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Synthesis and Characterization of 3′,4′-Anhydroadenosylcobalamin: A Coenzyme B_{12} Analogue with Unusual Properties

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Abstract: The question of how coenzyme B_{12}-dependent enzymes facilitate the cleavage of the Co−C bond of the cofactor is of interest. We have synthesized an analogue of 5′-deoxyadenosylcobalamin (AdoCbl[1]) designed to stabilize the 5′-deoxyadenosyl radical (5′-deoxyadenosine-5′-yl) that is produced upon homolysis of the Co−C bond. By replacement of the upper axial ligand of AdoCbl by a 3′,4′-anhydro-5′-deoxyadenosyl moiety, the radical formed on the nucleoside analogue is stabilized by allylic delocalization. The compound, 5′-deoxy- 3′,4′-anhydroadenosylcobalamin (3′,4′-anAdoCbl) was synthesized by chemical and enzymatic methods. The final step was coupling of cob(I)alamin and 3′,4′-anhydroATP catalyzed by CobA, an ATP: corrinoid adenosyltransferase. 3′,4′-anAdoCbl displays interesting properties. The compound has not been purified to homogeneity due to its thermal and oxygen sensitivity. It was characterized by UV−vis spectroscopy, ESI-MS, and NMR spectroscopy. The bond dissociation energy of the Co−C bond of the analogue was measured by radical trapping techniques. A significantly weaker bond (24 ± 2 kcal/mol) as compared to AdoCbl (30 kcal/mol) was observed, as was homolytic cleavage at ambient temperature. Photolysis experiments conducted under anaereobic conditions reveal no formation of cob(II)alamin, whereas the compound breaks down rapidly under aerobic conditions as measured by cob(III)alamin formation. We postulate that the weak Co−C bond is cleaved reversibly by photolysis, where recombination of the allylic radical and cob(II)alamin occurs efficiently in the absence of a radical scavanger. Activation of the coenzyme B_{12}-dependent enzymes diol dehydrase and ethanolamine ammonia-lyase was observed with the cofactor analogue. The measured activity was low and no formation of cob(II)alamin could be detected in the steady-state of the reaction for either enzyme. Comparative interactions of AdoCbl and 3′,4′-anAdoCbl with diol dehydrase and ethanolamine ammonia-lyase suggest that cleavage of the Co−C bond is facilitated by enzyme-coenzyme binding contacts that are remote from the Co−C bond.

Introduction

Enzymes that use coenzyme B_{12}, facilitate the cleavage of the Co−C bond of the cofactor to generate a 5′-deoxyadenosyl radical, which acts as an initiator in radical rearrangements.[2] Most coenzyme B_{12}-dependent enzymes catalyze a 1,2 hydrogen shift, where the other migratory substituent is either a heteroatom, as in the cases of diol dehydrase, ethanolamine ammonia-lyase, and lysine 5,6-aminomutase, or a carbon atom as for methylmalonyl CoA mutase and glutamate mutase.[3] One class of ribonucleotide reductases use AdoCbl to generate a transient thiyl radical in their catalytic mechanism.[4]

The Co−C bond of adenosylcobalamin is weak relative to most covalent bonds and displays a bond dissociation energy

(1) Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); 3′,4′-anAdoCbl, 5′-deoxy-3′,4′-anhydroadenosylcobalamin; B_{12s}, cob(I)-alamin; B_{12r}, cob(II)alamin; B_{12a}, cob(III)alamin; DMB, dimethylbenzimidazole; DDH, diol dehydrase; EAL, ethanolamine ammonia-lyase; TEMPO, 2,2,6,6,-tetramethylpiperidinyl-1-oxy; HPLC, high performance liquid chromatography; yADH, yeast alcohol dehydrogenase; ESI-MS, electrospray ionization mass spectrometry.

(2) Frey, P. A. *Chem. Rev.* **1990**, *90*, 1343−1357.

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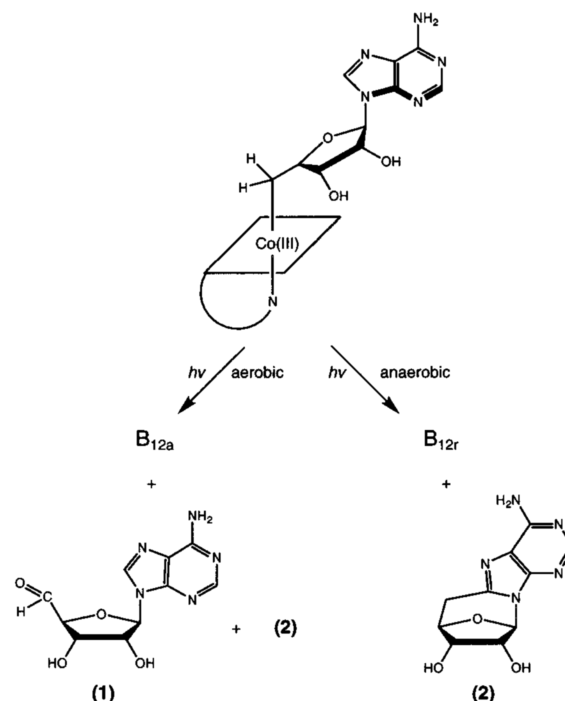
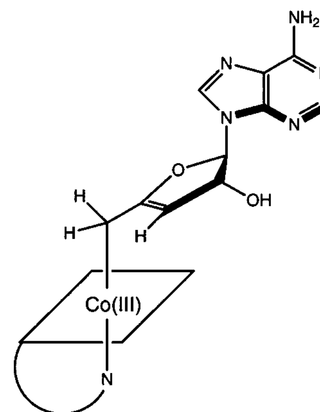
of about 30 kcal/mol.[5,6] Enzymes that use coenzyme B_{12} must facilitate the cleavage of the Co−C bond by as much as 12 orders of magnitude, which corresponds to a destabilization of the Co−C bond by ∼15 kcal/mol.[7] How this large rate enhancement is achieved has remained somewhat of an enigma, but a number of hypotheses have been put forward. These include a steric distortion by an upward flexing of the corrin ring that would weaken the Co−C bond,[8−12] the so-called trans steric effect, and the trans electronic effect which entails the importance of the lower axial ligand, dimethylbenzimidazole (DMB) in modulating the strength of the Co−C bond.[13,14] Both of these hypotheses are mainly based on model and structural studies of the coenzyme and different corrinoids. The importance of the lower axial ligand has been drawn into question by FT-Raman[15] and resonance Raman studies[16] on alkylcobalamins that show that the strength of the Co−C bond is affected very little by the lower ligand, and its role appears more complex, especially since some of the B_{12}-dependent enzymes replace the DMB moiety with a histidine residue of the enzyme.[17−19] Theoretical work,[20,21] enzymatic studies with cofactor analogues[22] and spectroscopic studies[23] of enzyme-bound AdoCbl have postulated direct bending or other distortion of the adenosyl moiety to cleave the bond.

The weak Co−C bond of AdoCbl is photolabile, and the homolytic cleavage of the bond generates the very unstable 5′-deoxyadenosyl radical that is quenched rapidly in solution. Hogenkamp and co-workers[24,25] have shown that anaerobic photolysis of AdoCbl yields cob(II)alamin (B_{12r}) and 5′-deoxy-8,5′-cycloadenosine **(2)** (Scheme 1). In the presence of oxygen, B_{12r} is oxidized to cob(III)alamin (B_{12a}), and the adenosyl radical is converted to **(2)** and adenosine-5′-carboxaldehyde **(1)** (Scheme 1). Here we report on the synthesis and characterization of an oxygen-sensitive AdoCbl analogue that displays an unusual

Scheme 1



Scheme 2



resistance to photolysis under anaerobic conditions. The compound, 3′,4′-anhydroadenosylcobalamin (Scheme 2) activates both diol dehydrase (DDH) and ethanolamine ammonia-lyase (EAL) and owing to allylic stabilization of the 5′-deoxyadenosyl radical should be very useful in studying the first step in the catalytic cycle of coenzyme B_{12}-dependent enzymes, the cleavage of the Co−C bond.

Experimental Section

Materials. AdoCbl, hydroxocobalamin (B_{12a}), KBH_4, alcohol dehydrogenase, and NADH were from Sigma. TEMPO and ethylene glycol were obtained from Aldrich. All other chemicals and reagents were commercial products of the highest purity.

Synthesis of 3′,4′-anhydroATP. This compound was synthesized by enzymatic phosphorylation of 3′,4′-anhydroadenosine as described in our previous communication.[26] MALDI-MS, calculated mass for $C_{10}H_{14}N_5O_{12}P_3$ = 489.0, found *m/e* M(H^+) = 490.0. ^1H NMR (200 MHz in D_2O) δ 8.17 (s, 1 H, H-2 or H-8), δ 8.13 (s, 1 H, H-2 or H-8), δ 6.42 (d, 1 H, H-1′); δ 5.54 (d, 1 H, H-3′); δ 5.15 (t, 1 H, H-2′); δ 4.57 (d, 2H, H-5′$_2$). ^{31}P NMR (81 MHz, referenced to 80% H_3PO_4.) δ −6.5 (γ-P); δ −10.2 (α-P); δ −21.5 (β-P).

(3) For recent reviews on B_{12}-dependent enzymes, see: *Chemistry and Biochemistry of B12*; Banerjee, R., Ed.; Wiley-Interscience: New York, 1999.
(4) Licht, S.; Gerfen, G. J.; Stubbe, J. *Science* **1996**, *271*, 477−481.
(5) Finke, R. G.; Hay, B. P. *Inorg. Chem.* **1984**, *23*, 3041−4043.
(6) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 4820−4829.
(7) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 8012−8018.
(8) Grate, J. H.; Schrauzer, G. N. *J. Am. Chem. Soc.* **1979**, *101*, 4601−4611.
(9) Chemaly, S. M.; Pratt, J. M.*J. Chem. Soc., Dalton. Trans.* **1980**, 2274−2281.
(10) Marzilli, L. G.; Toscano, J.; Randaccio, L.; Breschiani-Pahor, N. Calligaris, M. *J. Am. Chem. Soc.* **1979**, *101*, 6754−6756.
(11) Halpern, J.; Sook-Hui, K.; Leung, T. W. *J. Am. Chem. Soc.* **1984**, *106*, 8317−8118.
(12) Krautler, B.; Konrat, R.; Stupperich, E.; Gerald, F.; Gruber, K.; Kratky, C. *Inorg. Chem.* **1994**, *33*, 4128−4139.
(13) Ng, F. T. T.; Rempel, G. L.; Halpern, J. *J. Am. Chem. Soc.* **1982**, *104*, 621−623.
(14) Marzilli, L. G.; Summers, M. F.; Bresciani-Pahor, N.; Zangrando, E.; Charland, J. P.; Randaccio, L. *J. Am. Chem. Soc.* **1985**, *107*, 6880−6888.
(15) Puckett, J. M.; Mitchell, M. B.; Hirota, S., Marzilli, L.*Inorg. Chem.* **1996**, *35*, 4656−4662.
(16) Dong, S.; Padmakumar, R.; Banerjee, R.; Spiro, T. G. *J. Am. Chem. Soc.* **1996**, *118*, 9182−9183.
(17) Padmakumar, R.; Taoka, S.; Padmakumar, R.; Banerjee, R. *J. Am. Chem. Soc.* **1995**, *117*, 7033−7034.
(18) Zelder, O.; Beatrix, B.; Kroll, F.; Buckel, W. *FEBS Lett.* **1995**, *369*, 252−254.
(19) Chang, C. H.; Frey. P. A. *J. Biol. Chem.* **2000**, *275*, 106−114
(20) Christianson, D. W.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1985**, *107*, 2682−2686.
(21) Marques, H. M.; Brown, K. L. *Inorg. Chem.* **1995**, *34*, 3733−3740.
(22) Toraya, T.; Matsumoto, T.; Ichikawa, M.; Itoh, T.; Sugawara, T.; Mizuna, Y. *J. Biol. Chem.* **1986**, *261*, 9289−9293.
(23) Dong, S.; Padmakumar, R.; Banerjee, R.; Spiro, T. G. *J. Am. Chem. Soc.* **1999**, *121*, 7063−7070.
(24) Hogenkamp, H. P. C. *J. Biol. Chem.* **1963**, *238*, 477−480.
(25) Hogenkamp, H. P. C.; Ladd, J. N.; Barker, H. A. *J. Biol. Chem.* **1962**, *237*, 1950−1952.

(26) Magnusson, O. Th.; Reed, G. H.; Frey, P. A. *J. Am. Chem. Soc.* **1999**, *121*, 9764−9765.

Expression and Purification of ATP:Corrinoid Adenosyltransferase. An *Escherichia coli* overproducing strain JE2875 from a pT7-7 plasmid in BL21(DE3) cells was a generous gift of Prof. J. C. Escalante-Semerena, University of Wisconsin-Madison. Cells were grown in LB media, induced by 0.5 mM IPTG and harvested 6 h after induction. CobA was purified as described elsewhere.[27] The purified enzyme was precipitated by ammonium sulfate (70% saturation), resuspended in anaerobic Tris buffer (50 mM, pH 8), frozen and stored at −70 C.

Synthesis of 3′,4′-anAdoCbl. A 50 mL solution containing 0.2 mM 3′,4′-anhydroATP, 0.17 mM OH-cobalamin, 1.5 mM $MgCl_2$, 0.5 mM $CoCl_2$, and 50 mM Tris•HCl at pH 8 was deoxygenated under a stream of argon for 1 h. The sealed flask was transferred to a Coy anaerobic chamber, and 20 mg of KBH_4 was added to reduce cob(III)alamin to cob(I)alamin. The reaction was started by the addition of 20 mg of CobA, and its progress was monitored spectrophotometrically at 530 nm. After a 2 h incubation, the reaction mixture was cooled on ice and transferred to an Amicon pressure cell with a YM10 membrane. The protein was separated from the cobalamins and smaller molecules by this ultrafiltration method, and the filtrate was collected in a sealed flask under argon. The filtrate was subjected to a second ultrafiltration step using a YM1 membrane. This removed salts, and the retentate containing the product was collected, frozen, and stored in liquid nitrogen.

Photolysis. Anaerobic samples of AdoCbl and 3′,4′-anAdoCbl were prepared in the anaerobic chamber using solutions that were deoxygenated with O_2 free Argon. Aerobic samples were prepared using distilled water. Concentration of cobalamins was approximately 50 μM in each case. Samples in quartz cuvettes, fitted with Teflon stoppers, were kept on ice and irradiated with a 150 W tungsten lamp at a distance of 50 cm. At designated time points UV−vis spectra were aquired on a Hewlett-Packard, model 8452, diode array spectrophotometer. Anaerobic samples were monitored for cob(II)alamin formation by the decrease in absorbance at 525 nm, while aerobic samples were monitored for cob(III)alamin formation by increase in absorbance at 355 nm. Samples with TEMPO contained 20-fold excess (1 mM) of the radical trap. First-order rate constants (k_{obs}) where determined by fitting the data to a single-exponential equation (eq 1) using *Kaleidagraph*, where A_t is the absorbance at time t, A_o is the initial absorbance, and ΔA is the difference between the final and initial absorbance.

$$A_t = \Delta A[(1 - \exp(-k_{obs}t))] + A_o \quad (1)$$

Thermolysis Kinetics. Samples for kinetic runs were prepared on ice in the anaerobic chamber and put into Teflon-stoppered quartz cuvettes. Samples contained 55 μM 3′,4′-anAdoCbl and 2 mM TEMPO ($\geq$30 equiv) in anaerobic ethylene glycol. Samples were transferred to a circulating water bath (Fisher Scientific), thermally equilibrated for 2 min and incubated at the desired temperature. At designated time points, the cuvettes were quickly removed from the water bath and spectra acquired. Thermal equilibration was tested in control samples, and data points were excluded if they did not meet the criteria suggested by Brown and Evans.[28] Kinetic runs were measured at 4 °C intervals between 12 and 32 °C (±0.1 °C) by monitoring the decrease in absorbance at 530 nm. First-order rate constants were determined by single-exponential fits as described above. All fits gave correlation coefficients of $R^2 = 0.9998$ or better.

Nucleoside Products. Approximately 7 μmol of 3′,4′-anAdoCbl and 25 μmol of TEMPO were incubated at 25 °C in 20 mL of Argon flushed water for 2 h. The solution was concentrated by rotary evaporation and analyzed by HPLC using a C_{18} column (Phenomenex, 250 × 10 mm). A linear gradient of 40% to 100% MeOH in 40 min was employed. Two compounds eluting at 31 and 33 min were characterized by NMR spectroscopy as 3′-TEMPO-4′,5′-anhydroadenosine (1.1 μmol) and 5′-TEMPO-3′,4′-anhydroadenosine (5.3 μmol), respectively. The yield of each compound was estimated by the absorbance at 260 nm using the molar extinction coefficient of adenosine, 14,700 $M^{-1}cm^{-1}$. The total yield was ≥90%. ^{1}H NMR (400 MHz in d_6-DMSO) for 5′-TEMPO-3′,4′-anhydroadeosine: δ 8.16 (s, 1 H, H-2 or H-8); δ 8.12 (s, 1 H, H-2 or H-8), δ 7.33 (s, 2 H, NH_2), δ 6.26 (d, 1 H, H-1′, J = 2.5 Hz), δ 5.36 (d, 1 H, H-3′, J = 2.5 Hz), δ 5.15 (t, 1 H, H-2′, J = 2.5 Hz), δ 4.34 (ABq, 2 H, H-5′$_2$, J = 13 Hz, the inner lines are almost fully collapsed, and the outer lines are only ~5% of the intensity of the middle line), δ 1.25−1.53 (m, 6 H, TEMPO CH_2's), δ 1.13 (s, 6 H, TEMPO CH_3's), δ 1.05 (s, 3 H, TEMPO CH_3), δ 1.03 (s, TEMPO CH_3). ^{1}H NMR for 3′-TEMPO-4′,5′-anhydroadenosine: δ 8.27 (s, 1 H, H-2 or H-8), δ 8.17 (s, 1 H, H-2 or H-8), δ 7.32 (s, 2 H, NH_2), δ 6.04 (d, 1 H, H-1′, J = 4.5 Hz), δ 4.95 (t, 1 H, H-2′, J = 4.5 Hz), δ 4.73 (d, 1 H, H-3′, J = 4.5 Hz), δ 4.53 (s, 1 H, H-5′$_a$), δ 4.42 (s, 1 H, H-5′$_b$), δ 1.42 (s, 6 H, TEMPO CH_3's), δ 1.22 (s, 3 H, TEMPO CH_3), δ 1.17 (s, 3 H, TEMPO CH_3), δ 0.97−1.27 (m, 6 H, TEMPO CH_2's).

Enzyme Assays. Samples of EAL and DDH from *Salmonella typhimurium* were gifts from Dr. Vahe Bandarian and Dr. Andreas Abend, respectively. The enzymes were purified as described elsewhere.[29,30] Since the product of both reactions is acetaldehyde, a coupled assay using yeast alcohol dehydrogenase (yADH) was employed. Assays with EAL contained 50 mM Hepes, pH 7.5, 10 mM ethanolamine, 0.15 mM NADH, 45 IU yADH, 20 μM AdoCbl or 3′,4′-anAdoCbl and the appropriate amount of EAL. Assays with DDH contained 100 mM KP_i, pH 8, 125 mM propanediol, 23 mM Na-cholate, 0.15 mM NADH, 45 IU yADH, 20 μM AdoCbl or 3′,4′-anAdoCbl and the appropriate amount of DDH. Assays were performed at 25 °C.

Results and Discussion

(A) Synthesis and Purification of 3′,4′-anAdoCbl. The ATP:corrinoid adenosyltransferase (cobA) catalyzes the alkylation of the strong nucleophile, cob(I) alamin (B_{12s}) by the 5′-carbon af ATP to produce AdoCbl and tripolyphosphate.[27] We used this enzymatic reaction with 3′,4′-anhydroATP and B_{12s} as substrates to make 3′,4′-anAdoCbl. The reaction was carried out under strict anaerobic conditions in an anaerobic chamber since B_{12s} formed by reduction of cob(III)alamin is highly susceptible to oxygen. With a slight excess of 3′,4′-anATP the reaction proceeded to completion, as measured by the increase in absorbance at 530 nm.[31] It became apparent that the AdoCbl analogue was very labile and sensitive toward oxygen, which made purification of the compound a daunting process. All conventional chromatographic procedures were unsuccessful due to degradation of the product (also see ref 31). It appears that interaction of the compound with any resin leads to decompositon through cleavage of the Co−C bond, since cob(II)alamin is the only corrinoid species detected. With this restriction in mind we used ultrafiltration to partially purify the compound. Use of a YM 10 membrane separates the protein catalyst from the rest of the reaction mixture. Partial separation of small molecules from the product was done using a 1000 molecular weight cutoff membrane, which serves to concentrate the product while removing salts and smaller molecules. Care has

(27) Suh, S. J.; Escalante-Semerena, J. C. *J. Bacteriol.* **1995**, *177*, 921−925.

(28) Brown, K. L.; Evans, D. R. *Inorg. Chem.* **1994**, *33*, 6380−6387.

(29) Bandarian, V.; Reed, G. H. *Biochemistry* **1999**, *38*, 12394−12402.

(30) Abend, A.; Nitsczhe, R.; Bandarian, V.; Stupperich, E.; Retey, J. *Angew. Chem., Int. Ed.* **1998**, *37*, 625−627.

(31) Formation of 3′,4′-anAdoCbl can also be detected by HPLC using a C_{18} column with a H_2O/MeOH gradient containing 0.02% TFA. The analogue has a similar retention time as authentic AdoCbl (18 min) and can easily be separated from other corrinoid species (B_{12a}, etc.). However, partial degradation of the compound occurs on the column, since a substantial amount of B_{12a} is also eluted (11 min). Under the acidic elution conditions, 3′,4′-anAdoCbl is in the base-off form due to protonation of the lower axial DMB ligand. By using an HPLC system with a diode-array detector, we were able to show that the peak eluting in ~18 min is that of a base-off 3′,4′-anAdoCbl by comparison to a base-off spectrum of authentic AdoCbl (data not shown). The base-off cobalamin appears to be very labile as well as the base-on species. Absorption spectra acquired of the eluted peak revealed partial degradation to a mixture of aquo-cob(III)alamin and base-off 3′,4′-anAdoCbl (data not shown). This observation implies that purification of the base-off species could be very difficult, a suggestion that came up during the review of this paper.

(32) Giannotti, C. In *B12*; Dolphin, D., Ed.; Wiley-Interscience: New York, 1982; Vol. 1, pp 393−430.

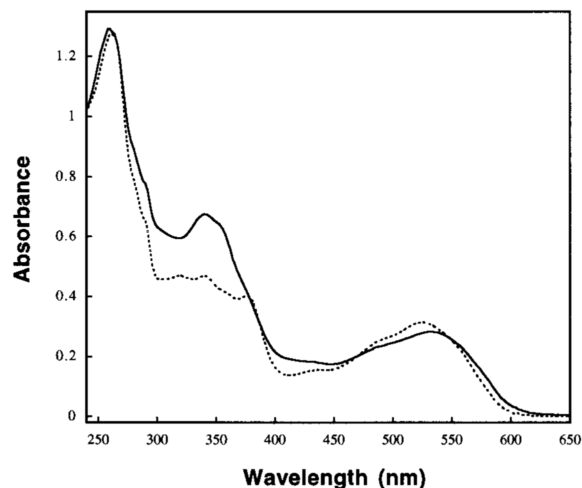


Figure 1. Absorption spectrum of 3′,4′-anAdoCbl (solid line) in comparison with AdoCbl (dotted line). Spectra were aquired at neutral pH under anaerobic conditions.

to be taken to run the reaction to near completion since separation of any contaminating corrinoids is not feasible by this method.

The UV−vis spectrum of partially purified 3′,4′-anAdoCbl is shown in Figure 1 in comparison with AdoCbl. The spectra look similar, except for an intensity difference of the γ-band at around 350 nm. This can partially be attributed to impurity in the preparation from B_{12a} (see part C). The difference can also be rationalized by the comparative properties of the axial ligands to the cobalt. It has been shown that there is a correlation between the difference in the σ-donor strength of the upper and lower axial ligands and the intensity of the γ-band. The closer matching of the σ-donors, the more intense the band becomes.[32] The lower ligand is in both cases a nitrogen donor (DMB); however, the allyl group of the 3′,4′-anhydroadenosyl moiety is a weaker σ-donor than the alkyl moiety of the adenosyl group and therefore a closer match to the N-donor.

Characterization of 3′,4′-anAdoCbl by 500 MHz ^{1}H NMR (D_2O) was done by comparing the aromatic region of the spectra to AdoCbl, an approach that has been used to access the purity of cobalamins.[33] Peaks in the high-field region of the spectra were difficult to interpret and compare to authentic AdoCbl, especially since the excess buffer components carried over from the synthesis are overlapping a part of the spectrum in that region. The abbreviations used for ^{1}H NMR assignments are: A, 5′-deoxyadenosyl; B, dimethylbenzimidazole base; R, ribose; C, corrin. The following chemical shifts were measured in the aromatic region: δ 8.17 (s, A2), δ 7.88 (s, A8), δ 7.16 (s, B7), δ 6.94 (s, B2), δ 6.28 (d, R1′), δ 6.21 (s, B4), δ 5.95 (s, C10), δ 5.88, (d, A1′). These values can be compared to the aromatic region of AdoCbl:[34] δ 8.19 (s, A2), δ 8.00 (s, A8), δ 7.16 (s, B7) δ 6.95 (s, B2), δ 6.26 (d, R1′), δ 6.24 (s, B4), δ 5.93 (s, C10), δ 5.56 (d, A1′). The key differences in the spectra are observed in the altered upper axial ligand where there is a shift from δ 8.00 to δ 7.88 for one of the adenine protons, and the anomeric proton on the ribose ring is shifted downfield by 0.32 ppm, from δ 5.56 to δ 5.88. Contamination by other corrinoid species was estimated to be $\leq$15% by comparison of the integrals for all the resonances in the aromatic region.

(33) Brasch, N. E.; Finke, R. G. *J. Inorg. Biochem.* **1999**, *73*, 215−219.
(34) Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, *108*, 4285−4294.
(35) Hogenkamp, H. In *B12*; Dolphin, D. Ed.; Wiley-Interscience: New York, 1982; Vol. 1, pp 295−323.

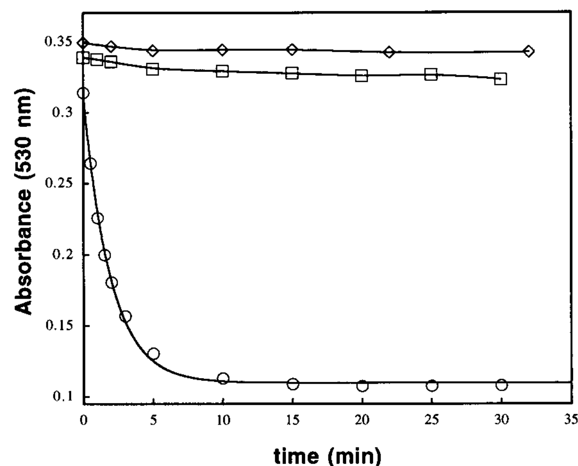


Figure 2. Photolysis of cobalamins under anaerobic conditions. 3′,4′-anAdoCbl in H_2O (□) and ethylene glycol (◇). Photolysis of AdoCbl in H_2O (○) yields a first-order rate constant of 0.5 min^{-1}.

A mass spectral analysis was obtained to further verify the chemical structure of the product. ESI-MS spectra revealed a small peak for 3′,4′-anAdoCbl plus K^+ at *m/z* 1600.5 (calcd 1600.7), while the largest peaks of the spectrum were due to fragmentation at the Co−C bond, giving $[(M + K) - 3',4'\text{-anAdo}]^+$ at $m/z = 1368.5$ (calcd 1368.5) and $[(M + K + H) - 3',4'\text{-anAdo}]^{2+}$ at $m/z = 684.4$ (calcd 684.5). These spectra were acquired using low-fragmentation voltage (100 V). At higher-fragmentation voltage the peak for the molecular ion disappeared, and only the fragments were observed. Spectra of AdoCbl acquired with a 100 V fragmentation voltage gave a strong signal for the molecular ion, while the peak for fragmentation at the Co−C bond was absent. This is an interesting observation and bears on results regarding the strength of the Co−C bond in these compounds as discussed later in the paper.

(B) Photolysis Experiments. The homolytic cleavage of the Co−C bond of AdoCbl can be induced by light. The products are B_{12r} and 5′-deoxy-8,5′-cycloadenosine, if the reaction is done anaerobically,[24] or B_{12a} and adenosine-5′-carboxaldehyde, when the reaction is conducted aerobically[25] (Scheme 1). Similar cyclic nucleosides and nucleoside aldehydes are also obtained upon photolysis of most purine-containing analogues of adenosylcobalamin.[35]

Photolysis experiments were conducted to examine the photolability of 3′,4′-anAdoCbl in comparison with AdoCbl. The experiments were done on ice using a tungsten lamp as a light source. Aerobic samples were monitored for cob(III)alamin formation at 355 nm. The analogue and AdoCbl yielded rate constants of 2.8 min^{-1} and 0.5 min^{-1}, respectively. Faster rate of photolysis for 3′,4′-anAdoCbl relative to AdoCbl is compatible with the expected weakness of the Co−C bond of the analogue. In fact, thermal cleavage of the Co−C bond of 3′,4′-anAdoCbl is readily observed at room temperature in the absence of light (see part C). Under anaerobic conditions, the rate constant for cob(II)alamin formation, as measured by a decrease in absorbance at 525 nm was 0.5 min^{-1} for AdoCbl; however, no formation of cob(II)alamin could be detected for 3′,4′-anAdoCbl (Figure 2). The failure to detect cob(II)alamin was tentatively attributed to the stability of the 3′,4′-anhydro-5′-deoxyadenosyl radical which apparently reacted with cob(II)alamin to regenerate 3′,4′-anAdoCbl instead of undergoing cyclization to 3′,4′-anhydro-5′,8-cycloadenosine.

To examine whether 3′,4′-anAdoCbl would be photolyzed, the experiment was conducted in the presence of 1 mM nitroxide

spin trap (TEMPO) to probe the reactivity of the allylic radical. Efficient trapping of the radical was observed concomitant with the formation of B_{12r}. The observed rate constants for the process were 0.5 min^{-1} and 1.5 min^{-1} for AdoCbl and 3′,4′-anAdoCbl, respectively. These results show that light induces homolysis of the Co−C bond, but it does not lead to a secondary reaction of the incipient allylic radical. Indeed, recombination of B_{12r} and the organic radical appears to be very efficient because no net formation of B_{12r} can be detected spectrophotometrically in the absence of dioxygen or TEMPO. Only in the presence of a radical scavanger is the allylic radical quenched. In addition, homolysis of 3′,4′-anAdoCbl in anaerobic ethylene glycol does not lead to B_{12r} formation, showing that the allylic radical does not abstract a hydrogen atom from the glycol (Figure 2). Both photolysis[35] and thermolysis of AdoCbl[6] in ethylene glycol lead to appreciable formation of 5′-deoxyadenosine due to hydrogen abstraction from the glycol. The allylic 3′,4′-anhydro-5′-deoxyadenosyl radical is however more stable than the primary 5′-deoxyadenosyl radical and does not abstract a hydrogen atom from ethylene glycol. Instead, it recombines with B_{12r} to reform the Co−C bond. The results can be rationalized on thermodynamic grounds by considering the estimated difference in bond dissociation energies of these compounds. The BDE's are approximately 97, 91, and 85 kcal/mol for RCH_2−H, HOC$(R)_2$−H, and CH_2=$CHCH_2$−H, respectively.[36] This predicts that while abstraction of a hydrogen atom from ethylene glycol by the primary adenosyl radical is feasible, the abstraction by the allylic radical is unfavorable.

The apparent inability of the allylic radical to cyclize can also be attributed in part to steric constraints. The vinyl group on the ribose causes flattening of the five-membered ring which may prevent the 5′-carbon from attacking the 8-postion of the adenine base. Spin delocalization is, of course, also contributing to the decrease in reactivity at the 5′-carbon. Taken together, the results are an example of the *persistent radical effect*.[37] The kinetic paths for cyclization and H-atom abstraction have been removed, and the two paramagnets recombine effectively, thereby suppressing any side reactions that could occur, such as dimerization of the allylic radical.

(C) Thermolysis of 3′,4′-anAdoCbl. Since oxygen is an efficient radical scavenger, sensitivity of 3′,4′-anAdoCbl in air is a clear indication of the lability of the Co−C bond. As mentioned above, the compound breaks down within minutes at room temperature in the presence of oxygen, as measured by the formation of B_{12a}. To get a quantitative assessment of the strength of the Co−C bond, we used the radical trapping technique developed by Finke and co-workers[5,6,38] to measure activation parameters and determine the bond dissociation energy of the compound. TEMPO has been shown to be a good spin trap and was successfully used by Hay and Finke to determine the bond dissociation energy of AdoCbl in ethylene glycol.[5,38] Equation 2a describes the kinetics for thermolysis of adenosylcobalamin using the TEMPO trapping technique[38]

$$\frac{-d[\mathrm{AdoB_{12}}]}{dt} = \{[K_{\mathrm{off\text{-}on}}/(K_{\mathrm{off-on}}+1)]k_{\mathrm{h,on}}\} \left\{\frac{k_c + k_a[\mathrm{HOCH_2CH_2OH}] + k_T\{\mathrm{TEMPO}]}{k_c + k_a[\mathrm{HOCH_2CH_2OH}] + k_T\{\mathrm{TEMPO}] + k_r[\mathrm{Co(II)B_{12}}]}\right\} [\mathrm{AdoB_{12}}] = k_{\mathrm{obs}}[\mathrm{AdoB_{12}}] \quad (2a)$$

where k_c is the rate constant for cyclization of the adenosyl radical, k_a refers to quenching by the solvent, ethylene glycol, k_r is the second-order rate constant for recombination of the radical with cob(II)alamin and k_T the rate constant for scavenging by the radical trap. K_{off-on} describes the equilibrium between the two forms of adenosylcobalamin where the lower axial, dimethylbenzimidazole ligand is either coordinated to the cobalt (base-on) or where the cobalt is five-coordinate (base-off). $k_{h,on}$ is the rate constant for homolysis of the base-on form only. The base-off form is more stable toward homolysis than the base-on AdoCbl. At ambient temperatures, the compound is predominantly in the base-on form, but at the elevated temperatures required to induce homolysis of AdoCbl,[5,38] a correction for this equilibrium needs to be employed due to the substantial amount of base-off AdoCbl. This kinetic equation can be simplified by considering that the allylic radical neither cyclizes nor is quenched by ethylene glycol as shown previously by the photolysis experiments, and more importantly by assuming that 3′,4′-anAdoCbl is exclusively in the base-on form at the relatively low temperatures required to induce homolysis, that is, $K_{off-on} \gg 1$. This latter assumption is verified by our observation that the absorption spectra of 3′,4′-anAdoCbl taken in ethylene glycol does not change between 12 and 32 °C, and thus the base-off form which has a different absorption spectra is not formed in this temperature range (data not shown). The kinetic equation (eq 2b) for thermolysis of 3′,4′-anAdoCbl can therefore be expressed in much simpler terms.

$$\frac{-d[\mathrm{anAdoB_{12}}]}{dt} = k_{\mathrm{h,on}}\left\{\frac{k_T\{\mathrm{TEMPO}]}{k_T\{\mathrm{TEMPO}] + k_r[\mathrm{Co(II)B_{12}}]}\right\}[\mathrm{anAdoB_{12}}] = k_{\mathrm{obs}}[\mathrm{anAdoB_{12}}] \quad (2b)$$

Conditions were established where the rate of homolysis was zero-order in TEMPO, making Co−C bond cleavage the rate-determining step (data not shown). In subsequent experiments, excess TEMPO was used (≥30 equiv) to determine the first-order rate constants for homolysis in ethylene glycol at temperatures ranging from 12 to 32 °C. A typical reaction profile is shown in Figure 3. The reaction is clean and shows the conversion to B_{12r} with isosbestic points at 327, 396, 492, and 597 nm, respectively. Notice the small shoulder at the end of the reaction. This represents the B_{12a} impurity in the preparation as discussed earlier. Using the $\Delta\epsilon_{520\ nm}$ of ~4 $mM^{-1}cm^{-1}$ for AdoCbl and B_{12r},[39] we can estimate that the preparation contains at least 85% of 3′,4′-anAdoCbl, an independent measurement that corroborates the NMR results discussed before. Kinetic data were fitted to a single-exponential equation to determine the rate constants (k_{obs}) at different temperatures. An Eyring plot (Figure 4) yielded the activation parameters $\Delta H^{\ddagger} = 29.5 \pm 0.4$ kcal/mol and $\Delta S^{\ddagger} = 27 \pm 2$ kcal/mol, respectively.[40]

To determine the bond dissociation energy of 3′,4′-anAdoCbl, we adopted the radical cage formalism developed for the determination of BDE of organotransition metal compounds in solution by Koenig et al.[41] This approach takes into account the formation of a caged radical pair in solution as described in general form in eq 3.[41]

(36) Carey, F. A.; Sundberg, R. J. *Advanced Organic Chemistry: Part A,* 3rd ed.; Plenum Press: New York, 1990; pp 683−684.

(37) Daikh, B. E.; Finke, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 2938−2943.

(38) Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1469−1481.

(39) Marsh, E. N. G.; Ballou, D. P. *Biochemistry* **1998**, *37*, 11864−11872.

(40) The reported values and errors were obtained by nonlinear regression fitting to the Eyring equation: $k = (kT/h)e^{\Delta S^{\ddagger}/R}\,e^{-\Delta H^{\ddagger}/RT}$. The linear plot is shown for clarity, and the parameters obtained by that method were identical within error to the ones obtained by the direct fitting procedure.

(41) Koenig, T. W.; Hay, B. P.; Finke, R. G. *Polyhedron* **1988**,*7*, 1499−1516.

Figure 3. Thermolysis of 3′,4′-anAdoCbl at 24 °C. The experiment was performed in ethylene glycol in the presence of 2 mM TEMPO. Spectra were aquired at the designated time points: 0 min, 5 min, 14, 28, and 70 min.

Figure 4. Eyring plot of k_{obs} for the thermolysis of 3′,4′-anAdoCbl. The values for k_{obs} (s^{-1}, ±3%) and temperatures (°C), respectively, are as follows: 9.93×10^{-5}, 12; 2.16×10^{-4}, 16; 4.55×10^{-4}, 20; 9.14×10^{-4}, 24; 1.83×10^{-3}, 28; 3.61×10^{-3}, 32.

$$\mathrm{M{-}L} \underset{k_c}{\overset{k_{1(soln)}}{\rightleftharpoons}} \underset{\text{cage pair}}{[\mathrm{M^\bullet\ {}^\bullet L}]} \underset{k_{1(soln)}}{\overset{k_d}{\rightleftharpoons}} \underset{\text{free radicals}}{[\mathrm{M^\bullet}] + [\mathrm{L^\bullet}]} \xrightarrow[k_T]{\mathrm{T^\bullet}} \mathrm{L{-}T} \quad (3)$$

The formalism describes the correction for solution determined activation parameters as follows: $\mathrm{BDE_{Co-C}} \cong \Delta H^{\ddagger}_{obs}(\mathrm{soln}) - F_c \cdot \Delta H^{\ddagger}_{\eta}$, where F_c is the fractional-cage efficiency (i.e., describes the efficiency of recombination of caged radicals) and $\Delta H^{\ddagger}_{\eta}$ is the activation enthalphy for viscous flow of the solvent used. This treatment neglects $\Delta H^{\ddagger}_{c}$ and sets $\Delta H^{\ddagger}_{d}$ equal to $\Delta H^{\ddagger}_{-1}$(soln), which can be considered equal to $\Delta H^{\ddagger}_{\eta}$.[41] The enthalphy for viscous flow of ethylene glycol in the temperature range employed is 6.5 kcal/mol.[42] Transient photolysis studies on AdoCbl have estimated a fractional-cage efficiency of 0.7−0.8.[43] Given the structural similarities between AdoCbl and the analogue in question, a value of 0.8 ± 0.2 is used for F_c. With these corrections in mind, the BDE of 3′,4′-anAdoCbl can be estimated as 24 ± 2 kcal/mol. On the basis of these data, it is clear that the Co−C bond of the analogue is weaker than in AdoCbl (BDE = 30 ± 2 kcal/mol) by approximately 6 kcal/mol.

Table 1. Activation Parameters for Co−C Bond Homolysis in Ethylene Glycol by the Nitroxide Spin Trapping Method

compound	k_h(s^{-1}) at 25 °C[a]	$\Delta H^{\ddagger}_h$ (kcal/mol)	$\Delta S^{\ddagger}_h$ (eu)	Co−C BDE (kcal/mol)
AdoCbl[b]	4×10^{-10}	34.5 ± 0.8	14 ± 1	30 ± 2
NpCbl[c]	3×10^{-4}	32.2 ± 0.6	33 ± 2	28 ± 2
3′,4′-anAdoCbl	1×10^{-3}	29.5 ± 0.4	27 ± 2	24 ± 2

[a] Calculated from $\Delta H^{\ddagger}_h$ and $\Delta S^{\ddagger}_h$ values. [b] From ref 38. [c] From ref 44.

In Table 1, a comparison is given for the activation parameters obtained in this study to those measured by Finke and co-workers by the same radical trapping method for AdoCbl and neopentylcobalamin (NpCbl).[38,44] NpCbl provides a good comparison to a sterically strained cobalamin with a weak Co−C bond which has been studied extensively as a model for Co−C bond cleavage.[8,9,28,44] In fact, NpCbl displays some similar behavior as 3′,4′-anAdoCbl, it is oxygen-sensitive and resistant to homolysis under strict anaerobic conditions in aqueous solution. However, NpCbl is quenched in ethylene glycol, whereas 3′,4′-anAdoCbl is quite stable (see part B). By comparing the data in Table 1, one can see that the rate of homolysis at 25 °C has increased by almost 10^7 compared to AdoCbl and is even 3-fold higher than NpCbl. The $t_{1/2}$ for 3′,4′-anAdoCbl is only 11 min at 25 °C. This incredible rate enhancement can be attributed to allylic stabilization of the radical product. It is worth noting that the free energy of activation is $\Delta G^{\ddagger}_{25°C}$ = 30.3 kcal/mol and $\Delta G^{\ddagger}_{25°C}$ = 21.5 kcal/mol for AdoCbl and the analogue, respectively. The difference of 8.8 kcal/mol approaches a value of ∼11 kcal/mol estimated for stabilization of an allylic radical relative to a primary alkyl radical.[36]

The difference in entropy of activation between AdoCbl and 3′,4′-anAdoCbl is of some interest. Restricted rotation of the allyl group as allylic resonance is set up in the transition state would be expected to cause a decrease in the entropy of activation compared to AdoCbl.[45] The opposite is actually observed, where $\Delta S^{\ddagger}$ is 13 eu higher for the analogue than AdoCbl. The reason for this is not known. However, a possible explanation for this unexpected finding can be provided by considering that the upper axial ligand may be interacting unfavorably with the corrin. Relief of ground-state strain in the transition state could therefore be more important than the opposing effect, the stiffening of the allyl group.

The products of the thermolytic reaction are B_{12r} as documented above and a trapped radical species (Scheme 3). To determine the nature of the nucleoside product, thermolysis of 3′,4′-anAdoCbl at 25 °C was performed in the presence of excess spin trap. The reaction mixture was separated by HPLC and two main nucleoside products isolated. Structural analysis by NMR shows that these products are 5′-TEMPO-3′,4′-anhydroadenosine (**1**) and 3′-TEMPO-4′,5′-anhydroadenosine (**2**), formed in a 4:1 ratio, respectively. The total yield was estimated to be >90% showing that trapping by TEMPO is very efficient. It is interesting to note that these results emphasize the allylic nature of the radical formed upon homolysis of the Co−C bond;

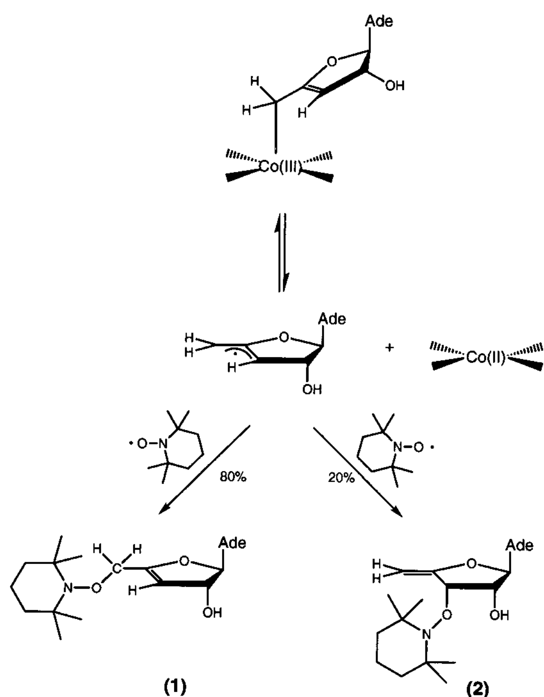
(42) This value is obtained from Andrade's law, $\ln \eta = A_\eta + (\Delta H^{\ddagger}_\eta + RT)/RT$. The viscosity of ethylene glycol at different temperatures was obtained from: Viswanath, D. S.; Natarajan, G. *Data book on the viscosity of liquids*; Hemisphere Publishing Corporation: New York, 1989.

(43) (a) Chen, E.; Chance, M. R. *J. Biol. Chem.* **1990**, *265*, 12987−12994. (b) Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1993**, *115*, 12152−12157. (c) Walker, L. A.; Shiang, J. J.; Anderson, N. A.; Pullen, S. H.; Sension, R. J. *J. Am. Chem. Soc.* **1998**, *120*, 7286−7292.

(44) Waddington, M. D.; Finke, R. G. *J. Am. Chem. Soc.* **1993**, *115*, 4629−4640.

(45) Benson, S. W. *Thermochemical Kinetics*, 2nd ed.; John Wiley and Sons: New York, 1976; p 100.

Scheme 3



however, the product ratio probably does not reflect the spin density at the 3′- and 5′-carbons since trapping at the 3′-position is the sterically less favored reaction.

(D) Activation of DDH and EAL. The coenzyme analogue was tested for activity with two B_{12}-dependent enzymes, diol dehydrase (DDH) and ethanolamine ammonia-lyase (EAL). Activity could be detected with both enzymes using a coupled assay with alcohol dehydrogenase by monitoring the oxidation of NADH spectrophotometrically. The specific activity of DDH with 3′,4′-anAdoCbl is 0.020 IU/mg compared to 90 IU/mg with AdoCbl, or 0.020%. Similarly, the activity of EAL is 0.024 IU/mg compared to 45 IU/mg with AdoCbl, or 0.053%. On the basis of these data, k_{cat} with 3′,4′-anAdoCbl as cofactor can be calculated to be 0.07 s^{-1} and 0.03 s^{-1} for DDH and EAL, respectively. The assays were done at 25 °C, and it is interesting to note that k_{obs} for thermolysis of 3′,4′-anAdoCbl at that temperature is $\sim$0.001 s^{-1}. This means that the enzymes do not have to provide more than about 30−70-fold rate enhancement for the cleavage of the Co−C bond. This is little compared to the $\sim 10^{12}$ rate enhancement required for AdoCbl.

Since our preparation of 3′,4′-anAdoCbl is not perfectly pure, a point of concern is the possibility that the low activity is due to AdoCbl as a trace contaminant. This issue was clarified by incubating a sample of 3′,4′-anAdoCbl in the dark at room temperature after exposure to air for 3 h. Authentic AdoCbl and other known active analogues are stable under these conditions, whereas 3′,4′-anAdoCbl undergoes thermal degradation due to irreversible cleavage of the Co−C bond as described in this paper. No enzymatic activity could be detected after this treatment, providing compelling evidence that the observed activity is not due to AdoCbl but to the action of 3′,4′-anAdoCbl as a cofactor.

The formation of B_{12r} was tested in enzymatic samples prepared anaerobically. No change in the UV−vis spectrum can be detected under catalytic conditions for either enzyme, that is, no build up of B_{12r} is observed in the steady-state of the reaction. The dominant chromophore in the steady-state of the reaction of DDH activated by AdoCbl is B_{12r}.[46] Therefore, despite the weaker Co−C bond in 3′,4′-anAdoCbl, it is not cleaved in the steady-state in the way that the stronger Co−C bond of AdoCbl is cleaved. This suggests that the enzymes cannot properly facilitate the cleavage of the Co−C bond and separate the incipient radical from the cobalt, as has to occur for catalysis to take place. One would expect the allylic radical to be stable enough to be detected, but efficient separation from cob(II)alamin is required to prevent recombination of the paramagnets. Also, hydrogen abstraction from the substrate should be slow, especially considering that ethylene glycol which is a substrate for EAL does not quench the allylic radical in solution (see part B). In view of the fact that the Co−C bond of the analogue is much weaker than for AdoCbl and the rate enhancement for its cleavage is very low, the data suggest that the upper axial ligand is not oriented properly in the active site of these enzymes, stressing the probable importance of a steric effect from the ligand. The 5′-deoxy-3′,4′-anhydroadenosyl moiety incorporates trigonal carbons at positions 3′and 4′and lacks the 3′-OH group of the 5′-deoxyadenosyl moiety. Its ring conformation and binding specificity therefore differ significantly from 5′-deoxyadenosyl.

Conclusions

We have synthesized and characterized a novel analogue of coenzyme B_{12}, designed to stabilize the 5′-deoxyadenosyl radical formed upon homolysis of the Co−C bond. This is an adaptation of our earlier strategy for radical stabilization of the 5′-deoxyadenosyl radical generated by reductive cleavage of *S*-adenosylmethionine in the reaction of lysine 2,3-aminomutase.[26] A substantial weakening of the Co−C bond of the analogue is observed which almost surely can be rationalized by the stability of the allylic radical formed by the homolytic cleavage of the bond. The small rate enhancement of Co−C bond cleavage in DDH and EAL along with the lack of formation of any radical species is suggestive of the adenosyl moiety being the key determinant for the mechanism by which B_{12}-dependent enzymes labilize the bond. The results also emphasize the fact that B_{12}-dependent enzymes need not only to displace the equilibrium of Co−C bond cleavage toward homolysis, but must also physically separate the fragments to prevent recombination. The coenzyme analogue should be an excellent tool for the study of other B_{12}-dependent enzymes, especially in determining the factors that facilitate the homolysis of the novel organometallic bond.

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(46) Wagner, O. W.; Lee, H. A. Jr.; Frey, P. A.; Abeles, R. H. *J. Biol. Chem.* **1966**, *241*, 1751−1762.