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Radical Synthesis Opens Access to Organometallic Aryl-Cobaltcorrins - 4-Ethylphenyl-cobalamin, a Potential 'Antivitamin-B₁₂'

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Abstract

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4-Ethylphenyl-cobalamin, a novel organometallic aryl-cobalamin, was synthesized in a radical reaction. This organometallic B₁₂ features a strong (Co-C)-bond, and represents a 'locked' form of vitamin B₁₂. This B₁₂-antimetabolite may be used in animal studies to induce 'functional' B₁₂-deficiency artificially. Such studies may help to clarify still controversial issues related to the pathophysiology of B₁₂-deficiency in humans.

Keywords

Antagonist; Arene ligands; Cobalamins; Organometallic bond; Radical reaction; Vitamins

Complete lack of B₁₂ (cobalamins) is lethal to humans and mammals,^[1, 2] which depend upon two organometallic B₁₂-cofactors, to maintain normal metabolism. The two B₁₂-cofactors, coenzyme B₁₂ (**AdoCbl**, 5'-deoxyadenosyl-cobalamin) and methyl-cobalamin (**MeCbl**), owe their cofactor function to the reactivity associated with their (Co-C)-bond.^[3–6] In contrast, vitamin B₁₂ (**CNCbl**, cyanocobalamin) is a physiologically inactive cyano-corrin, whose life sustaining effect as a (pro)vitamin in humans is due to its role as 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 *m*ethyl*m*alonic *a*ciduria type *C* and *h*omocystinuria), is the product of *cblC*, or 'MMACHC-gene'.^[8] CblC is remarkably versatile and efficient at removing cobalt-bound ligands of typical cobalamins, e.g. the cyanide ligand from **CNCbl**, to produce cob(II)alamin (see 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 B₁₂-cofactors from vitamin B₁₂ and leads to 'functional' B₁₂-deficiency with severe patho-physiological consequences.^[8]

On the other hand, B₁₂-deficiency is deliberately 'induced' and studied in laboratory animals, to elaborate on suspected but still unidentified contributions of B₁₂-deficiency to pathological phenomena, such as to degenerative diseases of the central and peripheral nervous systems.^[11] The usual approach to induce B₁₂-deficiency in laboratory mice requires 'total' gastrectomy by deep surgery,^[12] executed with serious physiological

interferences outside the B₁₂-specific metabolic questions. As a ‘sparing’ alternative to the surgical procedure, suitable vitamin B₁₂ antagonists (‘antivitamins-B₁₂’), if available, could be administered to intact (wild type) laboratory animals to provoke B₁₂-deficiency symptoms similar to those observed in patients with hampered uptake of B₁₂.^[1]

We have set out to find promising ‘antivitamins-B₁₂’, i.e. close analogues of vitamin B₁₂ that would be taken up by animals (and humans) by the proteins of B₁₂-transport^[13] without subsequent conversion into active B₁₂-cofactors.^[7] The required properties of such B₁₂-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⁺-aryl-cobalamins might have the desired structural properties and feature strong (Co-C)-bonds resistant to CblC. Surprisingly, Co⁺-aryl-corrins are still an unknown class of B₁₂-derivatives.^[5, 14] This situation is clearly a consequence of a lack of methods suitable for the synthesis of aryl-corrins.^[5, 14] As shown here, the aryl-cobalamin Co⁺-4-ethylphenyl-cob(III)alamin (**EtPhCbl**) could easily and efficiently be prepared by a cobalt-arylation that employs the radicaloid cob(II)alamin as a selective radical trap^[15, 16] of an aryl radical.

The organometallic cobalamin **EtPhCbl** was prepared in a one-pot reaction in aqueous solution, starting with 4-ethylphenyl diazonium BF₄ and aquo-cobalamin, (**H₂OCbl**) and proceeding via cob(II)alamin from formic acid reduction of **H₂OCbl**^[17–19] (see Scheme 2). 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 (see Scheme 3).

The expected structure of Co⁺-4-ethylphenyl-cob(III)alamin was confirmed, as follows (see Supp. Info): Its UV/Vis-spectrum resembled the spectra of organometallic B₁₂-derivatives in their ‘base-on’ form (see Supp. Info, Figure S2). The pseudo-molecular ion with the mass *m/z* = 1434.7 was compatible with the molecular formula C₇₀H₉₇CoN₁₃O₁₄P. ¹H-homonuclear and ¹H¹³C-hetero-nuclear NMR-spectra in D₂O (see Supp. Info, Figure S4) allowed the assignment of the signals of all 82 carbon-bound hydrogen atoms and of all 70 carbon atoms (see Supp. Info, Table S1). These spectra established **EtPhCbl** as a ‘base-on’ cobalamin carrying a π -bonded aryl-ligand at the ‘upper’ π -face of the cobalt-centre. ¹H-NMR data and ¹H, ¹H-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** (see Figure 1) was determined using synchrotron radiation, with diffraction data extending to a resolution of 0.8 Å. The corrin-bound Co(III)-centre sits in a pseudo-octahedral coordination sphere, with the typical (Co-N)-distances in the equatorial plane (see Supp. Info). The Co-atom is slightly out of the plane of the four corrin nitrogen atoms and is shifted by 0.029 Å towards the upper 4-ethyl-phenyl-ligand. The (Co-C)-distance to the aryl-ligand of **EtPhCbl** (1.981 Å) is slightly shorter than in alkyl-cobalamins (mean value of 2.003 Å) but longer than in vinyl-cobalamin (1.91 Å).^[22] The (Co-N)-distance to the trans-axial nucleotide ligand (2.230 Å) is also longer than in vinyl-cobalamin (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 π -ligand is oriented north-south relative to the corrin ring (torsion angle C5-Co-C1-C2 : 0.6°) and is nearly co-planar (3° tilt) with the dimethylbenzimidazole ligand. Consistent with the close non-bonded 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-chlorovinyl-cobalamin.^[22]

Exploratory experiments with the aryl-cobalamin **EtPhCbl** confirmed its expected unprecedented thermal stability and its resistance against protonolysis: Heating an aqueous solution of **EtPhCbl** (buffered at pH 7) at reflux for 24 hours did not lead to detectable cleavage of the (Co-C)-bond and decomposition to aquocobalamin (**H₂OCbl**). In slightly acidic aqueous solution base-on **EtPhCbl** was protonated to base-off **EtPhCbl-H⁺**, which exhibited a $pK_a = 3.7 (\pm 0.1)$ at 25 °C, similar to that of protonated coenzyme B₁₂ (**AdoCbl-H⁺**) (see Supp. Info, Figure S6). Formation of aquocobalamin (**H₂OCbl**) was not detected, when an acidic aqueous solution (pH 2) of **EtPhCbl** was heated at 95 °C for 6 hours (see Supp. Info, Figure S7). In aerated aqueous solution, the aryl-corrin **EtPhCbl** was sensitive to light, and its exposure to daylight gave **H₂OCbl** from cleavage of the (Co-C)-bond (see Supp. Info, Figure S11).

4-Ethyl-phenyl-cobalamin (**EtPhCbl**) bound with high affinity to two crucial human 'B₁₂-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 Supp. Info, Figure S14). 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 $IF \cdot Cbl \rightleftharpoons Cbl + IF \rightleftharpoons IF \cdot CBC$ (see Figure 2A). In addition, the dissociation kinetics was measured after pre-incubation of IF with either **EtPhCbl** or **CNCbl** followed by addition of the chasing B₁₂-derivative **CBC**. The process obeyed the scheme $IF \cdot Cbl \rightleftharpoons Cbl + IF \rightleftharpoons IF \cdot CBC$, where the first step was rate limiting. The initial slope corresponded to $k_- \cdot [IF \cdot Cbl]$ after conversion of fluorescence to the concentration of $IF \cdot CBC$ (see Figure 2B; the corresponding binding/dissociation data for TC are presented in Suppl. Info, Figure S14). Values of k_+ and k_- were assessed by computer fitting. The fast binding of **EtPhCbl** to IF ($k_+ = 7.9 \cdot 10^7 \text{ M}^{-1}\text{sec}^{-1}$) and to TC ($k_+ = 9.3 \cdot 10^7 \text{ M}^{-1}\text{sec}^{-1}$), as well as the slow dissociation from IF ($k_- < 5 \cdot 10^{-6} \text{ sec}^{-1}$) and TC ($k_- < 4 \cdot 10^{-6} \text{ sec}^{-1}$) resembled the analogous processes with **CNCbl**.^[24] The calculated equilibrium dissociation constant of **EtPhCbl** was equal to $K_d \approx 2 \cdot 10^{-14} \text{ M}$ for both IF and TC. Therefore, uptake and circulation of the aryl-cobalamin **EtPhCbl** and of **CNCbl** in humans are expected to have a similar efficiency.

The aryl-cob(III)alamin **EtPhCbl** also bound to CblC with a high binding affinity, as determined by isothermal calorimetry (ITC, $K_D = 20 \text{ nM}$, see Supp. Info, Figure S17). By analogy to **MeCbl**, tight binding of **EtPhCbl** to CblC required the aryl-cobalamin to adopt a 'base-off' form, as was deduced from the characteristic changes of the UV/Vis-absorption spectrum (see Figure 3A). Accommodation of the larger aryl-moiety was consistent with expectations from modelling 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 ($k_{obs} = 0.6 \text{ min}^{-1}$), the aryl-corrinoid **EtPhCbl** was resistant to removal of its cobalt-bound organometallic ligand by CblC (see Figure 3B) and a reliable dealkylation rate could not be estimated.

4-Ethylphenyl-cobalamin (**EtPhCbl**) is the first example of an organometallic aryl-cobalamin, a new group of Co(III)-corrins. It was prepared in a reaction postulated to generate the (Co-C)-bond by combination of an aryl radical and cob(II)alamin. This synthetic path, unprecedented in preparative B₁₂-chemistry, makes use of the extraordinary capacity of cob(II)alamin and related B₁₂-derivatives to capture ('slave in'^[16]) highly reactive radicals at the corrin-bound cobalt-centre.^[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 aryl-cobalamins. Indeed, the expected properties of the (Co-C)-bonds of organometallic aryl-Co(III)-corrins are likely to arouse interest in this class of compounds.

As was assumed on the basis of the reactivity of its aryl-ligand, the B₁₂-derivative **EtPhCbl** features a (Co-C)-bond that is stabilized against thermolysis and that is inert against acid- and nucleophile-induced cleavage (see Supp. Info, Figures S7, S8 and S12). On the other hand, the organometallic bond of **EtPhCbl** could be efficiently cleaved by light, which is consistent with the typical photo-sensitivity of organometallic B₁₂-derivatives.^[27] This feature of aryl-cobalamins will make laser photolysis with visible light an efficient tool for controlled ‘un-locking’ (with spatial and temporal control) of the masked vitamin properties of such ‘antivitamins-B₁₂’ in cell culture and animal studies.

Thus organometallic aryl-cobalamins represent a new group of chemically inert un-natural corrinoids, designed here as B₁₂-antagonists (‘antivitamins-B₁₂’). In tests with microorganisms, the previously used B₁₂-antagonists were cobalamins modified at the corrin moiety or at the nucleotide loop.^[28] These ‘B₁₂-antagonists’ were low affinity ligands, not suitable for efficient uptake by mammalian B₁₂-transport systems.^[24] In contrast **EtPhCbl** binds well to human B₁₂-transporters, as well as to the processing enzyme CblC. Therefore, the aryl-corrinoid **EtPhCbl** is potentially useful in mammals as an antagonist of vitamin B₁₂ and of the B₁₂-cofactors, as well as of cobalamins with proposed non-cofactor roles, such as nitroxyl-cobalamin.^[29–31] **EtPhCbl** shares the unique ‘base-on’ structure of the cobalamins, and it may fulfil (specific) conjectured non-cofactor functions of B₁₂, 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-B₁₂’ (‘B₁₂-antimetabolites’), such as **EtPhCbl**, may be particularly useful in studies where ‘functional’ B₁₂-deficiency should be induced artificially in (‘intact’ or wild type) laboratory animals, using a controlled, non-invasive procedure. In contrast to the result of the surgical method that disrupts the animals’ nutritional supply with cobalamins, application of **EtPhCbl** and of similar aryl-cobalamins as ‘antivitamins-B₁₂’ is expected to suppress B₁₂-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 patho-physiology of B₁₂-deficiency in humans.

Experimental Section

Materials and Methods

Aquocobalamin chloride *Roussel UCLAF*; water was purified using Epure, Barnstead Co; 4-ethylphenyl-diazonium tetrafluoroborate (see Supp. Info), sodium formate *puriss p.a.*: Fluka, methanol (MeOH) HPLC-grade: *BDH prolabo*, LiChrorep RP-18 (25–40µm): *Merck*. UV/Vis Spectra: Hitachi-U3000; $\lambda_{\max}$ (log) in nm. ESI-MS: Finnigan MAT95-S, positive-ion mode, spray voltage 1.4 kV, solvent MeOH.

Synthesis of Co -(4-ethylphenyl)-cobalamin (**EtPhCbl**) (see Supp. Info for further details). In 2 ml of distilled water 50.0 mg (36.2 µmol) of aquocobalamin chloride (H₂OCbl⁺Cl[−]) were dissolved and the mixture was degassed with argon, before 43.5 mg (0.65 mmol) of sodium formate were added. The solution was stirred for 30 min at RT, when 12.2 mg (55.5 µmol) of 4-ethylphenyl-diazonium tetra-fluoroborate were added. The mixture was stirred for another hour, and all further operations were done under protection from light. An orange-red raw product was precipitated and dried under high vacuum. **EtPhCbl** was isolated by RP-18 column chromatography. Purified red **EtPhCbl** was dissolved in 0.4 ml of water and 29.1 mg (20.3 µmol, 56% yield) of **EtPhCbl** crystallized after addition of acetone. UV/Vis: (c = 3.31 × 10^{−5} M, 50 mM phosphate buffer pH 7): $\lambda_{\max}$ (log) = 518 (3.80), 473 (3.65), 374 (3.88), 343(4.04), 281(4.16), 268 (4.18) nm (see Figure S1). MS: ESI pos,

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 Supp. Info for further CD- and NMR-spectral data, details of the crystal structure analysis, as well as for the methods used in protein-binding/dissociation studies and enzyme investigations. The supplementary crystallographic data for this paper have been deposited as CCDC 848663 at the Cambridge Crystallographic Data Centre.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Two projections of a model of the structure of **EtPhCbl** derived from x-ray crystal structure analysis

Figure 2.

Interaction of **EtPhCbl** with human Cbl-binding protein intrinsic factor (IF). **(A)** Binding of **EtPhCbl** to IF measured via competition with the fluorescent B₁₂-derivative **CBC** ($k_{+CBC} = 64 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$): IF was rapidly mixed with either **CBC** or a solution of **CBC** and **EtPhCbl**. An arrow indicates effect of competition between **CBC** and **EtPhCbl**, consistent with $k_{+EtPhCbl} = 79 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$. **(B)** Dissociation of **EtPhCbl** from IF: The pre-incubated complex of IF (1.0 μM) and either **EtPhCbl** or **CNCbl** (1.3 μM) was mixed with the 'chasing' B₁₂-derivative **CBC** (1.3 μM). From the increase of the fluorescence the dissociation rate constants of the complexes were calculated: $k_{-EtPhCbl} \approx 1.5 \cdot 10^{-6} \text{ s}^{-1}$ and $k_{-CNCbl} \approx 6 \cdot 10^{-7} \text{ s}^{-1}$ (see Supp. Info for further details).

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, open circles), whereas MeCbl is rapidly demethylated (closed circles) – see Suppl. Info for exptl. Details.

Scheme 1.

Left. Structural formulas of some relevant cobalamins: vitamin B₁₂ (R = CN, cyano-cobalamin, **CNCbl**), coenzyme B₁₂ (R = 5'-deoxyadenosyl, 5'-deoxyadenosyl-cobalamin, **AdoCbl**), methyl-cobalamin (R = methyl, **MeCbl**), aquo-cobalamin (R = H₂O, **H₂OCbl**, a cation), 4-ethylphenyl-cobalamin (R = 4-ethylphenyl, **EtPhCbl**). Right. Symbols 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.

Outline of the synthesis of **EtPhCbl** from **H₂OCbl** and 4-ethylphenyl diazonium tetrafluoroborate.

Scheme 3.

Outline of the proposed mechanism of the formation of **EtPhCbl** via a hypothetical one-electron reduction of 4-ethylphenyl diazonium tetrafluoroborate by cob(II)alamin with loss of dinitrogen, and followed by recombination of cob(II)alamin with the 4-ethylphenyl radical.