Side Chain Entropy and Activation of Organocobalamins for Thermal Homolysis: Thermolysis of Neopentyl-13-epi- and Neopentyl-8-epicobalamin in Neutral Aqueous Solution

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A complete product and kinetic analysis has been carried out for the thermolysis of neopentyl-8-epicobalamin (Np-8-epiCbl) and neopentyl-13-epicobalamin (Np-13-epiCbl), epimers of neopentylcobalamin, NpCbl, in which the orientation of the *d* or *e* propionamide side chain has been altered by epimerization at corrin ring C8 or C13, respectively. When the organic products of the aerobic thermolysis of Np-8-epiCbl or Np-13-epiCbl are treated with aniline in diethyl ether, the Schiff's base of pivalaldehyde is formed with a yield of approximately 50%. This observation is consistent with the disproportionation of the neopentyl peroxide radical to pivalaldehyde and neopentyl alcohol under aerobic thermolysis conditions. Anaerobic thermolysis in neutral aqueous solution in the presence of (4-hydroxy-2,2,6,6-tetramethylpiperidinyl)oxy, H-TEMPO, gives rise to quantitative yields of the *O*-alkylated neopentyl-H-TEMPO and 8- or 13-epicob(II)alamin. Moreover, examination of the anaerobic thermolysis of these complexes in the presence of 0.5−1.0 mM H-TEMPO showed that the rate constants for thermolysis are the same as those obtained in neutral aerobic solution, establishing that dissolved oxygen is a competent trap for the homolytically derived radicals. These results clearly reveal that the mode of thermal C−Co cleavage is homolytic as is known to be the case for NpCbl itself. Thermolysis kinetics were studied in aerobic, neutral aqueous solution spectrophotometrically and gave the following observed activation parameters: Np-8-epiCbl, $\Delta H^{\ddagger}_{obs} = 28.7 \pm 0.1$ kcal mol^{-1} and $\Delta S^{\ddagger}_{obs}$ 17.1 ± 0.2 cal mol^{-1} K^{-1}; Np-13-epiCbl, $\Delta H^{\ddagger}_{obs} = 28.2 \pm 0.1$ kcal mol^{-1} and $\Delta S^{\ddagger}_{obs} = 18.4 \pm 0.3$ cal mol^{-1} K^{-1}. UV−visible scanning experiments demonstrated that the base-off form of Np-8-epiCbl at pH 1.0 was at least 2 orders of magnitude less reactive than the neutral species. In addition, a complete examination of the pH-dependence for the thermolysis of Np-13-epiCbl demonstrated that the base-on species is about 10^3-fold more reactive than the protonated base-off species of this epimer. Rate constants for thermal decomposition in aerobic neutral aqueous solution have been corrected for the presence of a significant amount of the base-off species by measuring the thermodynamic parameters for the intrinsic base-off/base-on equilibrium over the temperature range of interest using an NMR method. The resultant activation parameters for the base-on species of both complexes are as follows: Np-8-epiCbl, $\Delta H^{\ddagger}_{on} = 29.3 \pm 0.2$ kcal mol^{-1} and $\Delta S^{\ddagger}_{on} = 22.4 \pm 0.7$ cal mol^{-1} K^{-1}; Np-13-epiCbl, $\Delta H^{\ddagger}_{on} = 29.7 \pm 0.2$ kcal mol^{-1} and $\Delta S^{\ddagger}_{on} = 24.0 \pm 0.6$ cal mol^{-1} K^{-1}. Comparison of these values with the currently accepted values for base-on NpCbl in aqueous solution ($\Delta H^{\ddagger}_{on} = 28.3 \pm 0.2$ kcal mol^{-1} and $\Delta S^{\ddagger}_{on} = 19.3 \pm 0.6$ cal mol^{-1} K^{-1}) shows that the enthalpy of activation for C−Co bond homolysis is essentially unchanged by side chain epimerization ($\Delta \bar{H}^{\ddagger}_{on} = 28.7 \pm 0.5$ kcal mol^{-1}) but that the entropy of activation is increased by 4.7 ± 0.8 cal mol^{-1} K^{-1} by epimerization at C13 and by 3.1 ± 0.9 cal mol^{-1} K^{-1} by epimerization at C8. These results are discussed in terms of the importance of side chain thermal motions in determining the entropy difference between the ground and the transition states of NpCbl for its thermal homolysis.

Introduction

5′-Deoxyadenosylcobalamin (AdoCbl,[1] coenzyme B_{12}, Figure 1) is well known to be involved in the catalysis of a number of enzymatic reactions in various organisms, the majority of which involve 1,2-intramolecular rearrangements in which an electrophilic group and a hydrogen on adjacent carbon atoms of the substrate exchange places. For those AdoCbl-requiring enzymes which have been most thoroughly studied (including diol dehydrase,[4] ethanolamine deaminase,[5] methylmalonyl-CoA mu-

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(1) (a) IUPAC-IUB nomenclature[2] is used throughout. Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); CNCbl, cyanocobalamin (vitamin B_{12}); CN-8-epiCbl, cyano-8-epicobalamin; CN-13-epiCbl, cyano-13-epicobalamin (neovitamin B_{12}); CN(H_2O)-13-epiCbi$^+$, cyanoaqua-13-epicobinamide;[3] NpCbl, neopentylcobalamin; NpCbi$^+$, neopentylcobinamide; Np-13-epiCbi$^+$, neopentyl-13-epicobinamide; α-ribazole 3′-phosphate, 1′-α-5,6-dimethylbenzimidazolyl-3′-phosphoribofuranose; α-ribazole-2′-phosphate, 1′-α-5,6-dimethylbenzimidazolyl-2′-phosphoribofuranose; Bzm, 5,6-dimethylbenzimidazole; NpBr, 1-bromo-2,2-dimethylpropane (neopentyl bromide); H-TEMPO, (4-hydroxy-2,2,6,6-tetramethylpiperidinyl)oxy; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; Np-H-TEMPO, *N*-(2,2-dimethylpropanoxy)-4-hydroxy-2,2,6,6-tetramethylpiperidine; H_2OCbl^+, aquacobalamin (B_{12a}); NBS; National Bureau of Standards. (b) All cobalamins discussed in this paper have the organic ligand coordinated to the cobalt at the upper (or β) face of the complex unless explicitly stated otherwise.

(2) *Biochemistry* **1974,** *13,* 1555.

(3) Like cyano(aqua)cobinamide (factor B) itself, cyano(aqua)13-epicobinamide is a mixture of two diastereomers, i.e., β-CN-α-(H_2O)-13-epiCbi$^+$ and β-H_2O-α-(CN)-13-epiCbi$^+$.

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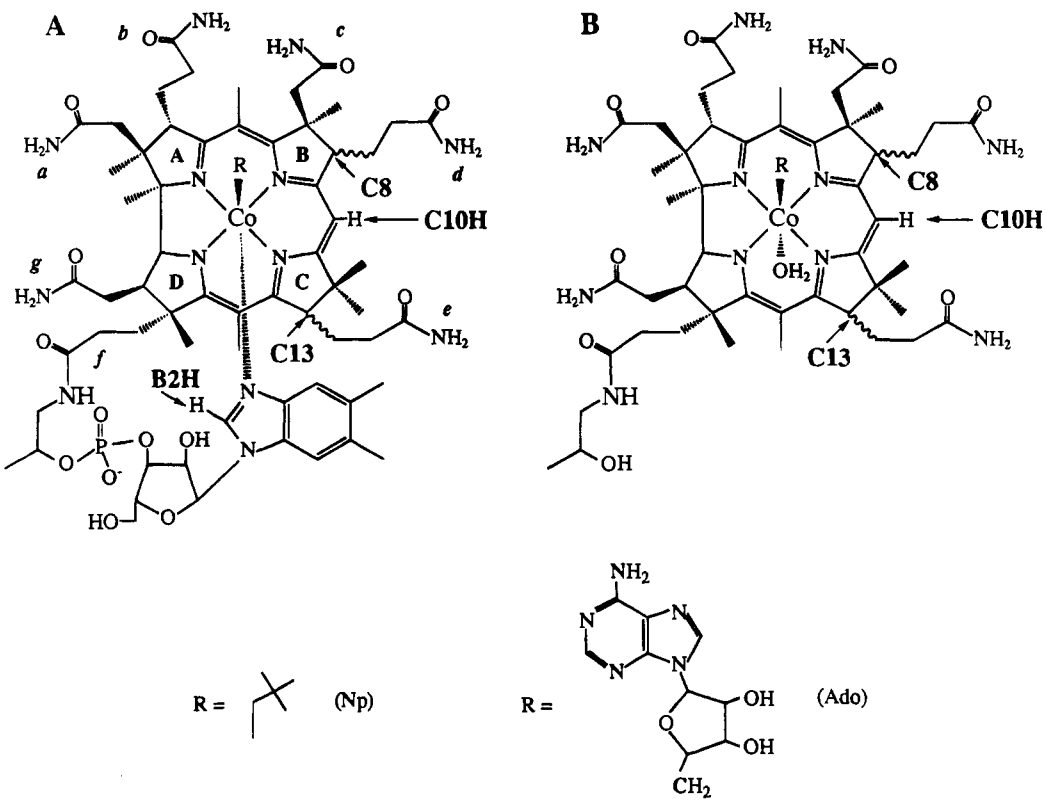

Figure 1. Structure of neopentylcobalamin and 5′-deoxyadenosylcobalamin (A) and the analagous cobinamides (B) denoting the positions of epimerization and the locations of B2-H and C10-H.

tase,[6] and ribonucleotide reductase[7]), isotopic labeling experiments as well as direct ESR observation of intermediates with unpaired electrons, have produced general agreement that the first step in the catalytic cycle involves the homolytic C−Co dissociation of the coenzyme (so-called "activation" of AdoCbl) to produce a 5′-deoxyadenosyl radical and a cob(II)alamin species. Binding of AdoCbl to such enzymes in the presence of substrates is now known to increase the rate of Co−C bond homolysis by as much as 12 orders of magnitude.[8] It is generally thought that conformational distortion of AdoCbl by the enzyme is responsible for this rate acceleration.[8b,9−16] Mechanisms have been proposed involving steric compression of the axial Co−N bond, lengthening of the axial Co−N bond (to stabilize the emerging Co^{II} oxidation state), corrin ring distortion induced by twisting the axial Co−N bond to rotate the Bzm moiety, flexing of the corrin ring, replacement of the axial Bzm by a functional group on the enzyme, and direct "pulling" or "bending" of the Co−C bond by interactions of the Ado ligand with the protein.[5e,10,11a,12,13,17−22] However, little is actually known about how these enzymes might achieve such distortions, or the mechanism by which conformational distortions might enhance Co−C bond cleavage in AdoCbl.

The central problem to resolve about the structure of the B_{12} coenzyme is how it can be kinetically stable under ordinary conditions, but capable of being readily activated by the appropriate enzymes. Considering the steric strain presumably responsible[12] for the large Co−C−C bond angle of 121−125°,[23,24] AdoCbl is extraordinarily stable indeed. Finke and Hay's data[8b] permit an estimation that at 37 °C, the half-time for thermolysis of AdoCbl is an astonishing 1.9 years.

Because of this stability and the fact that AdoCbl is susceptible to competing Co−C bond heterolysis due to the presence of the β oxygen substituent in its organic ligand,[8,25−27] a number of sterically strained RCbls, such as 2° alkyl-, benzyl-, and NpCbl, have been used as models for AdoCbl Co−C bond

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cleavage.[10,11,28-38] It is now abundantly clear that for compounds like benzylCbl and α- and β-NpCbl, in which the organic ligand lacks β hydrogens, thermolysis occurs exclusively via Co−C bond homolysis.[31,33,34,36,37] Furthermore, it now appears that even for compounds with β hydrogens, for which the final products may be an olefin, hydrogen and a Co^{II} complex, the initial event in thermolysis is Co−C bond homolysis.[39,40]

It is also well known that the base-off species of such RCbls, and the analogous cobinamide species in which the axial nucleotide has been removed by phosphodiester hydrolysis (Figure 1), are some 2−3 orders of magnitude less reactive than the base-on species.[8c,11,30,31,33,35,36] Termed the base-on effect (by Schrauzer and Grate[30]), our work with NpCbl[34] has shown that it is primarily due to a decrease in the (positive) entropy of activation in the base-off species, the enthalpies of activation of the base-on and base-off species being nearly the same for these complexes. The entropic nature of the base-on effect has in turn led to the suggestion[34] that steric interactions between the bulky organic ligand and the upwardly projecting *a*, *c*, and *g* acetamide side chains are responsible for the entropic activation of such base-on RCbls for thermal Co−C bond cleavage. Thus, if such steric interactions in the ground state interfere with the acetamide side chain rotational freedom of motion, relief of this interference in the transition state due to the incipient separation of the R• and Co^{II} moieties would provide an entropic driving force for reaction. The entropic stabilization of the base-off (and cobinamide) species is then seen as a consequence of a downward flexing of the corrin ring in the absence of the strongly donating Bzm ligand (as originally suggested by Schrauzer and Grate[30]), reducing the degree of interaction of the acetamide side chains with the organic ligand in the ground state, and hence reducing the entropic driving force for bond cleavage. In this view, the stability of AdoCbl might be attributed to the fact that the bulky adenosyl group lies over the "southern" half of the corrin,[23,24] constrained in the sterically open space between the C46 and C54 "sentinel" methyl groups on the C and D rings, respectively.[12] This conformation might stabilize the C−Co bond by sheltering the organic ligand from interactions with the *a*, *c*, and *g* acetamides which are attached to the opposite side of the corrin.[41] Such stabilization might not be able to occur with bulky, but less extended organic ligands such as neopentyl which might not be able to adopt a conformation which is sterically protected from the acetamides. As a result, NpCbl is some 10^5-10^6-fold more thermally labile than AdoCbl in neutral solution at 25 °C.[8,34,36]

We are consequently interested in studying the C−Co bond reactivity of RCbl analogs in which the number and/or structure of upwardly projecting side chains has been altered. Such alterations would be expected to influence the steric restriction of side chain mobility in the ground state and hence to alter the entropy of activation for C−Co bond cleavage. As a first step in this direction, we now report the synthesis, characterization, and thermolysis of neopentyl derivatives of CN-8-epiCbl[42,43] and CN-13-epiCbl.[44,45] These are analogs of CNCbl in which epimerization at C8 or C13 in the corrin ring (Figure 1) changes the disposition of the *d* or *e* propionamide side chain.

Experimental Section

Materials. 1-Bromo-2,2-dimethylpropane (neopentyl bromide, Np-Br[1]), (4-hydroxy-2,2,6,6-tetramethylpiperidinyl)oxy (H-TEMPO[1]), and trifluoroacetic acid (TFA[1]) were from Aldrich. CNCbl was from Roussell and trifluoroacetic anhydride (TFAA[1]) was from Fisher. Trifluoroacetic acid was dried by refluxing over P_2O_5 and was distilled from P_2O_5 immediately before use. Glass distilled water was used throughout and all work with organocobalt corrinoids was carried out in the dark (with the aid of flashlights) to prevent photolytic decomposition.

CN-8-epiCbl. This complex was prepared by a modification of the method of Rapp and Oltersdorf.[42] Its synthesis and characterization are a subject of another report.[43]

CN-13-epiCbl and CN-(H_2O)-13-epiCbi$^+$.[3] These complexes were prepared by a modification of the method of Bonnett *et al.*,[45] using CNCbl and dry TFA containing 5% (v/v) TFAA. The total yield of CN-13-epiCbl was 16% with 4% CN(H_2O)-13-epiCbi$^+$.

Np-13-epiCbl. This complex was prepared from CN-13-epiCbl by a method similar to the one employed by Schrauzer and Grate[30] for the preparation of NpCbl, using zinc reduction in methanolic ammonium bromide and NpBr and allowing the alkylation to proceed for 24 h. After HPLC separation, the yield was 85%.[46] Purity was >95% as assessed by HPLC. The compound was stored frozen at −20 °C. Neopentane (identified by its mass spectrum) was the only volatile product identified by GC/MS after anaerobic pyrolysis of solid Np-13-epiCbl. Although both the ^1H and ^{13}C NMR spectra of Np-13-epiCbl are badly broadened,[41] apparently due to exchange between the base-on and base-off species (vide infra), the methyl resonance of the Np ligand was clearly identifiable as a broad 9-proton singlet at −0.27 ppm and the C10 and R1 hydrogens could be located at 6.30 and 6.16 (J = 1.2 Hz) ppm, respectively, at 25.0 ° C. Np-13-epiCbl was further characterized by its uv-visible spectrum and quantitative analysis of its thermolysis products (vide infra).

Np-8-epiCbl. This complex was prepared from CN-8-epiCbl in a manner similar to that described for Np-13-epiCbl, except that NH_4Cl was used rather than NH_4Br and the alkylation was complete within 4 h. The yield was 50%. As described above, neopentane was identified by its mass spectrum after anaerobic pyrolysis of solid Np-8-epiCbl. Np-8-epiCbl was further characterized by its UV−visible spectrum and by quantitative analysis of the thermolysis products (vide infra).

Np-13-epiCbi$^+$. This compound was prepared similarly from CN-13-epiCbi$^+$ and NpBr using zinc in 10% acetic acid as the reductant. Formation of a product believed to be α-Np-13-epiCbi$^+$ [38] was rapid, but with time, formation of the β-Np-13-epiCbi$^+$ was observed. The latter was positively identified by comparison of its uv-visible spectrum to that of protonated, base-off β-Np-13-epiCblH$^+$. The yields of the alkylation products in the final reaction mixture (2 h) were 29% α-Np-

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13-epiCbi$^+$,[38] and 35% β-Np-13-epiCbi$^+$ (the reminder being 35% $(H_2O)_2$-13-epiCbi^{2+}) by HPLC. After purification by semi-preparative HPLC, the final yield for β-Np-13-epiCbi$^+$ was 34%. Anaerobic, solid state pyrolysis gave neopentane as the only volatile product by GC/MS. The product was further characterized by complete assignments of its ^{1}H and ^{13}C NMR spectra, described elsewhere.[41]

8-Epicob(II)alamin and 13-Epicob(II)alamin. Generation of the cobalt(II) species of the CNCbl epimers was effected by either formate reduction of the respective aquo complex[47] or reduction of the cyano complex using zinc wool in the presence of NH_4Cl. The UV−visible spectra were very similar to that obtained for cob(II)alamin with the following spectral characteristics. λ ($\epsilon \times 10^{-3}$ M^{-1} cm^{-1}): 8-epicob(II)-alamin, 472 (9.40); 13-epicob(II)alamin, 404 (7.53) , 466 (7.50), 520 (sh, 4.20).

1-(2,2-Dimethylpropanoxy)-2,2,6,6-tetramethyl-4-hydroxypiperidine (Np-H-TEMPO) and *N*-(2,2-dimethylpropylidene)benzenamine (the Schiff's base of pivalaldehyde and aniline) were prepared and characterized as described previously.[38]

Methods. Work requiring anaerobic conditions was carried out in a Vacuum Atmospheres glovebox under an atmosphere of nitrogen or argon with O_2 < 2 ppm. Oxygen was removed from water by purging in the glovebox with argon that had been passed through vanadous sulfate scrubbing towers.[48] Water was deemed free of O_2 if the UV−visible spectrum of cob(II)alamin in a Schlenk cuvette, persisted for at least 8 h.

Solid state pyrolysis was carried out as described previously.[49] Products were identified by GC/MS on a Finnigan 4500 instrument equipped with a 6 m × 2 mm Carbopak B/1% SP 1000 column or on a Finnigan INCOS 500 instrument equipped with a 60 m DB5 column as described previously.[38]

GC analyses of Np-H-TEMPO and *N*-(2,2-dimethylpropylidene)-benzenamine were carried out on a Varian 3700 GC instrument using a Restek 30 m DB5 column and a FID detector. The following temperature program was used for both analyses: 1 min at 60 °C (injector port 280 °C), increasing to 200 °C at 15 °C/min, then holding at 200 °C for 1 min prior to recycling. All pH measurements were made using a Radiometer PHM 84 pH meter and a Radiometer type C combination glass electrode with electrode, samples, standards, and rinse water incubated at the appropriate temperature. Identification and quantitation of the aerobic and anaerobic (trapped with H-TEMPO) products of Np-8-epiCbl and Np-13-epiCbl were carried out as described previously.[38]

Analytical and semi-preparative HPLC was carried out as described previously.[34,50] UV−visible spectra were obtained on a Cary 219 recording spectrophotometer equipped with a thermostatted 5 cell sample turret, and temperature was maintained by use of a Neslab RTE-220 circulating water bath. Concentrations of 13-epi-cobalt corrinoids were determined by UV−visible spectroscopy after conversion to the dicyano derivative, $(CN)_2$-13-epiCbl$^-$. The molar absorptivities of $(CN)_2$-13-epiCbl$^-$ were determined at 25.0 ± 0.1 °C using 8 sample solutions prepared from solid CN-13-epiCbl, which was dried at 80 °C under vacuum over P_2O_5 to a constant mass. The averaged molar absorptivity at the γ-band of $(CN)_2$-13-epiCbl$^-$ (366 nm) was found to be (2.14 ± 0.02) × 10^4 M^{-1} cm^{-1} and is in good agreement with that previously reported by Bonnett, *et al.* (2.06 × 10^4 M^{-1} cm^{-1}).[45] The UV−visible spectral parameters of all of the 13-epi-corrinoids used here are listed in Table SI (supplementary material). All 8-epi corrinoids were quantitated as the $(CN)_2$Cbl$^-$ species since gentle heating in the presence of excess cyanide gives rise to epimerization at C-8 back to the normal configuration.[43]

Temperature was measured using a YSI 702A thermistor probe[51] and a YSI 740AX signal conditioner or a Cole Parmer 93−100 thermistor device, as described previously.[37] Each thermistor probe−signal conditioner combination was carefully calibrated against NBS calibrated thermometers using stem corrections. Samples for spectrophotometric kinetic measurements (total volume 3.0 mL, in 1.000 cm path length quartz cuvettes) contained (1.0−2.0) × 10^{-5} M Np-8-epiCbl or Np-13-epiCbl, 0.1 M phosphate, acetate, or chloroacetate buffer, and sufficient KCl to adjust the ionic strength to 1.0 M. Reactions were initiated by addition of a small volume of Np−cobalt corrinoid stock solution to the sample cuvette which had been incubated in the spectrophotometer cell block for at least 30 min ($t_{1/2}$ for temperature equilibration was 3.4 min). Sample temperature was determined by thermistor measurement in a dummy sample cuvette before and after each kinetic run and the kinetic results were rejected unless the two measurements agreed to within <0.2 °C. Absorbance was measured as a function of time at 352 nm for a least 5 half-times, and the data were fitted to eq 1, where A_t is the absorbance at time t, A_0 is the

$$A_t = \Delta A[1 - \exp(-k_{obs}t)] + A_0 \quad (1)$$

initial absorbance, and ΔA is the difference between the final absorbance and A_0, using a non-linear least squares algorithm. For the neutral species of Np-8-epiCbl and Np-13-epiCbl, $k_{neut} = k_{obs}$ was determined as the weighted average of 5 kinetic runs at pH ≥ 7.5. For temperatures > 30 °C, for which $t_{1/2}$ < 40 min, the drop in temperature upon initiation of the reaction was minimized by pre-incubation of the Np-8-epiCbl or Np-13-epiCbl stock solution at the measurement temperature, addition of the stock solution directly to the cuvette in the cell block with a digital pipet, and mixing of the sample with a plastic rod which had also been pre-incubated at the measurement temperature.

In order to determine the pK_a for the base-on/base-off equilibrium, $K_{base\text{-}off}$,[49] for Np-13-epiCbl, rate constants for thermolysis of Np-13-epiCbl at various pH's between 0.5 and 7.5 were fitted to eq 2 by a

$$k_{obs} = \{(k_{off}[H^+] + k_{neut}K_{base\text{-}off})/([H^+] + K_{base\text{-}off})\} \quad (2)$$

weighted non-linear least squares algorithm. Rate constants as a function of pH and temperature are given in Table SII (supplementary material).

Kinetic measurements of the anaerobic thermolysis of Np-8-epiCbl and Np-13-epiCbl were carried out in the presence of 0.5−10.0 mM H-TEMPO in 0.1 M phosphate buffer (pH 7.5), ionic strength 1.0 M (KCl), at 35.0 ± 0.1 or 40.7 ± 0.1 °C. The solutions were prepared in a glove box and sealed in Schlenk cuvettes. The reactions were monitored at 466 nm (13-epicob(II)alamin) or 472 nm (8-epicob(II)-alamin). Since H-TEMPO reacts slowly with cobalt(II) corrinoids in water,[31] the data deviated slightly from first-order kinetics and were consequently fitted to eq 3, in which the slow reaction of 8- or 13-

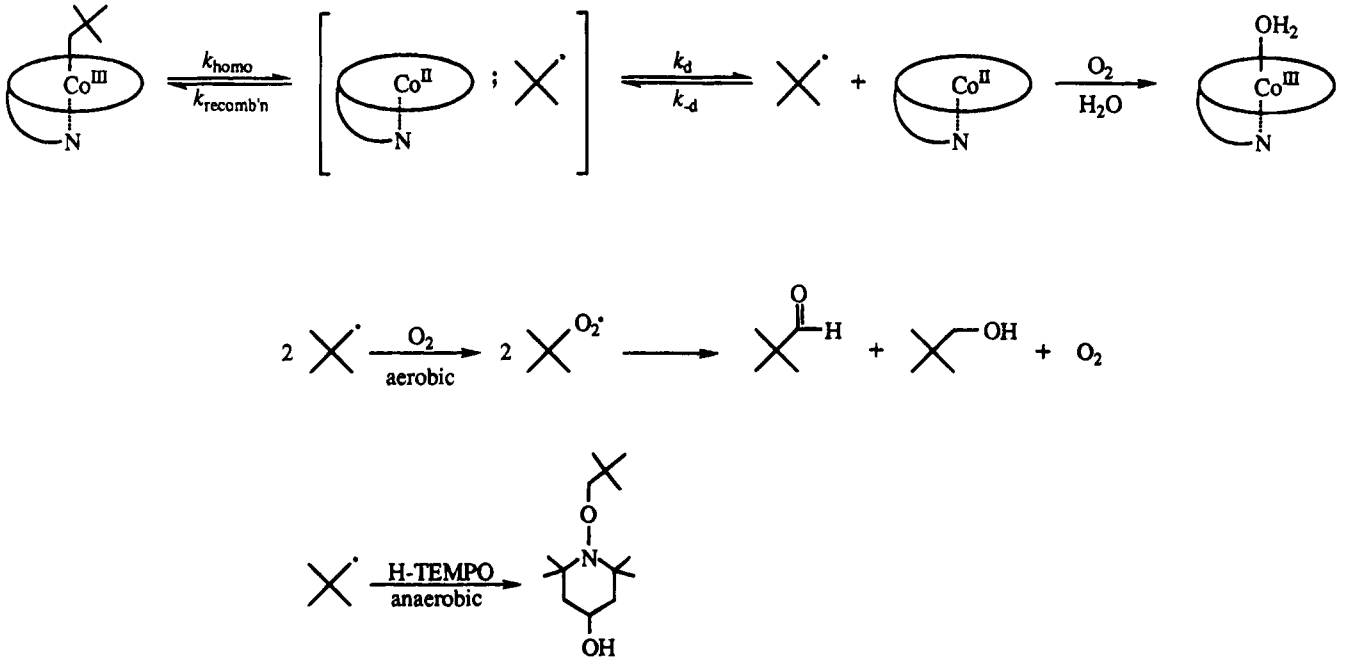
$$A_t = \Delta A[1 - \exp(-k_{obs}t)] + mt + A_0 \quad (3)$$

epicob(II)alamin with H-TEMPO is accounted for by a linear term. For the anaerobic thermolysis of Np-13-epiCbl in the presence of 1.0 mM H-TEMPO, k_{obs} was 2 orders of magnitude larger than the m term.

Anaerobic NMR samples of the neopentylcobalt epicorrinoids (23 to 35 mM) were prepared in 0.7 mL of "100%" D_2O, containing 0.1 M potassium phosphate buffer (pD = 8.2) and at ionic strength 1.0 M (KCl), following removal of exchangeable protons by dissolution in 99.9% D_2O and evaporation to dryness three times. ^{1}H NMR spectra were recorded on a GE QE-300 NMR spectrometer operating at 300.669 MHz. Typically, 160 scans were collected into 32K data sets using a spectral width of 3610 Hz. The data were processed using exponential multiplication of 0.5 Hz. Resonance frequencies were determined by digital fitting of the peaks of interest to Lorenzian line shapes. At least 12 min of temperature equilibration was allowed before data acquisition. The actual probe temperature (which differed from the temperature indicated on the instrument's VT pyrometer by as much as 6 ° C!) was measured using a dummy NMR sample containing the calibrated thermistor device as previously described.[37,52] The measured temperature equilibration half-time was ca. 1 min.

(47) Linn, D. E., Jr.; Gould, E. S. *Inorg. Chem.* **1988**, *27*, 1625.
(48) Meites, L.; Meites, T. *Anal. Chem.* **1948**, *20*, 984.
(49) Brown, K. L.; Hakimi, J. M.; Nuss, D. M.; Montejano, Y. D.; Jacobsen, D. W. *Inorg. Chem.* **1984**, *23*, 1463.
(50) Jacobsen, D. W.; Green, R.; Brown, K. L. *Methods Enzymol.* **1986**, *123*, 14.
(51) Manufacturers' tolerance for this thermistor probe is ± 0.1 °C, and the time constant is 1 s.
(52) (a) van Geet, A. L. *Anal. Chem.* **1968**, *40*, 2227. (b) van Geet, A. L. *Anal. Chem.* **1970**, *42*, 679.

Scheme 1



Results

Thermolysis Products. Thermolytic lability of the complexes benzylCbl,[31] α-,[38] and β-NpCbl[30,33,34,36] in either aqueous or ethylene glycol solution has been previously shown to be due to homolytic cleavage of the C−Co bond. Although it seems unlikely that epimerization of NpCbl at C8 or C13 would alter the course of thermolysis, it is necessary to verify this before comparing the reactivity of these epimers to that of NpCbl. Scheme 1 depicts the overall course of thermal decomposition of NpCbl in both aerobic and anaerobic solution. The initial step is the reversible homolytic cleavage of the C−Co bond of NpCbl to give a solvent caged radical pair. After diffusive separation of the pair, either radical can be trapped with an appropriate trap. The neopentyl radical reacts very rapidly ($k_2 = 2.65 \times 10^9$ M^{-1} s^{-1}) with molecular oxygen to form the neopentylperoxy radical.[53] This species disproportionates to pivalaldehyde and neopentyl alcohol, while the cob(II)alamin species is rapidly oxidized to aquocobalamin (H_2OCbl^+, B_{12a}). In the absence of oxygen, the neopentyl radical can be rapidly trapped with excess H-TEMPO.[36,37] The validity of Scheme 1 was tested by identifying and determining these anticipated reaction products.

The results for the determination of the thermolysis products of the NpCbl epimers are shown in Table 1. For the thermal decomposition of Np-8- or 13-epiCbl in aerobic, neutral aqueous solution pivalaldehyde and neopentyl alcohol are the expected products. Pivalaldehyde, was derivatized with aniline, as described previously,[38] to give the corresponding imine (Schiff's base). This product was identified and the yield was determined by NMR and GC by comparison with authentic material. As seen in Table 1, the yields of the Schiff's base for the epimers of NpCbl were 52 ± 5% and 52 ± 4%, respectively. These results confirm the formation of 0.5 equiv of pivalaldehyde from thermolysis in the presence of O_2.

Thermal decomposition of the Np-8- or Np-13-epi cobalt corrinoids in the absence of O_2, but with added H-TEMPO gives rise to the Np-H-TEMPO derivative which was also identified and the yield was determined using NMR and GC by comparison with authentic material. The yields of Np-H-TEMPO for the 8-epi and 13-epi complexes were determined to be 99 ± 4% and 96 ± 5%, respectively. Under these conditions, the epicob(II)alamin persists and its concentration can be determined by its UV−visible spectrum. As seen in Table 1, the epicob(II)alamins were formed essentially quantitatively from both NpCbl epimers. Taken together with the analytical results of the products derived from the organic ligands, these experiments demonstrate that Scheme 1 is indeed applicable to Np-8-epiCbl and Np-13-epiCbl thermolysis, and that the mode of carbon−cobalt bond cleavage is not altered by epimerization of the corrin macrocycle.

Table 1. Product Quantitation of Np-8- and Np-13-epiCbl in Neutral Aqueous Solution

compound	aerobic % imine[a]	anaerobic % Np-H-TEMPO[b]	anaerobic % epiCob(II)[c]
Np-8-epiCbl	52 ± 5	99 ± 4	103 ± 5
Np-13-epiCbl	52 ± 4	96 ± 5	95 ± 2

[a] Yield of *N*-(2,2-dimethylpropylidene)benzenamine (the imine of pivalaldehyde and aniline) determined by GC after reaction of the aerobic thermolysis product with aniline. The yield is expressed as percent of the total amount of NpCbl epimer present in solution. [b] Yield of Np-H-TEMPO determined by GC (as described above), from anaerobic thermolysis in the presence of excess H-TEMPO. [c] Yield of epicob(II)alamin from thermolysis in anaerobic solution in the presence of H-TEMPO, as determined from UV−visible absorption measurements.

Thermolysis Kinetics. The results of the kinetic analysis of the thermolysis of both NpCbl epimers in aerobic, neutral aqueous solution are summarized in Table 2. The temperature range employed was dictated by the observed thermal lability of each complex. An upper temperature limit was imposed on the experiments due to the methodology employed as described previously.[37] In short, for samples above ambient temperature, initiation of the reaction results in an unavoidable drop in sample temperature. The half-time for temperature reequilibration is 3.4 min for our apparatus, and thus sufficiently accurate rate constants may not be obtained when the reaction half-life is on the same order of the time required for temperature equilibration. For reactions near this upper limit, data obtained prior to temperature reequilibration were not used. Inclusion of rate

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Table 2. Rate Constants for the Thermolysis of Np-8-epiCbl and Np-13-epiCbl in Neutral Aqueous Solution[a]

Np-13-epiCbl			Np-8-epiCbl		
T, °C	$10^4\,k_{neut}$,[b] s^{-1}	$10^4\,k_{on}$,[c] s^{-1}	T, °C	$10^4\,k_{neut}$,[b] s^{-1}	$10^4\,k_{on}$,[c] s^{-1}
15.2	0.245 ± 0.002	0.337 ± 0.007	19.4	0.125 ± 0.002	0.647 ± 0.018
20.1	0.567 ± 0.003	0.813 ± 0.015	25.1	0.306 ± 0.003	1.63 ± 0.04
25.1	1.33 ± 0.02	1.98 ± 0.04	31.5	0.857 ± 0.004	4.73 ± 0.11
30.0	2.77 ± 0.04	4.35 ± 0.09	36.5	1.905 ± 0.007	10.74 ± 0.25
35.0	6.29 ± 0.05	10.4 ± 0.2	41.5	4.063 ± 0.008	23.4 ± 0.56
39.7	11.6 ± 0.03	20.2 ± 0.6	45.1	6.79 ± 0.10	39.43 ± 1.1
35.0	6.8 ± 0.1[d]		40.7	3.43 ± 0.04[d]	

[a] Ionic strength 1.0 M (KCl), pH 7.69−7.84 (depending upon temperature). [b] Weighted averages of five observed rate constants at each temperature ± the weighted standard deviations. [c] $k_{on} = k_{neut}/f_{on}$. [d] Rate constant for anaerobic thermolysis in the presence of excess H-TEMPO.

data at temperatures that exceeding this upper limit gives rise to underestimated rate constants.

Further confirmation of the applicability of Scheme 1 and the kinetic competence of dissolved oxygen as a radical trap was obtained by determining the rate constant for anaerobic thermolysis of the NpCbl epimers in the presence of excess H-TEMPO. It should be noted that H-TEMPO slowly oxidizes cob(II)alamin (B_{12r}) to H_2OCbl^+, as previously reported by Blau and Espenson.[31] This oxidation by H-TEMPO was also observed for both epimers of cob(II)alamin. However, the concentration dependence of this reaction could be exploited to provide conditions amenable to the needed analysis. Thus in (0.5−1.0) × 10^{-3} M H-TEMPO, the oxidation of the cob(II)-alamin epimers was much slower (≈100-fold) than the primary thermolysis reaction so that kinetic data (at 466 or 472 nm) could be successfully treated to extract the rate constant for the latter reaction as described in the Experimental Section. At even lower concentrations of H-TEMPO, quantitative accumulation of the cob(II)alamin epimers could be observed spectrophotometrically (vide supra).

The anaerobic kinetic results are included in Table 2. The agreement between the aerobic and anaerobic rate constants for Np-13-epiCbl thermolysis at 35 °C is quite good. For Np-8-epiCbl, the rate constant for aerobic thermolysis at 40.7 °C calculated from the activation parameters (3.60 × 10^{-4} s^{-1}) is in excellent agreement with the observed value (3.43 × 10^{-4} s^{-1}) under anaerobic conditions. These results demonstrate that dissolved oxygen is a kinetically competent trap for the radicals obtained from C−Co bond homolysis.

These observed rate constants do not represent the rate constants for thermolysis of the base-on species of the NpCbl epimers since significant amounts of the base-off species exist even at neutral pH (vide infra). Nonetheless, Eyring plots (not shown) are satisfactorily linear and gave the observed activation parameters $\Delta H^{\ddagger}_{obs}$ = 28.7 ± 0.1 kcal mol^{-1} and $\Delta S^{\ddagger}_{obs}$ = 17.1 ± 0.2 cal mol^{-1} K^{-1} for Np-8-epiCbl and $\Delta H^{\ddagger}_{obs}$ = 28.2 ± 0.1 kcal mol^{-1} and $\Delta S^{\ddagger}_{obs}$ 18.4 ± 0.3 cal mol^{-1} K^{-1} for Np-13-epiCbl . Our interest lies in obtaining accurate values for the activation parameters of the base-on species. In order to do this, it is important to thoroughly understand the temperature-dependence of the intrinsic base-off/on equilibrium.

Axial Nucleotide Coordination Equilibrium. Scheme 2 represents the two potential axial ligand equilibria available to the NpCbl epimers. The pH-independent off−on equilibrium, as measured by K_{meas}, is the predominant equilibrium under neutral conditions. The other equilibrium is a pH-dependent process in which reversible protanation of the benzimidazole nitrogen occurs and is governed by the acid dissociation constant, K_{Bz}. The overall acid dissociation constant, $K_{base\text{-}off}$, is then given by $K_{base\text{-}off} = K_{Bz}(1 + K_{meas})$.[49,54] An[55] independent measurement of pK_{Bz} for α-ribazole 3′-phosphate gave a value of 5.56 at 25.0 °C (I = 1.0 M, KCl).[56] Thus, under the conditions employed for the thermolysis kinetics at pH 7.69−7.84 (depending upon the temperature), the axial nucleotide is completely deprotonated and the only significant equilibrium is the pH-independent intramolecular association governed by K_{meas}.

The observed rate constant for thermolysis under neutral conditions, k_{neut}, represents a weighted average of the rate constants, k_{on}, for the base-on species, and k_{off}, for the base-off species as in eq 4, where f_{on} is the fraction of base-on species

$$k_{neut} = f_{on}k_{on} + (1 - f_{on})k_{off} \quad (4)$$

present at neutral pH (eq 5). Thus, in order to determine k_{on}, it is necessary to know f_{on} and k_{off}.[57]

$$f_{on} = K_{meas}/(1 + K_{meas}) \quad (5)$$

For Np-13-epiCbl, k_{off} was estimated at two temperatures from the pH-dependence of the observed rate constant for thermolysis. The data (Figure 2) were fitted to eq 2 by a non-linear least squares routine to give k_{neut} = 1.31 × 10^{-4} s^{-1}, k_{off} = 1.32 × 10^{-7} s^{-1}, and $pK_{base\text{-}off}$ = 4.91 at 25.1 °C (data not shown) and k_{neut} = 6.29 × 10^{-4} s^{-1}, k_{off} = 6.60 × 10^{-7} s^{-1}, and $pK_{base\text{-}off}$ = 4.88 at 35 °C. The results show that the base-off species is at least three orders of magnitude less reactive than the base-on species. Thus, as long as f_{on} at neutral pH exceeds *ca.* 0.05, the contribution to the observed reactivity from the base-off species is negligible, and $k_{on} = k_{neut}/f_{on}$.[57] Although the pH dependence of the thermolysis of Np-8-epiCbl was not studied as thoroughly, experiments in which the spectrum of this complex was scanned periodically over several days at 45 °C and pH 1 demonstrated that k_{neut} is at least 2 orders of magnitude larger than k_{off} for this epimer as well.

The temperature dependence of the base-off/on equilibrium for NpCbl in both methanol-d_4 and in D_2O under "neutral" conditions has been previously determined using a well-established NMR method.[36,37] The method depends on the sensitivity of the chemical shift of the C10 hydrogen to axial ligand substitution.[58] Thus, the temperature dependence of the difference in resonance frequency, $\Delta\nu_{obs}$, of the C10 hydrogen between neutral NpCbl and neutral $NpCbi^+$ can be used to determine the thermodynamic parameters associated with K_{meas}. This is shown in eq 6, where $\Delta\nu_0$ is the difference in resonance

$$\Delta\nu_{obs} = \{\Delta\nu_0 \exp[(\Delta S_{meas}/R) - (\Delta H_{meas}/RT)]\}/\{1 + \exp[(\Delta S_{meas}/R) - (\Delta H_{meas}/RT)]\} \quad (6)$$

frequency between the base-on and base-off species at absolute zero, and ΔH_{meas} and ΔS_{meas} are the enthalpy and entropy

(54) Unlike CH_3Cbl and $(CN)_2Cbl^-$ [55] no evidence is observed for a "tuck-in" species in which there is an intramolecular hydrogen-bond between the deprotonated B2 nitrogen and a *g*-amide hydrogen in unprotonated base-off NpCbl or its epimers.

(55) (a) Brown, K. L.; Pick-Siler, S. *Inorg. Chem.* **1988**, *27*, 3548. (b) Brown, K. L.; Brooks, H. B.; Gupta, B. D.; Victor, M.; Marques, H. M.; Scooby, D. C.; Goux, W. J.; Timkovich, R. *Inorg. Chem.* **1991**, *30*, 3430.

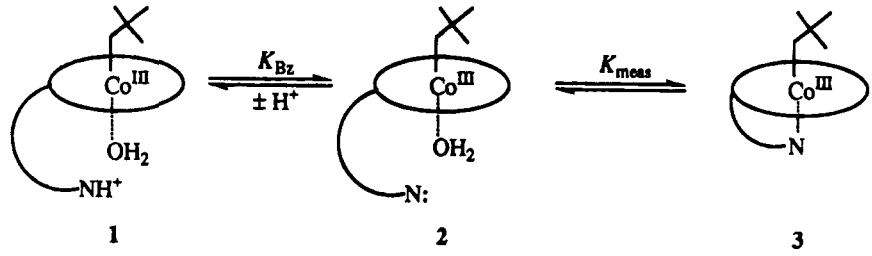
(56) Brown, K. L. *J. Am. Chem. Soc.* **1987**, *109*, 2277.

(57) It is implicitly assumed that the rate constants for thermolysis of the protonated (**1**) and deprotonated (**2**) base-off species are the same.

(58) (a) Brodie, J. D.; Poe, M. *Biochemistry* **1972**, *11*, 2534. (b) Cockle, S. A.; Hensens, O. D.; Hill, H. A. O.; Williams, R. J. P. *J. Chem. Soc., Dalton Trans.* **1975**, 2633.

(59) (a) Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, *108*, 4285. (b) Bax, A.; Marzilli, L. G.; Summers, M. F. *J. Am. Chem. Soc.* **1987**, *109*, 566.

Scheme 2



1 2 3

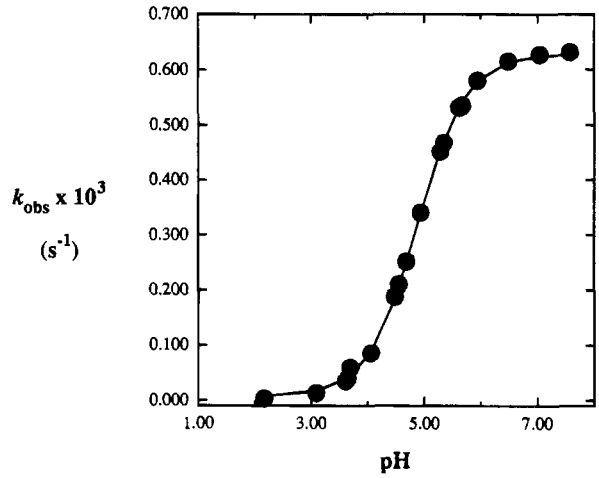


Figure 2. pH–rate profile for Np-13-epiCbl at 35.0 °C. Data points represent single determinations, except for those obtained under neutral conditions which represent weighted averages of several determined rate constants (see text). The solid line represents a fit of the data to eq 2 using a weighted non-linear least-squares regression. The determined parameters were $k_{neut} = 6.29 \times 10^{-4}$ s^{-1}, $k_{off} = 6.60 \times 10^{-7}$ s^{-1}, and $K_a = 1.32 \times 10^{-5}$.

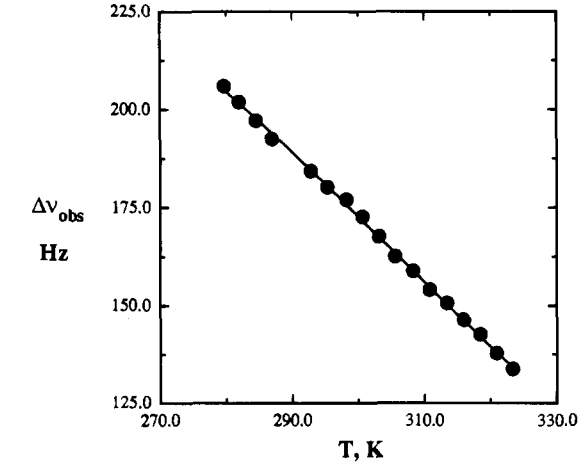


Figure 3. Plot of $\Delta\nu_{obs}$, the magnitude of the difference in ^{1}H NMR resonance frequency between the C10 hydrogen of Np-13-epiCbl and Np-13-epiCbi$^+$. The solid line is a non-linear least squares regression from which the values of $\Delta\nu_0 = 263.2 \pm 1.2$ Hz, $\Delta H_{meas} = -5.0 \pm 0.3$ kcal mol^{-1}, and $\Delta S_{meas} = -15.5 \pm 0.7$ cal mol^{-1} K^{-1} were obtained.

associated with K_{meas}. Figure 3 shows the data obtained for Np-13-epiCbl under neutral conditions and over an approximately 40-deg temperature range encompassing the range over which the kinetic measurements were made. A non-linear least squares fit to eq 6 gave $\Delta\nu_0 = 263.2 \pm 1.2$ Hz, $\Delta H_{meas} = -5.0 \pm 0.3$ kcal mol^{-1}, and $\Delta S_{meas} = -15.5 \pm 0.7$ cal mol^{-1} K^{-1}.

For Np-8-epiCbl, the C10H resonance overlapped with the R1H resonance preventing accurate location of its resonance frequency. Consequently, the B2H resonance, which also shows a large chemical shift difference between the base-on and base-off species, was used instead. Here, the difference in resonance frequency for the B2H between Np-8-epiCbl and the base-off but unprotonated dicyano species, (CN)$_2$-8-epiCbl$^-$,[60] as a function of temperature was used in conjunction with eq 6 (data not shown) to give $\Delta\nu_0 = 296 \pm 5$ Hz, $\Delta H_{meas} = -1.1 \pm 0.2$ kcal mol^{-1}, and $\Delta S_{meas} = -6.6 \pm 0.9$ cal mol^{-1} K^{-1}.

With the thermodynamic parameters for both NpCbl epimers in hand, values of f_{on} (eq 5)[61] and hence k_{on}, the rate constant for thermolysis of the base-on species, could be calculated. The latter are listed in Table 2. Activation parameters were then obtained from the Eyring plots (Figure S1, supplementary material) using weighted linear least squares fits as recently described,[37] and are listed in Table 3 along with those previously determined for NpCbl. As described previously,[37] the stated uncertainties in the activation parameters for the base-on species represent standard deviations ($\pm 1\sigma$) which were correctly propagated from the standard deviations of the measured quantities, k_{neut}, $\Delta\nu_{obs}$, and T.

Discussion

The results obtained from the products analysis of the thermal decomposition of the Np-8- and Np-13-epiCbl clearly show that C–Co bond homolysis is the only significant mode of decomposition and that Scheme 1 is in fact operative for these complexes as it is for NpCbl.[36,37] Competence of molecular oxygen as a radical trap has also been established by examining the anaerobic kinetics of thermal decomposition in the presence of excess H-TEMPO. Agreement between the rate constants obtained under aerobic and anaerobic conditions (Table 2) serves to validate Scheme 1.

Schrauzer and Grate[30] first observed the large difference in thermal reactivity of the base-on and base-off species of NpCbl and suggested that coordination of the pendant nucleotide results in conformational changes in the macrocyclic system which greatly increase reactivity. Their observations, moreover, demonstrated that the base-off protonated species (as well as NpCbi$^+$) was some 10^3-fold less reactive than the species observed under neutral conditions. These results have been confirmed in recent work,[34,36] and it should be noted that a substantial decrease in thermal reactivity of AdoCbl upon removal of the axial nucleotide has also been observed.[8] The current work shows that this is also the case for the NpCbl epimers, and the observed reactivity in neutral solution can be attributed solely to the base-on species.

In RCbls with bulky organic ligands, the intramolecular equilibrium constant for formation of the base-on species is sufficiently low so that significant amounts of base-off species may exist even at neutral pH and at near-ambient temperatures.[30,34,36,37] This is clearly the case for the NpCbl epimers as

(60) Thermal epimerization of (CN)$_2$-8-epiCbl$^-$ to (CN)$_2$Cbl$^-$ [43] limited the upper temperature at which measurements could be made to about 35 °C.

(61) f_{on} varies from 0.193 ± 0.01 at 19.4 °C to 0.171 ± 0.01 at 45.1 °C for Np-8-epiCbl, while this parameter varies from 0.724 ± 0.014 at 15.2 °C to 0.575 ± 0.008 at 39.7 °C for Np-13-epiCbl.

Table 3. Comparison of the Activation Parameters for Base-Off/On Equilibria[a] and Corrected Activation Parameters in Neutral Aqueous Solution for Thermolysis[b] of Base-On NpCbl, Np-8-epiCbl, and Np-13-epiCbl

compound	$\Delta H^{\ddagger}_{meas}$,[a] kcal mol^{-1}	$\Delta S^{\ddagger}_{meas}$,[a] cal mol^{-1} K^{-1}	$\Delta H^{\ddagger}_{on}$,[b] kcal mol^{-1}	$\Delta S^{\ddagger}_{on}$,[b] cal mol^{-1} K^{-1}
NpCbl[c]	-4.1 ± 0.1	12.5 ± 0.3	28.3 ± 0.2	19.3 ± 0.6
Np-8-epiCbl	-1.1 ± 0.2	-6.6 ± 0.9	29.3 ± 0.2	22.4 ± 0.7
Np-13-epiCbl	-5.0 ± 0.3	-15.5 ± 0.7	29.7 ± 0.2	24.0 ± 0.6

[a] From eq 6 (see text and Figure 3). [b] From slopes and intercepts of Eyring plots (see text and Figure S1 of supplementary material). [c] Reference 37.

well. The thermodynamic parameters (Table 3) measured by the NMR method[36,37] show that, at 25 °C, K_{meas} is larger for the C13 epimer (2.5) than for NpCbl (1.7), while the C8 epimer has the weakest axial nucleotide binding (0.25). These results are consistent with a recent study of the effects of epimerization on the base-off/on equilibrium of RCbls, in which the equilibrium constant for formation of the base-on species was always found to decrease in the order R-13-epiCbl > RCbl > R-8-epiCbl.[62] In the case of the R-13-epiCbls, in which the *e* side chain is displaced, the increase in the nucleotide binding constant is attributable to the loss of close contacts between the *e* side chain methylene groups and the B2 and R5 hydrogens of the nucleotide in the base-on RCbl.[62] For the R-8-epiCbls, in which the *d* side chain is displaced, the decreased affinity of the axial nucleotide for the cobalt atom can be attributed to the loss of a hydrogen bond between the *d* side chain propionamide and the B1 nitrogen of the nucleotide of the base-on RCbl, previously observed by ^{1}H and ^{15}N NMR studies.[62,63]

As seen in Table 3, epimerization of NpCbl at C8 or C13 has little or no effect on the enthalpy of activation for C−Co bond homolysis. The weighted average value is 29.2 ± 0.6 kcal mol^{-1} ($\pm 1\sigma$) or 29.2 ± 1.3 kcal mol^{-1} ($p < 0.05$), which easily encompasses all three values. While the original X-ray crystal structure of CN-13-epiCbl[64] suggested a substantial difference in the C−Co bond distance compared to that of CNCbl[65] (d_{C-Co} = 1.92 Å for CNCbl but 1.74 Å for CN-13-epiCbl), a recently obtained crystal structure for the C13 epimer[66] shows that the C−Co bond distances for these two complexes are virtually identical. Similarly, the C−Co bond distance of CN-8-epiCbl[43] (1.91 Å) is also virtually identical to that of CNCbl. These structural studies suggest that epimerization at C8 or C13 does not effect ground state C−Co bond enthalpies and would therefore be unlikely to effect enthalpies of activation for C−Co bond homolysis.

The situation is quite different for the entropy of activation for homolysis. Here, epimerization at C13 causes a 4.7 ± 0.8 cal mol^{-1} K^{-1}, or 24%, increase in activation entropy while epimerization at C8 causes a 3.1 ± 0.9 cal mol^{-1} K^{-1} increase (both $\pm 1\sigma$). Using 95% confidence limits, the increase for Np-13-epiCbl (4.7 ± 2.5 cal mol^{-1} K^{-1}) is clearly significant, while the increase for Np-8-epiCbl (3.1 ± 2.9 cal mol^{-1} K^{-1}) is only barely so.

Both the original[64] and new[66] X-ray structures of CN-13-epiCbl show that the *e* side chain in this epimer is "upwardly" pseudo-axial, so that it brackets the β axial ligand position along with the pseudo-axial *a* and *c* side chains. This is not the case in CN-8-epiCbl,[43] where the epimerized *d* side chain is pseudo-equatorial and remote from the β axial ligand. It thus seems likely that the 24% increase in the entropy of activation of Np-13-epiCbl relative to NpCbl is the result of steric interactions between the now "upwardly" projecting *e* side chain and the bulky organic ligand. Since the effect is entirely in the entropy of activation, it is extremely unlikely that such steric interactions lead to a distortion of the C−Co bond in the ground state, which would necessarily have enthalpic consequences. We conclude that the increased entropy of activation for C−Co bond homolysis in Np-13-epiCbl probably indicates a decreased difference between the transition and ground state free energies due to the side chain thermal motions and their steric interactions with the organic ligand, both of which do make a contribution to the entropy changes associated with C−Co bond cleavage. This conclusion is in concert with that recently drawn from a study of the effects of alteration of the steric bulk of the carbonyl substituent on the *c* side chain of NpCbl which will be reported elsewhere.[67]

In the case of the C8 epimer, although the X-ray structure of CN-8-epiCbl shows that the epimerized *d* side chain is quite remote from the β axial ligand position, there is an increase in the "upward" fold of the corrin ring relative to CNCbl.[43] Thus, the smaller increase in entropy of activation of NpCbl thermolysis upon C8 epimerization could conceivably be due to increased steric interactions between the β face acetamide side chains and the bulky Np ligand brought about by this upward folding of the corrin. In order to assess the likely importance of such an effect, a study of the dependence of the steric interactions of acetamide chains with β organic ligands on the corrin ring fold by molecular mechanics is currently in progress.

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Supplementary Material Available: An Eyring plot for the aerobic thermolysis of Np-13-epiCbl and tables of UV−visible spectral parameters for the 13-epicorrinoids and for thermolysis of Np-13-epiCbl as a function of pH and termperature (3 pages). Ordering information is given on any current masthead page.

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