Vitamin B_{12}

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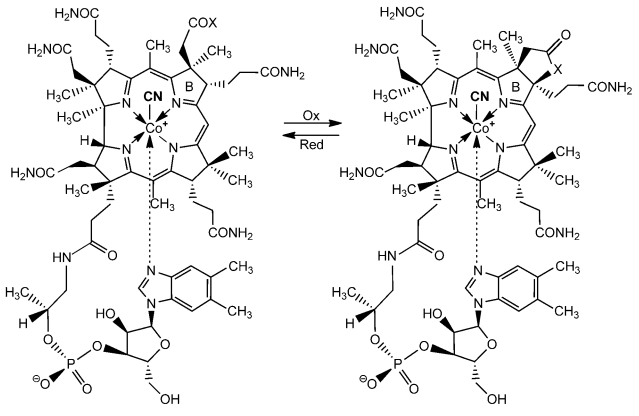
Reconstitution of the B_{12} Macrocycle by Radical Ring Closure of a Blue Secocorrin**

Markus Ruetz, Sergey N. Fedosov, and Bernhard Kräutler**

Dedicated to Professor François Diederich on the occasion of his 60th birthday

The cobyric acid moiety of vitamin B_{12} (**1**) is fascinating as a highly decorated and stereochemically complex corrin,[1–4] and its total synthesis[5,6] is considered by many to be the highlight of organic synthesis.[7] Natural corrins feature a characteristic set of substituents and "strategically" placed methyl groups, seen as a shield against degradation. Indeed, B_{12} cofactors are well equipped for their tasks in the catalysis of "difficult" organometallic enzyme chemistry,[8–11] and they are strikingly stable and resistant even under the conditions of free radical reactions.[12] However, the enolizable 8-position in ring B of the natural cobalt corrin complexes remains their "Achilles' heel": oxidation of vitamin B_{12} (**1**) affects the 8-position and efficiently furnishes corrinoid analogues, such as the lactone **2** and the lactam **3** (Scheme 1).[13] Furthermore, a blue corrinoid was formed by a puzzling partial degradation of vitamin B_{12} (**1**) at ambient temperature in aqueous bicarbonate solution. The blue compound was identified as the secocorrrin **4** (Scheme 2), in which the peripheral C–C bond between the saturated 7- and 8-positions of ring B had been cleaved.[14]



1 X = NH_2
7 X = OH
2 X = O
3 X = NH

Scheme 1.

As reported here, the carbonyl function at C8 and the double bond at C7 at the site of the unprecedented C–C bond rupture in the blue secocorrin **4** lend themselves to reductive reconstitution of the corrin ligand by a remarkable path: Treatment of the blue 8-oxo-7,8-*seco*-cobalamin-c-acid **4** with $NaBH_4$ in an oxygen-free aqueous solution led to a change of color from blue to brown within minutes and gave, after workup with air oxidation, red 8_β-hydroxy-7-epicobalamin-c-acid (**5**, 84% yield), in which the corrin ring was completely reconstituted. The UV/Vis and CD spectra of **5** (Figure 1) matched those of vitamin B_{12} (**1**), indicating structural similarity. The ESI mass spectrum of **5** exhibited a pseudomolecular ion at m/z 1372.4, which was 2 mass units more than that of the blue secocorrinoid **4**, and 17 more than **1**. Comparison of the ^{1}H NMR spectra of **5** with those of **4** and **1** showed the expected differences in the chemical shift values for protons associated with ring B. The ^{1}H and ^{13}C signals of all the H and C atoms, except for the cyanide carbon atom (see Table S1 in the Supporting Information), were assigned from spectra of **5** in D_2O or in H_2O/D_2O (9:1). The low-field region of the ^{1}H NMR spectrum of **5** showed high similarity to that of vitamin B_{12} (**1**),[15] and differed from the spectrum of the blue secocorrin **4**, for example, by the absence of the signal (of the C7^1-vinyl proton of **4**) at 6.16 ppm. An HMBC spectrum showed correlations between the protons of the d side chain and C7, as well as between protons of the c side chain and C8. This established the reconstitution of the C7–C8 bond of ring B in **5**. However, the signal of C8 of **5** had a ^{13}C chemical shift value of 86.3 ppm (i.e. 28.1 ppm lower field than the chemical shift of C8 in **1**), consistent with a bound hydroxy group. From ROESY spectra of **5** the relative configurations at C7 and C8 were deduced (see Figure S5 in the Supporting Information). ^{1}H,^{1}H correlations between the nucleotide base and the c and d side chains indicate the attachment of both of these groups at the ("lower") α face of the corrin ring. From the spectral data the structure of **5** was deduced as the Co_β-cyano-8_β-hydroxy-7-epicobalamin-c-acid (**5**, Scheme 2).

When a solution of the 7,8-secocobalamin **4** in methanol was reduced at −0.6 V (vs. 0.1N calomel electrode) under N_2 atmosphere, the color changed from blue to brown, consistent with the presence of Co^{II} corrins. A brown reduction mixture was still present after 4 h of electrolysis, when 1.8 F mol^{-1} had

[*] Mag. M. Ruetz, Prof. Dr. B. Kräutler
Institut für Organische Chemie
und Centrum für Molekulare Biowissenschaften (CMBI)
Universität Innsbruck, Innrain 80/82, 6020 Innsbruck (Austria)
E-mail: bernhard.kraeutler@uibk.ac.at
Homepage: http://www.uibk.ac.at/organic/en
Dr. S. N. Fedosov
Department of Engineering Science, Aarhus University
Science Park, Gustav Wieds Vej 10, 8000 Aarhus C (Denmark)
E-mail: snf@mb.au.dk

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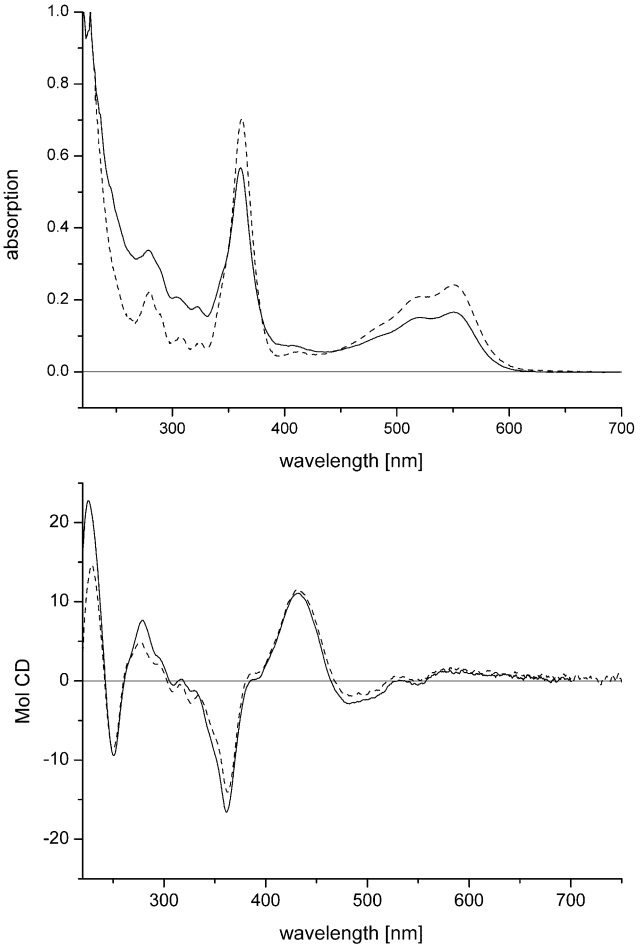
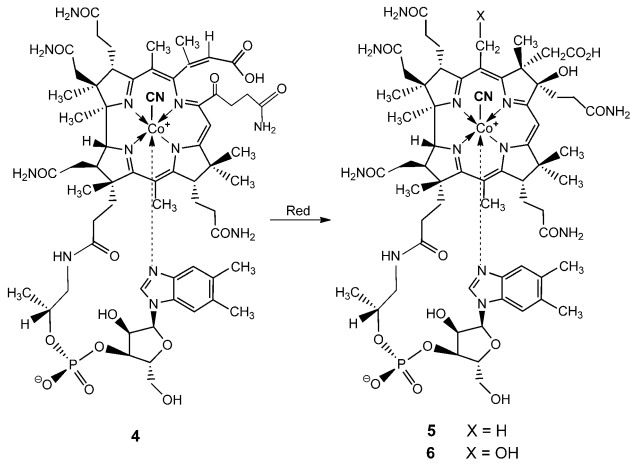


Figure 1. UV/Vis spectra (top) and CD spectra (bottom) of **5** (solid lines) and **1** (dashed lines) in water. UV/Vis spectra: $c(\mathbf{5}) = 2.57\times10^{-5}$ M; $c(\mathbf{1}) = 3.2\times10^{-5}$ M. CD spectra: $c(\mathbf{5}) = 2.57\times10^{-5}$ M; $c(\mathbf{1}) = 2.4\times10^{-5}$ M.



Scheme 2.

been consumed. Exposure of the reaction mixture to air resulted in an immediate color change to red. Two red compounds with nearly identical UV/Vis spectra (about 80% crude total yield) were detected by HPLC and separated by reversed-phase chromatography. The less polar, minor fraction was identified (by HPLC, UV/Vis, and NMR spectroscopy) as 8_β-hydroxy-7-epicobalamin-c-acid (**5**). The pseudomolecular ion, at m/z 1388.4, in an ESI mass spectrum of the more polar compound **6** indicated the presence of one more oxygen atom than in **5**. The ^{1}H NMR spectrum of **6** showed a new AB system at 4.93/4.94 ppm, but the singlet of the methyl group C51 was absent. Otherwise, the spectrum of **6** was similar to that of **5**, including the noted lack of a signal for the proton at C8. Likewise, ROESY correlations of the two methylene protons of the c side chain (at 3.35/3.57 ppm) occurred with protons of the d side chain. Thus, the relative configuration at the 7- and 8-positions in **6** was the same as in **5**. The new AB system (at 4.93/4.94 ppm) correlated with the proton of the c side chain at 3.57 ppm, the methyl group at C7A, and the proton at C3. Thus, the new AB system was located at C51, explaining the absence of a methyl group singlet for C51. In summary, in the major product of the electrochemical reduction of **4**, two hydroxy groups were present, one of them at C51. The red corrinoid **6** was, hence, deduced to be the Co_β-cyano-51,8_β-dihydroxy-7-epicobalamin-c-acid (**6**), with an intact corrin ring and with the c side chain attached at the α-face of the corrin macrocycle (Scheme 2).

To examine the mechanistic basis for the remarkable formation of **6**, electrochemical reduction of **4** was conducted in CD_3OD. The corresponding products (**[D$_n$]-5** und **[D$_m$]-6**) were analyzed for their isotopic label (see the Supporting Information). According to the ^{1}H NMR and ESI mass spectra of **[D$_n$]-5**, it consisted of 45% **[D$_1$]-5** and 55% **[D$_2$]-5**, with nearly 100% deuterium label at C51 and 45% at C81.

The corrin moiety of vitamin B_{12} has thermodynamic features[1] that give it an intriguing propensity for restoration under suitable reaction conditions (e.g. reduction of the B_{12} oxidation product **2** gives B_{12}-c-acid **7**[5,16]). As shown here, the reductive closure of ring B of the blue secocorrin **4** provided the corrin **5** and opened a new and efficient path to the B_{12} macroring. In related reductive formation of C–C bonds in cobalt or nickel complexes of 5,6-secocorrins, the corrin ring of the cobyrinic acid derivatives could also be reconstituted.[17,18] Template effects of transition-metal ions and redox communication between the tetrapyrrolic ligand and the coordinated metal center were used strategically to assist the crucial ring-forming steps to corrins.[19,20] Indeed, in the original approaches by Eschenmoser and Woodward for the total synthesis of vitamin B_{12} (**1**) the formation of the highly substituted C–C bond between rings A and D of the corrin ring was considered to be particularly difficult,[5,6] and a remarkable photochemical ring-closing reaction was developed to master that synthetic challenge.[21]

The site of the cleavage of ring B in **4** features two unsaturated carbons (C7 and C8) that display a remarkably short "nonbonding" interaction (2.75 Å)[14] (Figure 2). Reductive closure of ring B apparently occurs without conformational reorientation of the groups attached at C7 and C8. Reduction of **4** with $NaBH_4$ gives the corrin **5**, without incorporation of a hydrogen atom from the reducing reagent.[22] This reaction may, thus, occur by electron- and proton-transfer reactions and radical intermediates. This

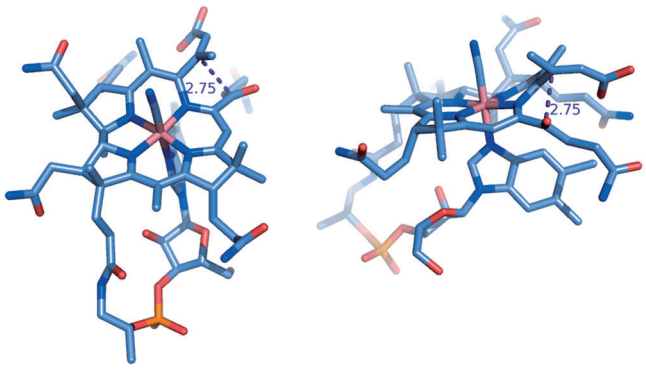


Figure 2. Two views of the structure of the blue secocorrin **4** according to X-ray analysis.[14]

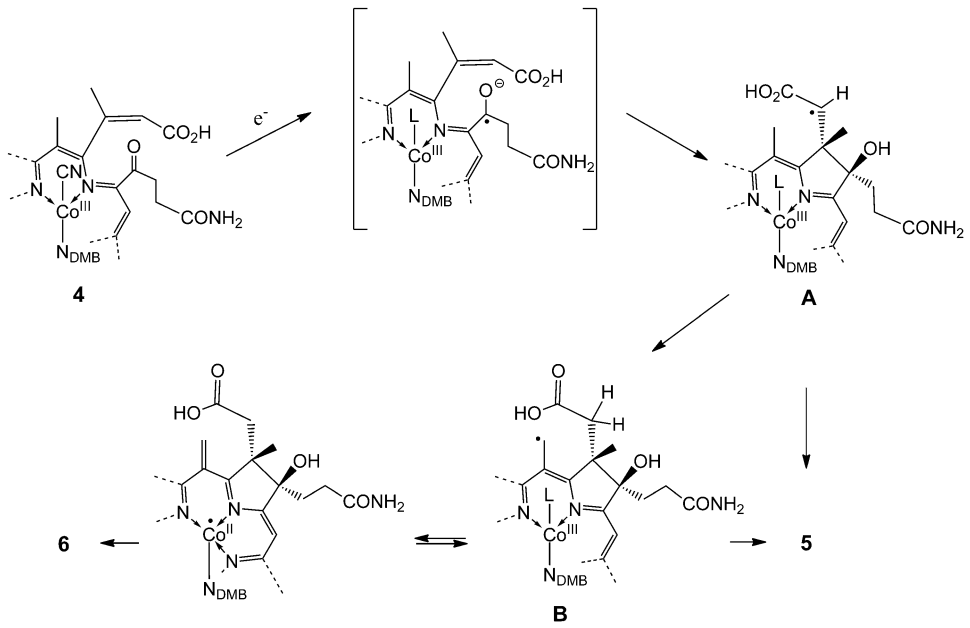
mechanistic aspect was scrutinized by electrochemical reduction of the secocorrin **4**. This approach not only gave the corrinoid reduction product **5**, but **6** also a corrinoid analogue of **5**, in which the methyl group at the 5-*meso* position was hydroxylated. The modification observed at the C51 position of **6** is reminiscent of earlier experiences in (adventitious) reactions at the methyl groups at the two *meso* positions of cobyrinic acid derivatives, which were rationalized by radical mechanisms.[23,24]

As depicted in Scheme 3, a radical mechanism is likely to account for the formation of the two corrinoid products, **5** and **6**, under the conditions of the reported electrosynthetic experiment. Radical **A** is considered a possible, short-lived intermediate on the way to **5** and **6**. It may come about by one-electron reduction of the ligand π system of blue secocorrin **4** and rapid radical bond formation between C7 and C8. Indeed, the structure of **4** shows short nonbonding contacts at C7 and C8 and is already highly preorganized for formation of the C–C bond (see Figure 2). Thus, the bond-forming process and electron transfer might be concerted. The carbon-centered radical at C7[1] of **A** would then be suitably positioned to abstract an H atom from the nearby methyl group C51, giving the stabilized radical **B**. The π-delocalized radical **B** could furnish **6** by electron transfer to the cobalt center and addition of a solvent nucleophile at C51 (MeOH or H_2O). Formation of **[D$_n$]-5** from experiments in CD_3OD also supported such a mechanistic scenario: **[D$_n$]-5** (partially) deuterated at C51 is likely to form via radical **B** and abstraction of a D atom from (the methyl group of) CD_3OD.

8_β-Hydroxy-7-epicobalamin-c-acid (**5**) was shown by NMR analysis to have five of the seven side chains bound to the lower face of the corrin moiety. The capacity of **5** to bind the three human proteins of specific B_{12} transport, that is, intrinsic factor (IF), transcobalamin (TC), and haptocorrin (HC),[25] was tested. The ability of these B_{12}-binding proteins to discriminate between vitamin B_{12} and its analogues decreases in the order IF > TC > HC for all corrinoids tested so far (see Ref. [26]). The kinetics of the binding process of **5** was measured using the fluorescent conjugate of vitamin B_{12} CBC.[27] Attachment of this reporter ligand to B_{12}-binding proteins leads to an increased fluorescence quantum yield of CBC (see Refs. [26,27]). The competition between the B_{12} analogue **5** and CBC for interaction with the B_{12}-binding proteins was analyzed by means of the time dependence of the fluorescence of CBC, shown in Figure 3.

The binding process (Figure 3), in which the complex E·CBC was traceable by fluorescence, followed a binding scheme in which two ligands compete for a site at E (where E = IF, HC or TC): E·**5**←E→E·CBC. We assumed the binding transitions to be effectively irreversible within the time interval of 0.2 s, and the binding rate constants of the B_{12} analogue **5** were calculated by computer fitting and appeared to be very low for all three B_{12}-binding proteins (Table 1). Binding of **5** to IF was hardly detectable in our setup ($k_{+1} \leq 0.1 \times 10^6\,M^{-1}s^{-1}$). Vitamin B_{12} c-acid (**7**) bound under the same conditions by one to two orders of magnitude faster ($k_{+c} = 2.7/6.1/6.0 \times 10^6\,M^{-1}s^{-1}$ for IF/TC/HC); see comparative data in Figure 3 and Ref. [14] for more details. Inefficient primary binding of the B_{12} analogue **5** is probably caused by spatial incompatibility of its reoriented carboxylate group with Glu379 of IF, as well as Gln389 of TC and Gly391 of HC,[28] which belong to the protein sites involved in primary attachment of vitamin B_{12} (**1**).[26]

In a second set of experiments, the dissociation of the test ligand **5** was studied after



Scheme 3. Electrochemical reduction of 7,8-*seco*cobalamin **4**. Suggested radical intermediates on the way to Co_β-cyano-8_β-hydroxy-7-epicobalamin-c-acid **5** and Co_β-cyano-51,8_β-dihydroxy-7-epicobalamin-c-acid **6**.

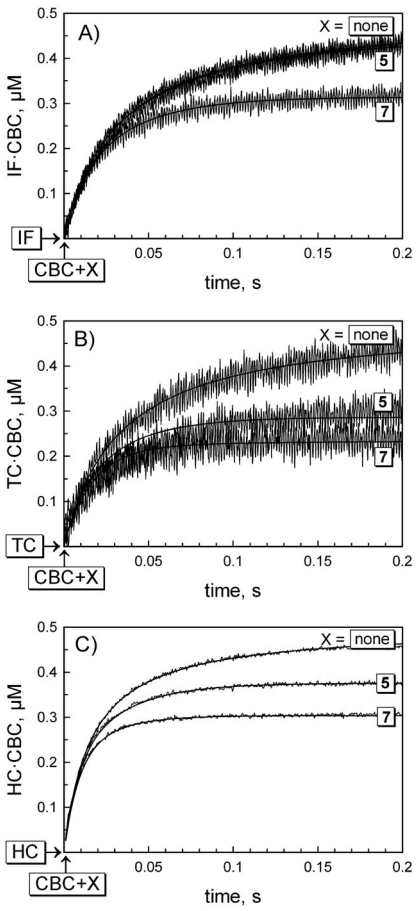


Figure 3. Binding kinetics of the B_{12} analogue **5** in competition with the fluorescent B_{12} conjugate CBC. Final conditions in all cases: 0.5 μM protein, 0.5 μM CBC, 4.5 μM test ligand X (**5** or **7**), 21 °C, pH 7.5. Thick solid lines show the simulations with calculated values for binding of the test ligand X (**5** or **7**) to A) intrinsic factor (IF), B) transcobalamine, C) haptocorrin.

Table 1: Calculated rate constants for the interaction between **5** and the specific B_{12}-binding proteins IF, TC, and HC (="E"), and deduced apparent dissociation constants K_d^{app}(**5**), based on the overall binding scheme shown in Equation (1).

Protein (E)	E·CBC←E k_{+C} [$M^{-1}s^{-1}$]	E⇄E_1·**5** $k_{+1,-1}$ [$M^{-1}s^{-1}$],[s^{-1}]	E_1·**5**⇄E_2·**5** $k_{+2,-2}$ [s^{-1}]	K_d^{app}(**5**)[a] [M]
IF	$64/50\times10^6$	$k_{+1}\approx0.1\times10^6$ $k_{-1}=3$	$k_{+2}=0.6$ $k_{-2}=0.7$	16×10^{-6}
TC	61×10^6	$k_{+1}=3.5\times10^6$ $k_{-1}=0.06$	$k_{+2}=0.002$ $k_{-2}=0.0004$	2.8×10^{-9}
HC	125×10^6	$k_{+1}=2.5\times10^6$ $k_{-1}=0.04$	$k_{+2}=0.01$ $k_{-2}=0.02$	1.1×10^{-8}

[a] The apparent dissociation constant of **5**. $K_d^{app}(\mathbf{5}) = K_{1/2} = 1/[k_{+1}/k_{-1}+(k_{+1}/k_{-1})\cdot(k_{+2}/k_{-2})]$.

its binding to the protein E, followed by exposure to CBC (see Figure S8 in the Supporting Information). The reaction was consistent with the mechanism given in Equation (1), where the complex E·CBC was traced over time. Notations E_1·**5** and E_2·**5** stand for the primary protein–ligand complex (=E_1·**5**) with dissembled domains α···**5**·β; and for the secondary complex (E_2·**5**) with fully assembled domains α·**5**·β.[25,28]

$$\underset{(+\mathbf{5})}{\text{E·CBC}} \rightleftharpoons \text{CBC} + \text{E} + \mathbf{5} \rightleftharpoons \underset{(+\text{CBC})}{\text{E}_1\text{·}\mathbf{5}} \rightleftharpoons \underset{(+\text{CBC})}{\text{E}_2\text{·}\mathbf{5}} \qquad (1)$$

The rate constants of primary binding were calculated by computer fitting of the data (see Table 1 and Figure S8 in the Supporting Information). The overall affinities, and likewise the binding rates, of the B_{12} transport proteins for the corrin **5** showed a "wrong" order (i.e. TC > HC > IF), with TC being the best binder for this B_{12} ligand. All other B_{12} analogues fall within the "normal" order of affinities (i.e. HC > TC > IF), where HC is the strongest binder. Amazingly, the analogue **5** bound better to TC, and the protein–ligand contacts tightened over 30 min of incubation. During this time, the percentage of highly dissociable complex E_1·**5** decreased from 75% (2 min) to 15% (32 min), and the more "stable" complex E_2·**5** appeared instead. This shift was expressed by an increasing amplitude of the slow dissociation phase (Figure S8 in the Supporting Information).

Stabilization of the B_{12} analogue **5** in the binding site of TC caused a 6-fold increase in the overall equilibrium affinity (K_d^{app}). Yet, the rate of dissociation decreased by a factor of 120 and became very slow compared to IF and HC. Retarded dissociation of **5** from TC might be caused, among other reasons, by formation of a hydrogen bond between the C8-OH group of **5** and His173.[28] This residue is present only in TC and probably occupies the space above the C8-OH group of **5** in the complex TC·**5** (based on the data of TC·B_{12}).[28] The remarkably slow dissociation of **5** makes the low-affinity complex TC·**5** sufficiently long-lived (when preformed in vitro) to be delivered to the tissues through blood circulation.

The efficient formation of the red corrin Co_β-cyano-8_β-hydroxy-7-epicobalamin-c-acid (**5**) from the blue secocorrinoid B_{12} degradation product **4** illustrates the striking propensity of the corrin macroring of vitamin B_{12} to be restored under proper (reducing) conditions (for a less dramatic example, see Ref. [29]). A fast radical process appears to afford the ring-closure reaction **4**→**5**, which produces a 7-epicobalamin, a new type of a vitamin B_{12} analogue. In **5**, five (of the seven) side chains protrude from the "lower" face of the B_{12} macrocycle. Binding of **5** to the B_{12} transport protein intrinsic factor (IF) is particularly weak (K_d = 16 μM), and **5** shows an unprecedented order of affinities (TC > HC > IF). The B_{12} analogue **5** binds much weaker than vitamin B_{12} (**1**)[25] and the B_{12}-c-acid (**7**),[14] reflecting the capacity of these specific and strong B_{12} binders to discriminate between vitamin B_{12} and its close relatives. At the typical physiological B_{12} concentrations in the human body, the 7-epicobalamin **5** would mostly exist unbound by any of the three specific B_{12}-binding proteins. This is the first example of a B_{12} analogue that retains the major elements of the B_{12} structure, and yet it is hardly bound by any of these B_{12}-binding proteins.

Experimental Section

General: 7,8-Secocobalamin **4**[14] and B_{12}-c-acid (**7**)[13,16] were prepared as described in Refs. [14,16]; see the Supporting Information for further general information.

Synthesis of **5**: Inside a glove box, compound **4** (0.43 mg, 0.31 μmol) was dissolved in 0.3 mL of oxygen-free water. $NaBH_4$ (ca. 0.05 mg) was added to this solution, and the color of the solution changed from blue to brown within minutes. After 15 min the flask was removed from the glove box and the reaction was stopped by exposure to air. After workup (for details see the Supporting Information) compound **5** (0.36 mg,0.26 μmol; 84% yield) was obtained as a microcrystalline precipitate, which was characterized as follows: UV/Vis (H_2O, $c = 2.6 \times 10^{-5}$ M): λ_{max} ($\log \varepsilon$) = 551 (3.81), 519(3.77), 407(3.46), 361(4.34), 322(3.85), 306(3.91), 278(4.12) nm. CD (H_2O, $c = 2.6 \times 10^{-5}$ M): λ_{max}, λ_{min} ($\Delta\varepsilon$) [nm ($mol^{-1} cm^3 cm^{-1}$]: 581 (1.4), 530 (0.1), 482.5 (−2.9), 432.5 (11.1), 361.5 (−16.6), 317.5 (0.2), 309.5 (−0.5), 278.5 (7.6), 250.5 (−9.4) (see Figure 1). ^1H and ^{13}C NMR: see Table S1 in the Supporting Information. ESI-MS (MeOH): 1410.4 (16, $[M+K]^+$), 1394.4 (18, $[M+Na]^+$), 1374.4 (33), 1373.4 (73), 1372.4 (100, $[M+H]^+$), 1354.4 (22, $[M-H_2O]^+$ 1226.3 (18, $[M-C_9H_9N_2]^+$), 1014.3 (17, $[M-C_{14}H_{18}N_2O_7P]^+$.

Electrosynthesis of **6**: Inside a glove box, a solution of compound **4** (1.1 mg, 0.80 μmol) in 3.0 mL of electrolyte solution (0.1 M TBAHFP in MeOH) was placed in the cathode compartment of an electrolysis cell. Reduction at −0.6 V (vs. 0.1 N calomel electrode) at a mercury electrode consumed 1.8 F mol^{-1} after 4 h. Then the electrolysis was stopped and the solution was worked up (for details, see the Supporting Information). A crude red product was obtained, which consisted of two red compounds initially, according to HPLC. The two compounds were separated by preparative HPLC. After desalting and precipitation (see the Supporting Information), **5** (0.18 mg, 0.15 μmol; 17.8% yield) and **6** (0.55 mg, 0.39 μmol; 48.7% yield) were obtained as microcrystalline powders. The latter sample was characterized as follows: UV/Vis (H_2O, $c = 2.12 \times 10^{-5}$ M): λ_{max} ($\log\varepsilon$) = 546.5 (3.83), 520(3.78), 402(3.53), 358(4.29), 320(3.84), 304(3.89), 279-(4.14) nm. ^1H NMR: see Table S1 in the Supporting Information. ESI-MS (MeOH): 1410. (22, $[M+Na]^+$), 1390.5 (50), 1389.5 (83), 1388.5 (100, $[M+H]^+$), 1242.4 (26) $[M-C_9H_9N_2]^+$), 1030.4 (35) $[M-C_{14}H_{18}N_2O_7P]^+$.

Binding and dissociation experiments with the proteins of B_{12} transport: The reactions were performed in 0.2 M sodium phosphate buffer, pH 7.5, 21 °C. The binding kinetics were monitored with a stopped-flow spectrofluorometer DX.17 MV (Applied Biophysics, UK) using the fluorescent response from the B_{12}-conjugate CBC,[25,27] excitation 525 nm, emission >550 nm, slit 1.5 mm, voltage 380—400 V, band pass of 18.6 nm, light path of 1 cm. Binding reactions were started by rapid mixing of the B_{12}-binding protein E (HC, TC, or IF) with either CBC alone or with CBC and B_{12}-analogue **5**. Maximal amplitude of the fluorescent response (E + CBC) corresponded to 0.5 μM of the E·CBC complex. It was affected by the presence of the nonfluorescent competing B_{12}-analogue **5**. All reactions recorded in Figure 3 and Figure S8 in the Supporting Information represent the average of three to six individual curves. For further details see the legend of Figure 3).

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