Vitamin B_{12} Antimetabolites

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Access to Organometallic Arylcobaltcorrins through Radical Synthesis: 4-Ethylphenylcobalamin, a Potential "Antivitamin B_{12}"**

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A complete lack of vitamin B_{12} (cobalamins) is lethal to humans and mammals,[1,2] which depend upon two organometallic B_{12} cofactors to maintain normal metabolism. The two B_{12} cofactors, coenzyme B_{12} (AdoCbl, 5′-deoxyadenosylcobalamin) and methylcobalamin (MeCbl), owe their cofactor function to the reactivity associated with their Co–C bonds.[3–6] In contrast, vitamin B_{12} (CNCbl, cyanocobalamin) is a physiologically inactive cyanocorrin, whose life-sustaining effect as a (pro)vitamin in humans is due to its role as a metabolic precursor of AdoCbl and MeCbl.[7] Indeed, when CNCbl and other cob(III)alamins are taken up by the cells of healthy animals and humans, the enzyme CblC degrades these Co^{III}-corrins to cob(II)alamin, the substrate in the biosynthesis of AdoCbl and MeCbl.[7] The protein CblC, also named MMACHC (for methylmalonic aciduria type C and homocystinuria), is the product of *cblC* (the "MMACHC gene").[8] CblC is remarkably versatile and efficient at removing cobalt-bound ligands from typical cobalamins, for example, the cyanide ligand from CNCbl, to produce cob(II)alamin (Scheme 1).[7] The mechanisms employed by CblC are either a nucleophilic displacement of cobalt-bound organic groups or, alternatively, the reductive dissociation of Co^{III}-bound inorganic ligands.[9,10] Impairment of CblC by mutations blocks the path to the B_{12} cofactors of vitamin B_{12} and leads to "functional" vitamin B_{12} deficiency, which has severe pathophysiological consequences.[8]

On the other hand, vitamin B_{12} deficiency is deliberately "induced" and studied in laboratory animals to elaborate on suspected but still unidentified contributions of such a deficiency to pathological phenomena, such as to degenerative diseases of the central and peripheral nervous systems.[11] The usual approach to induce vitamin B_{12} deficiency in laboratory mice requires "total" gastrectomy,[12] which has serious physiological interferences remote from the B_{12}-specific metabolic questions. An alternative to the surgical procedure would be to administer suitable vitamin B_{12} antagonists ("antivitamins B_{12}"), if available, to intact (wild-type) laboratory animals to provoke vitamin B_{12} deficiency symptoms similar to those observed in patients with a reduced uptake of vitamin B_{12}.[1]

We set out to find promising "antivitamins B_{12}", that is, close analogues of vitamin B_{12} that would be taken up by animals (and humans) by the proteins of vitamin B_{12} transport[13] without subsequent conversion into active B_{12} cofactors.[7] The required properties of such vitamin B_{12} antagonists could be found in (non-natural) cobalamins with high thermolytic stability and inertness towards degradation by the "tinkering" enzyme CblC. We conjectured that Co_β-arylcobalamins might have the desired structural properties and feature strong Co–C bonds resistant to CblC. Surprisingly, Co_β-arylcorrins are still an unknown class of vitamin B_{12} derivatives.[5,14] This situation is clearly a consequence of a lack of methods suitable for the synthesis of arylcorrins.[5,14] As shown here, the arylcobalamin Co_β-4-ethylphenylcob(III)alamin (EtPhCbl) could be easily and efficiently prepared by a cobalt arylation that employs the radicaloid cob(II)alamin as a selective radical trap[15,16] of an aryl radical (Scheme 2).

The organometallic cobalamin EtPhCbl was prepared in a one-pot reaction in aqueous solution, starting with 4-ethylphenyldiazonium tetrafluoroborate and aquocobalamin (H_2OCbl), and proceeding via cob(II)alamin by reduction of the H_2OCbl with sodium formate.[17–19] Dark red crystalline EtPhCbl was obtained in 56% yield. We inferred a process in which cob(II)alamin would reduce the diazonium ion to generate the 4-ethylphenyl radical,[20,21] which would subsequently be trapped rapidly and efficiently by cob(II)alamin (Scheme 3).

The expected structure of Co_β-4-ethylphenylcob(III)alamin was confirmed, as follows (see the Supporting Information): Its UV/Vis spectrum resembled the spectra of organometallic vitamin B_{12} derivatives in their "base-on" form (see Figure S2 in the Supporting Information). The pseudomolecular ion with the mass $m/z = 1434.7$ was compatible with the molecular formula $C_{70}H_{97}CoN_{13}O_{14}P$. ^1H homonuclear and $^1H,^{13}C$ heteronuclear NMR spectra recorded in D_2O (see Figure S4 in the Supporting Information) allowed the assignment of the signals of all the 82

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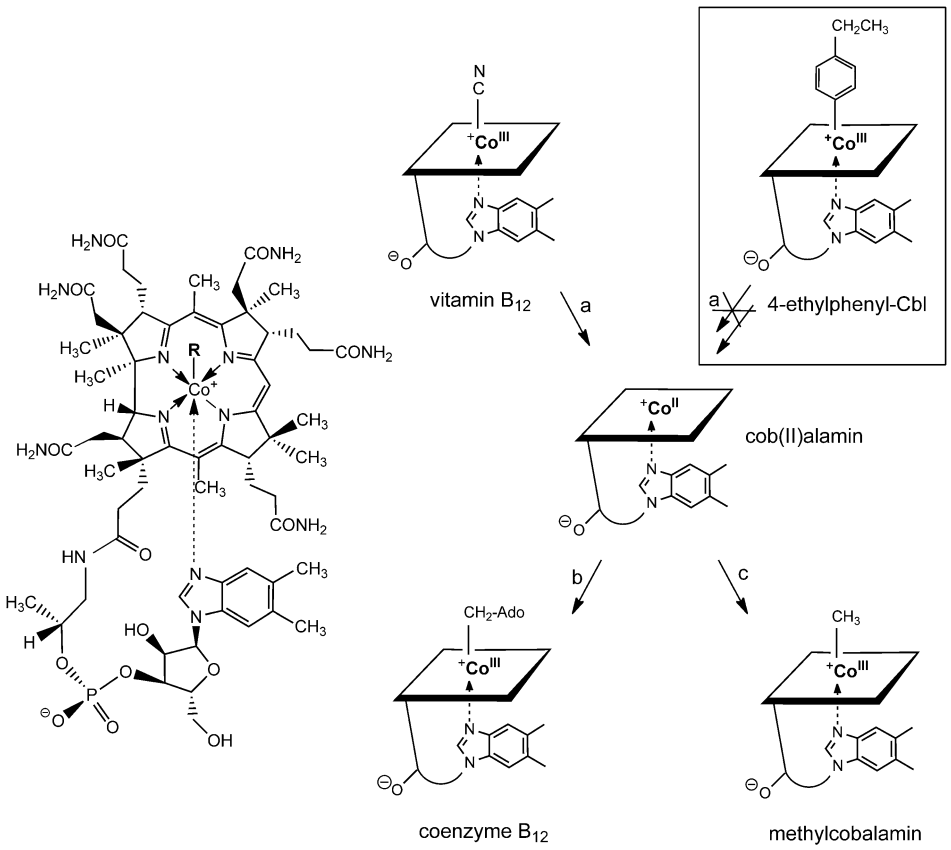
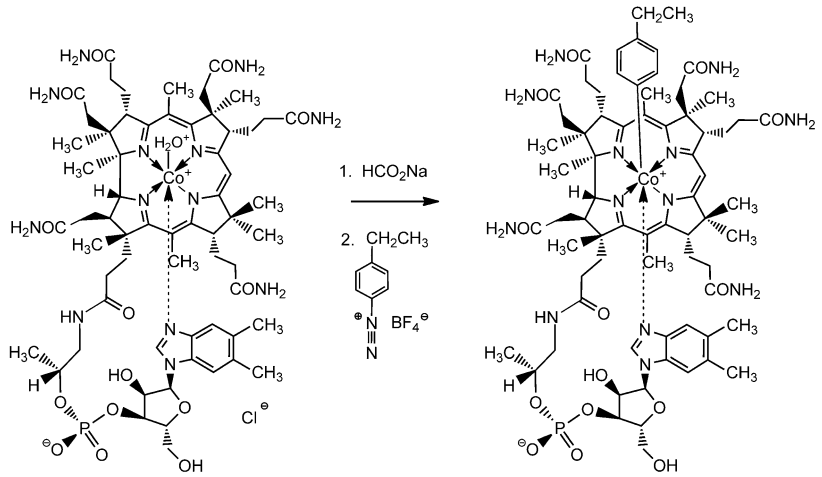


Scheme 1. Left: General structural formulas of some relevant cobalamins: vitamin B_{12} (R = CN, cyanocobalamin, CNCbl), coenzyme B_{12} (R = 5′-deoxyadenosyl, 5′-deoxyadenosylcobalamin, AdoCbl), methylcobalamin (R = methyl, MeCbl), aquocobalamin (R = H_2O, H_2OCbl, a cation), 4-ethylphenylcobalamin (R = 4-ethylphenyl, EtPhCbl). Right: Schematic representation of exemplary cobalamins and their biosynthetic conversions in mammals a) by the enzyme CblC, b) by adenosyltransferase, and c) by methionine synthase. EtPhCbl is resistant to conversion by CblC into cob(II)alamin.

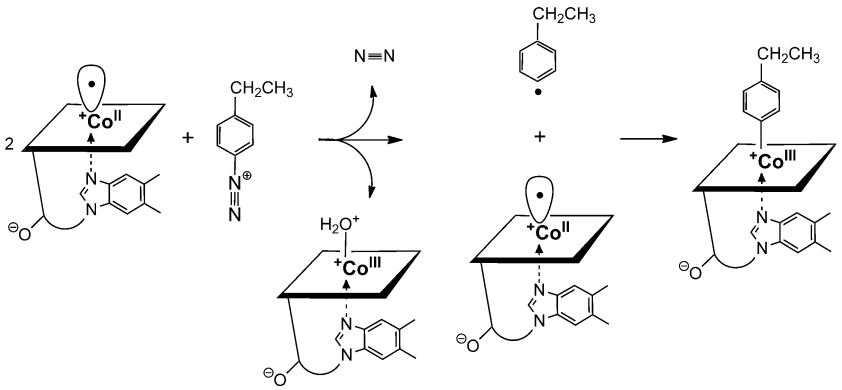


Scheme 2. Synthesis of EtPhCbl from H_2OCbl and 4-ethylphenyldiazonium tetrafluoroborate.

carbon-bound hydrogen atoms and of all the 70 carbon atoms (see Table S1 in the Supporting Information). These spectra established EtPhCbl as a "base-on" cobalamin carrying a σ-bonded aryl ligand at the "upper" β face of the cobalt center. ^{1}H NMR spectroscopic data and ^{1}H,^{1}H NOE correlations suggested the planar phenyl group to be oriented "north-south", and to undergo rapid rotations around its Co–C bond at ambient temperatures.

Crystals of EtPhCbl were grown from aqueous acetone. The crystal structure of EtPhCbl (Figure 1) was determined using synchrotron radiation, with diffraction data extending to a resolution of 0.8 Å. The corrin-bound Co^{III} center sits in a pseudooctahedral coordination sphere, with typical Co–N distances in the equatorial plane (see the Supporting Information). The cobalt atom is slightly displaced out of the plane of the four corrin nitrogen atoms and is shifted by 0.029 Å towards the upper 4-ethylphenyl ligand. The Co–C_β distance to the aryl ligand of EtPhCbl (1.981 Å) is slightly shorter than in alkylcobalamins (mean value: 2.003 Å), but longer that in vinylcobalamin (1.91 Å).[22] The Co–N_α distance to the *trans*-axial nucleotide ligand (2.230 Å) is also longer than in vinylcobalamin (2.165 Å).[22] These axial bond distances in EtPhCbl point to a strong "inverse" *trans* effect in this cobalamin.[23] The phenyl ring of the β ligand is oriented north-south relative to the corrin ring (torsion angle C5-Co-C1β-C2β: 0.6°) and is nearly coplanar (3° tilt) with the dimethylbenzimidazole ligand. Consistent with the close nonbonded interactions of the phenyl and corrin moieties, the fold angle of the latter is reduced to a value of 7.6(1)°, close to the "record" (5.9°) in *cis*-chlorovinylcobalamin.[22]

Exploratory experiments with the arylcobalamin EtPhCbl confirmed its expected unprecedented thermal stability and its resistance to protonolysis: Heating an aqueous solution of EtPhCbl (buffered at pH 7) at reflux for 24 h did not lead to detectable cleavage of the Co–C bond and decomposition to aquocobalamin (H_2OCbl). In slightly acidic aqueous solution, base-on EtPhCbl was protonated to base-off EtPhCbl-H^+, which exhibited a pK_a value of 3.7 ± 0.1 at 25 °C, similar to that of protonated coenzyme B_{12} (AdoCbl-H^+; see Figure S6 in the Supporting Information). The formation of aquocobalamin (H_2OCbl)



Scheme 3. Proposed mechanism for the formation of EtPhCbl through a hypothetical one-electron reduction of 4-ethylphenyldiazonium tetrafluoroborate by cob(II)alamin with loss of dinitrogen, followed by recombination of cob(II)alamin with the 4-ethylphenyl radical.

binding/dissociation data for TC are presented in Figure S14 of the Supporting Information). Values of k_+ and k_- were assessed by computer fitting. The fast binding of EtPhCbl to IF ($k_+ = 7.9 \times 10^7\,\text{M}^{-1}\,\text{s}^{-1}$) and to TC ($k_+ = 9.3 \times 10^7\,\text{M}^{-1}\,\text{s}^{-1}$), as well as the slow dissociation from IF ($k_- < 5 \times 10^{-6}\,\text{s}^{-1}$) and TC ($k_- < 4 \times 10^{-6}\,\text{s}^{-1}$) resembled the analogous processes with CNCbl.[24] The calculated equilibrium dissociation constant of EtPhCbl was $K_d \approx 2 \times 10^{-14}\,\text{M}$ for both IF and TC. Therefore, the uptake and circulation of the arylcobalamin EtPhCbl and CNCbl in humans are expected to have a similar efficiency.

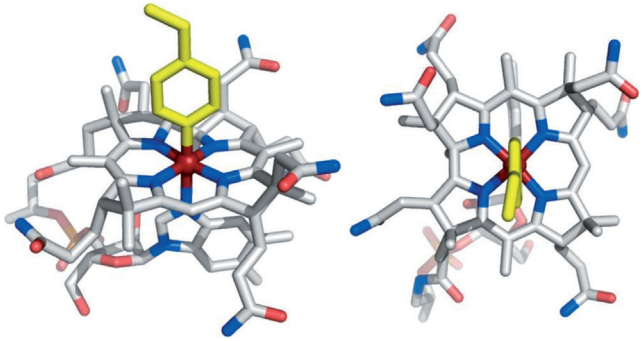


Figure 1. Two projections of a model of the structure of EtPhCbl derived from X-ray crystal-structure analysis. Gray: C atoms of the corrin unit, yellow: C atoms of the phenyl ligands, dark red: Co atom, blue: N atoms, light red: O atoms.

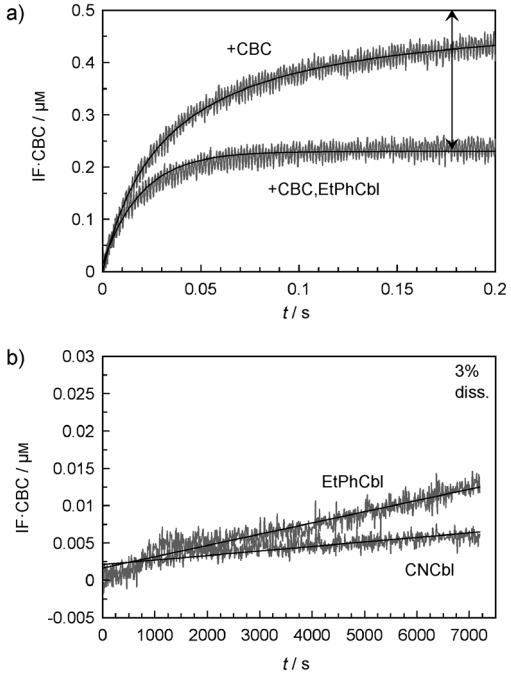


Figure 2. Interaction of EtPhCbl with IF. a) Binding of EtPhCbl to IF measured by competition with the fluorescent B_{12} derivative CBC ($k_{+\text{CBC}} = 64 \times 10^6\,\text{M}^{-1}\,\text{s}^{-1}$): IF was rapidly mixed with either CBC or a solution of CBC and EtPhCbl. The arrow indicates the effect of competition between CBC and EtPhCbl, consistent with $k_{+\text{EtPhCbl}} = 79 \times 10^6\,\text{M}^{-1}\,\text{s}^{-1}$. b) Dissociation of EtPhCbl from IF: The preincubated complex of IF (1.0 μM) and either EtPhCbl or CNCbl (1.3 μM) was mixed with the "chasing" B_{12} derivative CBC (1.3 μM). The dissociation rate constants of the complexes were calculated from the increase in the fluorescence: $k_{-\text{EtPhCbl}} \approx 1.5 \times 10^{-6}\,\text{s}^{-1}$ and $k_{-\text{CNCbl}} \approx 6 \times 10^{-7}\,\text{s}^{-1}$ (see the Supporting Information for further details).

was not detected when an acidic aqueous solution (pH 2) of EtPhCbl was heated at 95 °C for 6 h (see Figure S7 in the Supporting Information). The arylcorrin EtPhCbl was sensitive to light in aerated aqueous solution, and its exposure to daylight gave H_2OCbl through cleavage of the Co–C bond (see Figure S11 in the Supporting Information).

4-Ethylphenylcobalamin (EtPhCbl) bound with high affinity to two crucial human "B_{12}-transporter" proteins, intrinsic factor (IF) and transcobalamin (TC), as was tested by competition experiments with CBC, a fluorescent conjugate of CNCbl (see Figure 2 and Figure S14 in the Supporting Information). The binding of CBC to a Cbl-specific protein increases the emission of the conjugate.[13] The presence of a competing cobalamin decreases the fluorescence, which becomes 50% at equal binding rate constants, and [Cbl] = [CBC] according to the scheme IF·Cbl ← Cbl + IF + CBC → IF·CBC (see Figure 2a). In addition, the dissociation kinetics were measured after preincubation of IF separately with EtPhCbl or CNCbl followed by addition of the "chasing" B_{12} derivative CBC. The process obeyed the scheme IF·Cbl → Cbl + IF + CBC → IF·CBC, where the first step was rate-limiting. The initial slope corresponded to k_-[IF·Cbl] after conversion of the fluorescence to the concentration of IF·CBC (see Figure 2B; the corresponding

The arylcob(III)alamin EtPhCbl also bound to CblC with a high binding affinity, as determined by isothermal calorimetry (ITC, $K_D = 20$ nM; see Figure S17 in the Supporting Information). By analogy to MeCbl, tight binding of EtPhCbl to CblC required the arylcobalamin to adopt a base-off form, as was deduced from the characteristic changes in the UV/Vis absorption spectrum (Figure 3a). Accommodation of the

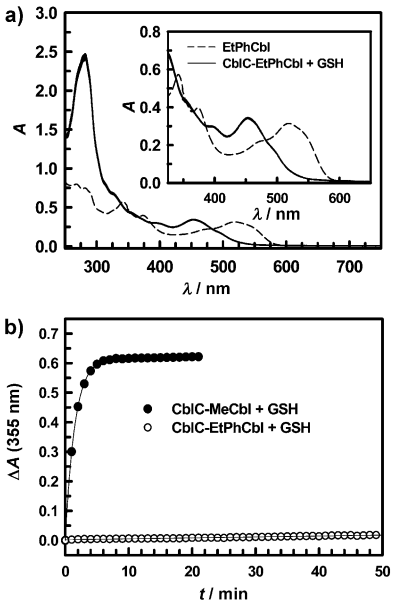


Figure 3. a) EtPhCbl is bound to CblC as a base-off form, as seen in the characteristic spectral changes and hypsochromic shift of the absorbance maxima. b) EtPhCbl is processed very slowly by CblC in the presence of glutathione (GSH, ○), whereas MeCbl is rapidly demethylated (●); see the Supporting Information for experimental details.

larger aryl moiety was consistent with expectations from modeling studies using the crystal structures of CblC with bound MeCbl as a template.[25] However, in contrast to the rapid demethylation of MeCbl by glutathione catalyzed by CblC ($k_{obs} = 0.6\ min^{-1}$), the arylcorrinoid EtPhCbl was resistant to removal of its cobalt-bound organometallic ligand by CblC (Figure 3B), and a reliable dealkylation rate could not be estimated.

4-Ethylphenylcobalamin (EtPhCbl) is the first example of an organometallic arylcobalamin, a new group of Co^{III} corrins. It was prepared in a reaction postulated to generate the Co–C bond through combination of an aryl radical and cob(II)alamin. This synthetic path, unprecedented in preparative vitamin B_{12} chemistry, makes use of the extraordinary capacity of cob(II)alamin and related B_{12} derivatives to capture ("slave in"[16]) highly reactive radicals at the corrin-bound cobalt center.[16,26] Ongoing work in our laboratory is focused on testing and extending the scope of the method developed here for the synthesis of other arylcobalamins. Indeed, the expected properties of the Co–C bonds of organometallic Co^{III} arylcorrins are likely to arouse interest in this class of compounds.

As was assumed on the basis of the reactivity of its aryl ligand, the vitamin B_{12} derivative EtPhCbl features a Co–C bond that is stabilized against thermolysis and is inert to acid- and nucleophile-induced cleavage (see Figures S7, S8, and S12 in the Supporting Information). On the other hand, the organometallic bond of EtPhCbl could be efficiently cleaved by light, which is consistent with the typical photosensitivity of organometallic vitamin B_{12} derivatives.[27] This feature of arylcobalamins will make laser photolysis with visible light an efficient tool for the controlled "unlocking" (with spatial and temporal control) of the masked vitamin properties of such "antivitamins B_{12}" in cell culture and animal studies.

Thus, organometallic arylcobalamins represent a new group of chemically inert non-natural corrinoids, designed here as vitamin B_{12} antagonists ("antivitamins B_{12}"). In tests with microorganisms, the previously used vitamin B_{12} antagonists were cobalamins modified at the corrin moiety or at the nucleotide loop.[28] These "B_{12} antagonists" were low affinity ligands, not suitable for efficient uptake by mammalian vitamin B_{12} transport systems.[24] In contrast, EtPhCbl binds well to human vitamin B_{12} transporters, as well as to the processing enzyme CblC. Therefore, the arylcorrinoid EtPhCbl is potentially useful in mammals as an antagonist of vitamin B_{12} and of the B_{12} cofactors, as well as of cobalamins with proposed non-cofactor roles, such as nitroxylcobalamin.[29–31] EtPhCbl shares the unique base-on structure of the cobalamins, and it may fulfil (specific) conjectured non-cofactor functions of vitamin B_{12}, which are associated with the base-on form of the cobalamins. Among such functions are the specific direct gene-regulatory interaction of base-on cobalamins with RNA,[32–34] and alternative modes of transcriptional[35] or posttranscriptional regulation.[36,37]

"Antivitamins B_{12}" ("B_{12} antimetabolites"), such as EtPhCbl, may be particularly useful in studies where functional vitamin B_{12} deficiency needs to be induced artificially in ("intact" or wild-type) laboratory animals, by using a controlled, non-invasive procedure. In contrast to the result of the surgical method that disrupts the animals nutritional supply of cobalamins, the application of EtPhCbl and of similar arylcobalamins as "antivitamins B_{12}" is expected to suppress B_{12}-dependent enzymes, leaving other (suspected and still elusive) physiological roles intact. Such studies could help to elucidate suspected roles of cobalamins in degenerative brain and nerve diseases,[11] and, thus, assist in clarifying some important (and currently controversial) issues related to the pathophysiology of vitamin B_{12} deficiency in humans.

Experimental Section

Aquocobalamin chloride (Roussel UCLAF); water was purified using Epure (Barnstead Co); 4-ethylphenyldiazonium tetrafluoroborate (see Supporting Information), sodium formate puriss p.a. (Fluka), methanol HPLC grade (BDH prolabo), LiChroprep RP-18 (25–40 μm; Merck). UV/Vis spectra: Hitachi-U3000. ESI-MS: Finnigan MAT95-S, positive-ion mode, spray voltage 1.4 kV

Synthesis of EtPhCbl (see the Supporting Information): Aquocobalamin chloride ($H_2OCbl^+Cl^-$; 50.0 mg, 36.2 μmol) was dissolved in distilled water (2 mL) and the mixture was degassed with argon, before sodium formate (43.5 mg, 0.65 mmol) was added. The solution was stirred for 30 min at RT, before adding 4-ethylphenyldiazonium tetrafluoroborate (12.2 mg, 55.5 μmol). The mixture was stirred for another hour, and all further operations were carried out in the absence of light. An orange-red raw product was precipitated and dried under high vacuum. EtPhCbl was isolated by RP-18 column chromatography. The purified red EtPhCbl was dissolved in water (0.4 mL), and addition of acetone led to the crystallization of 29.1 mg (20.3 μmol, 56% yield) of EtPhCbl. UV/Vis: ($c = 3.31 \times 10^{-5}$ M, 50 mM phosphate buffer pH 7): λ_{max} ($\log\varepsilon$) = 518 (3.80), 473 (3.65), 374 (3.88), 343(4.04), 281(4.16), 268 nm (4.18; see Figure S1 in the Supporting Information). MS: positive ion ESI (MeOH): m/z (%) =

1458.7 (14), 1457.7 (36), 1456.7 (40, $[M+Na]^+$), 1436.7 (41), 1435.7 (84), 1434.7 (100, $[M+H]^+$); see Supporting Information for further CD and NMR spectroscopic data, details of the crystal-structure analysis, as well as for the methods used in the protein-binding/dissociation studies and enzyme investigations. CCDC 848663 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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