_2Cl_2/CH_3OH, to remove inorganic salts. The dissolved product mixture was purified by column chromatography (dimensions: 3 × 15 cm; silica gel 60 (50 g); CH_2Cl_2/CH_3OH 5:1) and by using a rotatory evaporator and water-aspirator vacuum to remove the solvents from the homogeneous fractions of **5**. By using high vacuum, the solid white residue of **5** was dried (227 mg, 757 μmol, 74%). M.p. 85 °C (recrystallized from H_2O); R_f = 0.58 (CH_2Cl_2/CH_3OH 5:1); ^1H NMR ([D_6]DMSO): δ = 2.02–2.25 (m, 2H; 5′), 3.57–3.99 (m, 2H; 6′), 4.03 (m, $J \approx 4.5$ Hz, 1H; 4′), 4.12 (m, $J_1 \approx J_2 \approx J_3 \approx 5.6$ Hz, 1H; 3′), 4.69 (m, $J_1 \approx J_2 \approx J_3 \approx 5.6$ Hz, 1H; 2′), 5.26 (d, J = 5.4 Hz, 1H; OH3′), 5.47 (d, J = 5.9 Hz, 1H; OH2′), 5.86 (d, J = 5.4 Hz, 1H; L′), 7.27 (brs; NH_2), 8.13 (s, 1H; A2), 8.32 ppm (s, 1H; A8); ^{13}C NMR ([D_6]DMSO): δ = 36.2 (t; 5′), 42.2 (t; 6′), 73.0/73.3 (d; 2′ and 3′), 80.9 (d; 4′), 87.9 (d; L′), 119.4 (s; A5), 140.3 (d; A8), 149.5 (s; A4), 152.8 (d; A2), 156.3 ppm (s; A6); IR (KBr): $\tilde{\nu}$ = 3422 (vs), 3218 (s), 2926 (m), 2855 (w), 1647 (vs), 1605 (s), 1578 (m), 1478 (m), 1421 (m), 1383 (w), 1333 (m), 1300 (m), 1248 (w), 1205 (w), 1177 (w), 1128 (m), 1093 (w), 1055 cm^{-1} (m); UV/Vis (CH_3OH, c = 6.54 × 10^{-4} M): λ_{max} ($\log\varepsilon$) = 260 nm (4.15 $dm^3 mol^{-1} cm^{-1}$); CD (CH_3OH, c = 1.92 × 10^{-4} M): $\lambda_{max/min}$ ($\Delta\varepsilon$) = 267 (−0.59), 259 (sh) (−0.50), 228 nm (+0.27 $dm^3 mol^{-1} cm^{-1}$); MS (EI pos.): m/z (%): 299.1 (2) [M^+] ($C_{11}H_{14}ClN_5O_3$), 264.1 (6), 194.1 (8), 180.1 (7), 179.1 (6), 178.1 (23), 166.1 (18), 165.1 (18), 164.1 (95) [M−adenine]$^+$, 136.1 (100) [adenine+H]$^+$, 135.2 (95), 108.0 (29), 91.0 (52).

Preparation of 5′-(2-chloroethyl)-5′-deoxyadenosine (7): With protection from air, HMPT (4.8 mL) was added into a dry 25 mL 2-necked round-bottom flask, which was flushed with argon and cooled to 0 °C (external ice bath). Then, freshly distilled thionyl chloride (595 μL, 8.2 mmol, 976 mg) was added and the yellow solution was stirred for 20 min. After addition of dry 5′-(2-hydroxyethyl)-5′-deoxyadenosine (**8**; 604 mg, 2.05 mmol), the resulting mixture was stirred at 0 °C for 1 h and then overnight at room temperature. The reaction mixture was poured on to ice, 25% aqueous NH_3 (4 mL) was added, and the solvents were removed by using a rotatory evaporator and water-aspirator vacuum. Monitoring by TLC (silica gel 60; CH_2Cl_2/CH_3OH 5:1) indicated complete conversion. The concentrated aqueous solution of the product was stored in a refrigerator at 4 °C to yield a microcrystalline white precipitate, which was dried under high vacuum to give pure **7** (425 mg, 1.35 mmol). The solvents were removed from the mother liquor under high vacuum and the solid residue was purified by column chromatography (dimensions:

3×15 cm; silica gel 60 (50 g); CH_2Cl_2/CH_3OH 6:1). By using a rotatory evaporator and water-aspirator vacuum the solvents were removed from the homogeneous fractions of **7** to provide (after drying under high vacuum) a second crop of solid white **7** (89 mg). **7** was obtained in an overall yield of 514 mg (1.64 mmol, 80%). R_f=0.42 (CH_2Cl_2/CH_3OH 5:1); ^{1}H NMR ([D$_6$]DMSO): δ=1.60–1.90 (m, 4H; 5′ and 6′), 3.60–3.70 (m, 2H; 7′), 3.84 (m, J_1=4.4 Hz, 1H; 4′), 4.05 (m, $J\approx$4.5 Hz, 1H; 3′), 4.63 (m, $J\approx$4.9 Hz, 1H; 2′), 5.17 (d, J=4.9 Hz, 1H; OH3′), 5.43 (d, J=5.4 Hz, 1H; OH2′), 5.83 (d, J=5.4 Hz, 1H; 1′), 7.26 (br s, 2H; NH_2), 8.13 (s, 1H; A2), 8.29 ppm (s, 1H; A8); ^{13}C NMR ([D$_6$]DMSO): 28.9 (t; 6′), 30.6 (t; 5′), 45.5 (t; 7′), 73.2 (d; 3′), 73.4 (d; 2′), 83.2 (d; 4′), 87.9 (d; 1′), 119.4 (q; A5), 140.1 (d; A8), 149.5 (q; A4), 152.8 (d; A2), 156.2 ppm (q; A6); IR (KBr): $\tilde{\nu}$=3331 (vs), 3146 (vs), 2932 (m), 1674 (vs), 1645 (vs), 1607 (vs), 1576 (m), 1476 (m), 1445 (w), 1422 (m), 1385 (m), 1337 (m), 1300 (m), 1246 (m), 1206 (m), 1177 (w), 1125 (m), 1094 (m), 1067 cm^{-1} (m); UV/Vis (CH_3OH, $c=8.03\times10^{-4}$ M): λ_{max} ($\log\varepsilon$)=260 nm (4.09 dm^{3}mol^{-1}cm^{-1}); CD (CH_3OH, $c=1.75\times10^{-3}$ M): $\lambda_{max/min}$ ($\Delta\varepsilon$)=265 (−0.86), 233 nm (+0.05 dm^{3}mol^{-1}cm^{-1}); MS (CI pos., isobutane): m/z (%): 316.0 (29), 315.0 (12), 314.0 (100) $[M+H]^+$ ($C_{12}H_{17}ClN_5O_3$), 278.1 (6), 136.0 (14).

Preparation of homocoenzyme B_{12} (2): Aquocobalamin chloride (**4**; 50 mg, 36.2 μmol) was dissolved in 0.1 M TBAHFP$_6$ in CH_3OH (approximately 4 mL). The red solution was placed into the cathode chamber of a two-compartment electrolysis cell and was stirred magnetically. It was reduced at an Hg electrode at a potential of −1.1 V versus a 0.1 N calomel electrode. After 4 h of reduction and under protection from light, 5′-chloromethyl-5′-deoxyadenosine (**5**; 16.2 mg, 54.2 μmol, 1.5 equiv) was added, while the cathode was kept at the same potential. The reduction was continued for 6 h (10 h total time of electrolysis), after which time the current had dropped to 0.02 mA and 6.7 C had been consumed. The mixture was transferred into a separating funnel charged with water (15 mL; with protection from light) and extracted three times with an equal volume of dichloromethane. The aqueous layer was transferred into a round-bottom flask and the solvents were removed under vacuum. The residue was taken up in a minimal amount of water, and acetone was added dropwise until a slight turbidity appeared. After storage in a refrigerator overnight, crystalline homocoenzyme B_{12} (**2**; 54.3 mg, 94%) was obtained. The purity of recrystallized **2** was checked by running an ^{1}H NMR spectrum (500 MHz, c=5 mM, D$_2$O, 26 °C, 4096 scans), in which ^{13}C satellites could be observed as reference signals. R_f=0.22 (CH_3OH/H_2O 9:1); ^{1}H NMR and ^{13}C NMR data (D$_2$O): see Table 1; IR (KBr): $\tilde{\nu}$=3407 (vs), 3202 (s), 2963 (m), 2928 (m), 1665 (vs), 1657 (vs), 1562 (m), 1489 (m), 1476 (m), 1404 (m), 1350 (w), 1306 (w), 1223 (m), 1155 (w), 1107 (w), 1069 cm^{-1} (m); UV/Vis (H_2O, $c=4.42\times10^{-4}$ M): λ_{max} ($\log\varepsilon$)=519 (3.90), 500 (sh) (3.88), 442 (sh) (3.60), 376 (3.97), 344 (4.07), 318 (4.10), 289 (4.21), 263 nm (4.46 dm^{3}mol^{-1}cm^{-1}); CD (H_2O, $c=4.42\times10^{-4}$ M): $\lambda_{max/min}$ ($\Delta\varepsilon$)=553 (−6.9), 488 (+7.9), 435 (−3.6), 387 (+6.5), 359 (−7.1), 336 (+0.4), 299 (+6.2), 273 (+6.5), 257 (−2.0), 241 nm (+2.7 dm^{3}mol^{-1}cm^{-1}); MS (FAB pos., NOBA): m/z (%): 1597.4 (7), 1596.4 (20), 1595.4 (40), 1594.4 (37) $[M+H]^+$ ($C_{73}H_{103}CoN_{18}O_{17}P$), 1331.4 (42), 1330.4 (100) $[M-C_{11}H_{13}N_5O_3]^+$.

Preparation of bishomocoenzyme B_{12} (3): Aquocobalamin chloride (**4**; 50 mg, 36.2 μmol) was dissolved in 0.1 M $LiClO_4$ in water/*tert*-butanol (1:1; approximately 5 mL). The red solution was placed into the cathode chamber of a two-compartment electrolysis cell and was stirred magnetically. It was reduced at an Hg electrode at a potential of −1.1 V versus a 0.1 N calomel electrode. After 11 h of reduction, 6.0 C was consumed (about 1.7 F mol^{-1}). Under protection from light, 5′-(2-chloroethyl)-5′-deoxyadenosine (**7**; 19.0 mg, 60.6 μmol, 1.67 equiv) was added as a suspension in electrolyte solution, while the cathode was kept at the same potential. The reaction was continued overnight for 15 h, after which time the mixture was transferred into a round-bottom flask (with protection from light). The solvents were evaporated (under vacuum and at room temperature), then the residue was dissolved in a small amount of water and precipitated with acetone. The red solid (containing a small amount of starting material) was purified by column chromatography with RP18 (15.6 g) in a 2 cm×8.5 cm column and with an acetonitrile/water eluent (gradient from 8% to 18% acetonitrile in steps of 1%, 30 mL of each). The product fraction was evaporated, the residue was taken up in a minimal amount of water, and acetone was added dropwise until a slight turbidity appeared. After storage in a refrigerator overnight, crystalline bishomocoenzyme B_{12} (**3**; 45.0 mg, 77.3%) was obtained. The purity of recrystallized **3** was checked by observation of ^{13}C satellites as the reference in a ^{1}H NMR spectrum (500 MHz, c=5 mM, D$_2$O, 26 °C, 4096 scans). R_f=0.32 (CH_3OH/H_2O 9:1); ^{1}H NMR and ^{13}C NMR data (D$_2$O): see Table 1; IR (KBr): $\tilde{\nu}$=3413 (vs), 3214 (s), 2961 (w), 2926 (m), 1855 (w), 1636 (vs), 1568 (m), 1489 (m), 1476 (m), 1405 (w), 1255 (w), 1157 (w), 1082 cm^{-1} (w); UV/Vis (H_2O, $c=3.08\times10^{-4}$ M): λ_{max} ($\log\varepsilon$)=512 (3.95), 445 (sh) (3.63), 377 (sh) (3.96), 346 (4.11), 316 (4.13), 289 (4.22), 263 nm (4.47 dm^{3}mol^{-1}cm^{-1}); CD (H_2O, $c=3.08\times10^{-4}$ M): $\lambda_{max/min}$ ($\Delta\varepsilon$)=554 (−5.3), 496 (+8.6), 437 (−5.1), 387 (+5.8), 329 (−2.4), 299 (+5.2), 271 (+7.6), 247 nm (+1.6 dm^{3}mol^{-1}cm^{-1}); MS (FAB pos., glycerin): m/z (%): 1608 (24) $[M+H]^+$ ($C_{74}H_{105}CoN_{18}O_{17}P$), 1331 (100), 1330.3 (80) $[M-C_{12}H_{15}N_5O_3]^+$.

X-ray crystal structure analysis of homocoenzyme B_{12}: Crystals of **2** were grown from water/acetone. A crystal specimen was immersed in hydrocarbon oil, picked up with a rayon loop, and quickly cooled to cryotemperature by immersing in liquid nitrogen. Diffraction experiments were carried out on the EMBL beamline BW7b instrument, at DESY in Hamburg (Germany), equipped with a MAR345 imaging-plate detector and a gas-stream low-temperature (103(2) K) device.

Indexing of diffraction images, intensity integration, and data scaling were performed with the Denzo/Scalepack programs.[83] The structure was solved by direct methods to yield the Co atoms plus most remaining atoms of the structure. Missing atoms (mostly in the solvent region) were located in subsequent electron-density maps. Full-matrix least-squares refinement on F^2 was performed with the program SHELXL-97.[84] No absorption correction was applied to the data. Scattering factors including real and imaginary dispersion corrections were taken from the 'International Tables of Crystallography'. Hydrogen atom positions were calculated and refined as 'riding' on their respective non-hydrogen atoms. Methyl torsion angles were chosen to maximize the electron density at

Table 6. Crystallographic data for homocoenzyme B_{12} (**2**)

	Homocoenzyme B_{12} (**2**)
empirical formula	$C_{73}H_{102}N_{18}O_{17}PCo$
H_2O sites	29
sum of partial occupancies	17.5
acetone sites	3
formula weight	2083.1
crystal system	monoclinic
space group	$P2_1$
unit cell dimensions:	
a [Å]	14.896(3)
b [Å]	24.544(5)
c [Å]	15.119(3)
β [°]	111.35(3)
V [Å^3]	5148(2)
Z	2
ρ_{calcd} [g cm^{-3}]	1.344
μ [mm^{-1}]	0.27
F(000)	2230
crystal size [mm^3]	0.4×0.3×0.05
θ range for data collection [°]	2.2–29.8
wavelength [Å]	0.8431
reflections collected	30267
data reduction programs	Denzo/Scalepack
independent reflections	8903
R(int)	0.047
completeness to θ=29.8°	98.7%
data/restraints/parameters	8890/168/1340
final R indices (all data):	
R_1	0.0585
wR_2	0.1640
largest difference peak/hole [e Å^{-3}]	0.69/−0.67

the three calculated hydrogen-atom positions and were allowed to refine within the program. An analogous procedure was applied to the two ribose hydroxy groups. The isotropic adp for each hydrogen atom was set to 1.5 times the equivalent isotropic adp of the adjacent non-hydrogen atom. The solvent electron density was modeled with 3 acetone molecules and 29 partially occupied H_2O sites. Data pertaining to data collection and structure refinement and crystallographic residuals at the close of the refinement are given in Table 6.[85]

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