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Partial Synthesis of Coenzyme B_{12} from Cobyric Acid

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Dedicated to Prof. Albert Eschenmoser on the occasion of his 92nd birthday

Here we report the direct chemical synthesis of coenzyme B_{12} (AdoCbl) from Co_β-5'-deoxyadenosylcobyric acid (AdoCby) and the preparation of the latter from crystalline CN,H_2O-cobyric acid (CN,H_2OCby). AdoCby is a suggested common key intermediate in the biosynthesis of AdoCbl and of other cobamides in microorganisms. AdoCby was thoroughly characterized by spectroscopic means, including homo-nuclear and hetero-nuclear NMR, as such data are not available in published work. AdoCbl was prepared from AdoCby in one-step in over 85% yield, by covalent attachment in aqueous solution of the integral B_{12}-nucleotide moiety using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC*HCl) and *N*-hydroxybenzotriazole (HOBt) as coupling reagents. By the same procedure crystalline vitamin B_{12} (CNCbl) was also prepared in 92 % yield from CN,H_2OCby. Coordination of the B_{12}-nucleotide base at the Co_α-face of AdoCby or of CN,H_2OCby was indicated to assist in the efficient covalent coupling at the activated *f*-side chain function to furnish the complete corrinoids AdoCbl and CNCbl.

Keywords: Amide bond • biomimetic synthesis • cobalamin • porphyrinoid • vitamin B_{12}

Introduction

Nucleotide containing 'complete' B_{12}-derivatives, like vitamin B_{12} (cyanocobalamin, CNCbl, **1**), coenzyme B_{12} (5'-deoxyadenosylcobalamin, AdoCbl, **2**) and methylcobalamin (MeCbl) play indispensable roles in the metabolism of various microorganisms, animals and humans.[1-4] Keys to their ubiquitous biological functions are their special structures, their organometallic chemistry, their redox chemistry and photochemistry.[5-7] However, only certain prokaryotes have developed the capacity to biosynthesize natural corrinoids.[8, 9] So far, two pathways of their biosynthesis have been elucidated, one aerobic (oxygen dependent), and the other one anaerobic (oxygen independent). For both of these routes the light sensitive organometallic 5'-deoxyadenosyl-cobyric

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acid (AdoCby, **3**) is a suggested key intermediate, from which coenzyme B_{12} (AdoCbl, **2**) is 'completed' by formal biosynthetic attachment of the B_{12}-nucleotide moiety of the cobalamins.[8, 9]

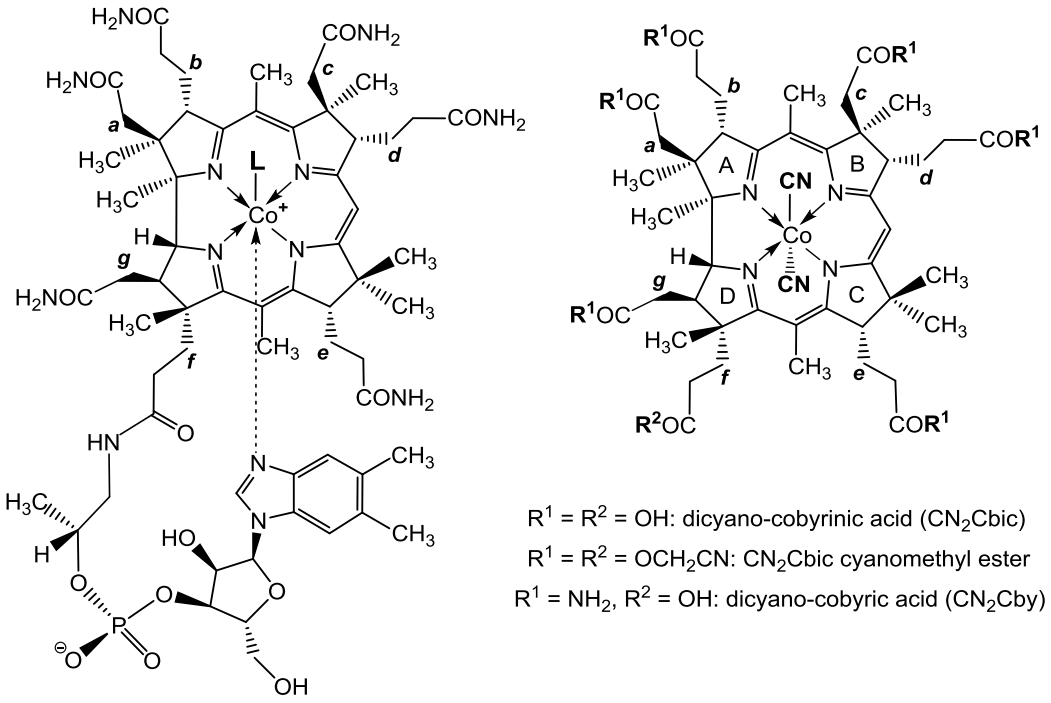

Figure 1. Structural formulas (left) of the cob(III)alamins vitamin B_{12} (CNCbl, L = CN, **1**) and coenzyme B_{12} (AdoCbl, L = 5'-deoxy-5'-adenosyl, **2**) and (right) dicyano-Co(III)-forms of cobyrinic acid, of its activated hepta-cyanomethyl ester and of cobyric acid (CN_2Cbic, CN_2Cbic cyanomethyl ester and CN_2Cby, respectively).

The unique structures of cyano,aquo-cobyric acid (CN,H_2O-Cby, **4**) and vitamin B_{12} (CNCbl, **1**) (see **Figure 1**) are known from pioneering single crystal x-ray analysis in the laboratories of D.C.Hodgkin,[10, 11] following the isolation of CNCbl (**1**) from natural sources.[12, 13] CNCbl (**1**) was subsequently also prepared in about 40% yield by chemical attachment of the B_{12}-nucleotide (*R*-2-amino-1-methylethyl)-3'-(α-ribazolyl)-diphosphate (**5**) at the *f*-acid function of CN,H_2O-Cby (**4**), activated with ethyl chloroformate.[14] Hence, CN,H_2O-Cby (**4**) became the key target of the (formal) total synthesis of vitamin B_{12} (**1**), and was the product of its total synthesis by Eschenmoser and coworkers [15, 16] and by Woodward and coworkers in the 1970es.[17] The Woodward group then reported the preparation of CNCbl (**1**) in 73% yield from synthetic CN,H_2O-Cby (**4**) by condensation with synthetic (*R*-2-amino-1-methylethyl)-3'-(α-ribazolyl)-diphosphate (**5**), thus actually also completing the total synthesis of vitamin B_{12} (**1**).[18]

In a fundamentally different approach, Bartels and Eschenmoser prepared crystalline vitamin B_{12} (**1**) in 44% yield from undifferentiated, activated cobyrinic acid (cyano,acetato-cobyrinic acid heptacyanomethyl ester, see Figure 1) via highly selective covalent attachment of the B_{12} nucleotide portion (*R*-2-amino-1-methylethyl)-3'-(α-ribazolyl)-diphosphate (**5**) at the 'natural' *f*-side chain, followed by ammonolysis of the six remaining activated ester groups.[19] The amazing selectivity of

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this B_{12}-reconstitution experiment was rationalized by pre-coordination of the nucleotide moiety to the cobalt center of the (α,β)-isomeric mixture of the CN,acetato-cobyrinic acid hepta-ester, allowing for an outstandingly selective condensation of the pre-coordinated B_{12}-nucleotide (**5**) at the *f*-side chain. This type of a reconstitution of vitamin B_{12} (**1**) delineated a 'primitive' (non-enzymatic) path from 'incomplete' and undifferentiated cobyrinic acid to the specific structure of 'complete' corrinoids, such as the cobalamins.[19, 20]

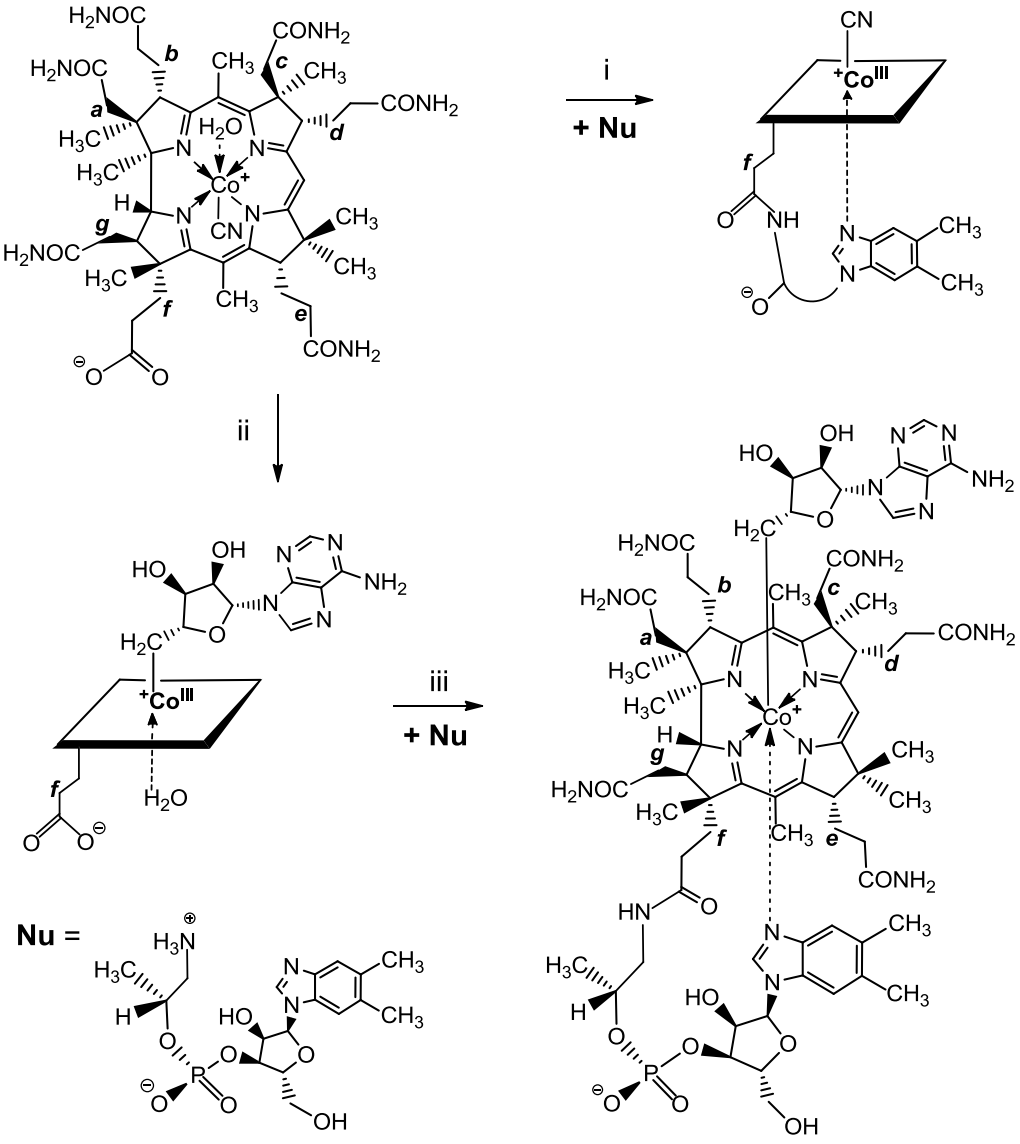

Figure 2. Outline of the partial syntheses of coenzyme B_{12} (AdoCbl, **2**) and of vitamin B_{12} (CNCbl, **1**) from cyano-aquo-cobyrinc acid (CN,H_2OCby, **4**) in aqueous solution. i) HOBt, EDC*HCl, H_2O; ii) = $NaBH_4$, 5'-iodo-5'-deoxyadenosine, MeOH/10 mM phosphate buffer pH=7 (1:1), argon; iii) HOBt, EDC*HCl, H_2O (see text for further details).

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Crystalline coenzyme B_{12} (AdoCbl, **2**) was isolated in the early 1960es as corrinoid product of a 'guided' biosynthesis in *Clostridium tetanomorphum*.[21] The genuine cofactor of this organism is the crystallization resistant pseudocoenzyme B_{12}, as was also discovered in the Barker laboratories.[22] X-ray analysis of crystals of AdoCbl (**2**) revealed its enigmatic organometallic nature.[23] AdoCbl (**2**) was subsequently prepared by partial chemical synthesis, via reduction of aquocobalamin (H_2OCbl) or vitamin B_{12} (CNCbl, **1**), followed by adenosylation at the cobalt center.[24, 25]

Herein, we report the partial synthesis of Co_β-5'-deoxyadenosyl-cobyric acid (AdoCby, **3**) from aquo,cyano-cobyric acid (CN,H_2O-Cby, **4**) and the direct preparation of AdoCbl (**2**) and CNCbl (**1**) in aqueous solution via covalent attachment of the B_{12}-nucleotide moiety (*R*-2-amino-1-methylethyl)-3'-(α-ribazolyl)-diphosphate, **5**) to AdoCby (**3**) or CN,H_2OCby (**4**), respectively (see **Figure 2**). Furthermore, the cobyric acid derivatives AdoCby (**3**) and CN,H_2OCby (**4**) were tested qualitatively for their capacity to coordinate N-methyl-imidazole in aqueous medium, in order to gain insights into the coordination tendency of imidazoles to such Cbys. Along the lines of Eschenmoser's pioneering experiments,[19] such a pre-cordination of the benzimidazole base of the B_{12}-nucleotide moiety would increase the effective molarity [26] of the latter for condensation reactions at the *f*-side chain of cobyric acids. This would represent a relevant factor in accelerating the amide-forming reaction via a properly pre-organized intramolecular path, and leading to an increase of the chemical yield of the desired cobalamins.

Results and Discussion

Preparation of Co_β-5'-deoxyadenosyl-cobyric acid (AdoCby, 3) from CN,H_2O-cobyric acid (4).

2.00 mg of crystalline Co_αCN,$Co_\beta H_2O$-Cby (**4**) were dissolved in MeOH/potassium phosphate buffer (10mM, pH=7, see Supporting Information, **Figure S1**). The Co(III)-corrinoid was reduced under anaerobic conditions using an excess of sodium borohydride and transformation into cob(I)yric acid was verified by a UV/Vis-spectrum (see Supp. Info **Figure S2**). 1.2 equivalents of 5'-iodo-5'-deoxyadenosine were added under protection from air and daylight, and an immediate color change to orange was observed (and formation of AdoCby (**3**) – see Supporting Information, **Figure S2**). From the reaction mixture, 1.85 mg of AdoCby (**3**, 74%) was isolated as orange powder.

The UV/Vis-spectrum of Co_β-5'-deoxyadenosyl-cobyric acid (AdoCby, **3**, see **Figure 3**) showed absorption maxima at 457, 378 and 303 nm, similar to published spectra of AdoCby (**3**),[27] and of Co_β-5'-deoxyadenosylcobinamide (AdoCbi).[28] The CD-spectrum of AdoCby (**3**) (see Supp. Info **Figure S3**) displayed the band structure of AdoCbi.[29] A (positive ion) ESI-MS spectrum of AdoCby (**3**) showed the pseudo-molecular ion $[C_{55}H_{77}CoN_{15}O_{11}]^+$ as base-peak at m/z=1182.4 and its sodium adduct $[C_{55}H_{76}CoN_{15}NaO_{11}]^+$ at m/z=1204.4 (see Supporting Information, **Figures S4** and **S5**).

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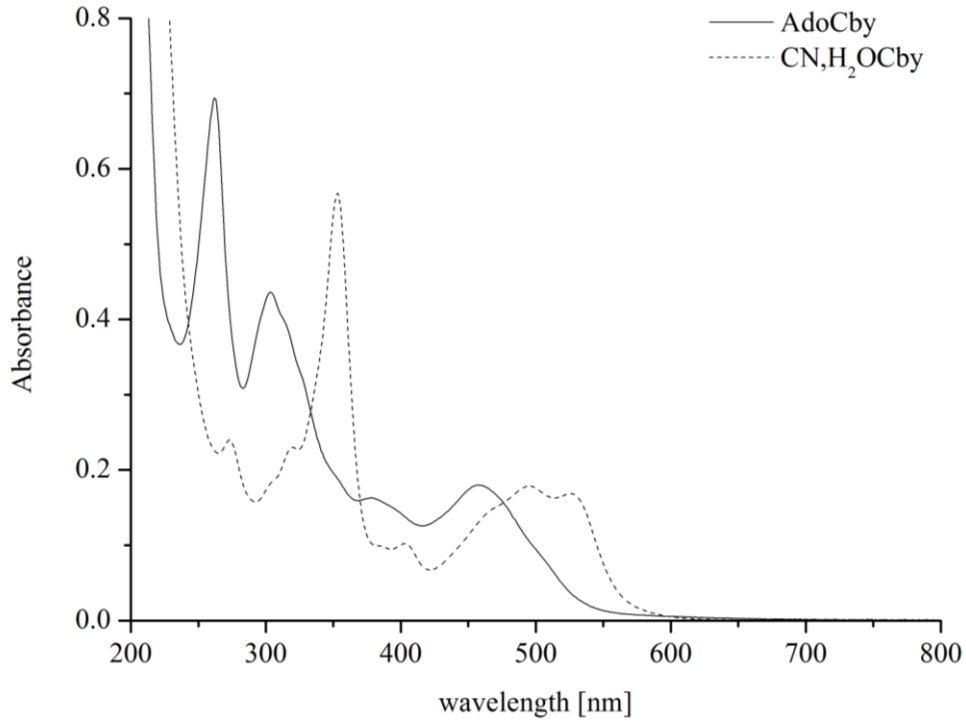

Figure 3. UV-Vis spectra of Co_β-5′-deoxyadenosylcobyric acid (AdoCby, **3**, c = 2.5 x 10^{-5} M) and of CN,H_2O-cobyric acid (CN,H_2OCby, **4**, c = 2.5·10^{-5} M) in H_2O.

A 500 MHz ^{1}H-NMR spectrum of AdoCby (**3**) in D_2O showed resonances for all carbon-bound H atoms, including the characteristic doublet- and triplet-like signals at high field for Co-bound H_2C5RL of the Ado ligand (see **Figure 4**). In the low field region, the protons HC2L and HC8L of the adenine base were detected at 8.20 ppm and 8.01 ppm, respectively. The proton signals of the ribose moiety could be assigned on the basis of a set of continuous ^{1}H, ^{1}H-COSY correlations from the C5RL protons at high field (at 0.35 and 0.50 ppm) all the way to HC1RL at 5.59 ppm (see Supporting Information, **Figure S8**). The two diastereotopic protons at C5RL were assigned to H_{re} at 0.35 ppm (H_AC5RL, *dd*, *J* = 9.5 Hz, *J* = 8.8 Hz) and to H_{si} at 0.50 ppm (H_BC5RL, *d*, *J* = 8.8 Hz), in analogy to assignments in ^{1}H-NMR spectra of coenzyme B_{12} (**2**). The ^{1}H,^{1}H-COSY spectrum shows a large coupling for H_AC5RL (H_{re}) ↔ HC4RL (9.5 Hz) and a small unresolved coupling for H_BC5RL (H_{si}) ↔ HC4RL, consistent with torsion angles (-169° and 75°, respectively) observed in the crystal of coenzyme B_{12} (**2**).[2] Furthermore, H_BC5RL (H_{si}) exhibits strong ^{1}H,^{1}H-ROESY correlations with HC3RL, HC4RL and HC19 and a weak ^{1}H,^{1}H-ROESY correlation to H_2C81. H_AC5RL (H_{re}), on the other hand, shows a strong correlation with HC3RL but none to HC19. These data indicate the adenosyl group attached at the β-face of the cobalt-center of AdoCby (**3**) (see Supporting Information, **Table S2**). ^{1}H,^{1}H NOE spectra, furthermore, indicated a similar positioning of the 5′-deoxyadenosyl moiety above the rings C and D (see Supp. Info **Figure S7**), as observed in AdoCbi [28] and coenzyme B_{12} (**2**).[30,31] The major NOE cross peaks HC8L↔H_3C12B, HC1RL↔H_3C17B, HC1RL↔H_3C12B, HC4RL↔H_3C12B, HC4RL↔HC19 and H_2C5RL↔HC19 found here in the spectra of AdoCby (**3**), provide evidence for very similar conformations of its adenosyl moiety as deduced for AdoCbi [28], as well as in AdoCbl (**2**) "base on" [30] and protonated "base off" forms.[31] Such NOE correlations were interpreted as a dynamic equilibrium between two major conformations, one of them similar to that found in the crystal structure, and the other with the adenosyl group rotated 50° counterclockwise.[31]

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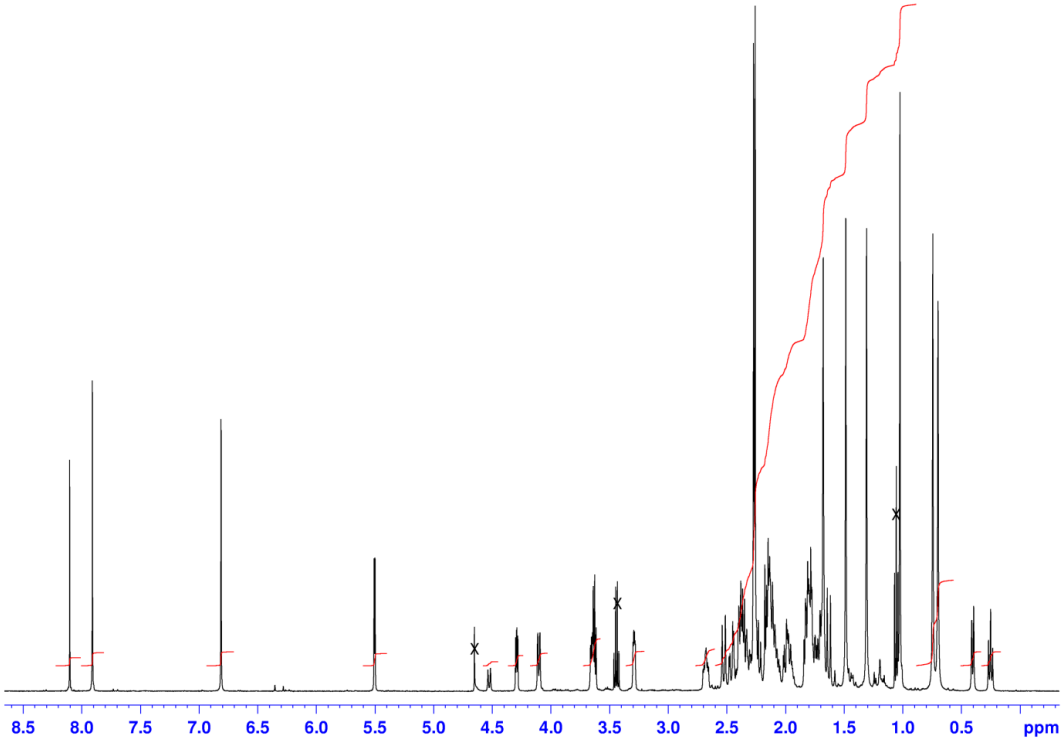

Figure 4. 500 MHz ^{1}H-NMR spectrum of AdoCby (**3**, 7.0 x 10^{-3} M in D_2O, 298 K, with suppression of the HDO signal); x marks residual solvent signals.

With few exceptions, the chemical shifts of corresponding protons and ^{13}C-atoms in the NMR spectra of AdoCby (**3**) and AdoCbi are very similar (see Supp. Info **Table S1**). However, the H_2C172 protons are shifted up-field by about 0.45 ppm in the AdoCby (**3**) spectrum. Likewise, among the carbon atoms of AdoCby (**3**) and of AdoCbi, only C173 is shifted significantly downfield by 2.8 ppm in AdoCby (**3**), reflecting the different carboxylic acid function in AdoCby (**3**) and in AdoCbi, a free acid group vs. a carboxamide, respectively. Interestingly, in spite of their very similar environment, the H_2C5RL protons of the adenosyl-moiety of AdoCby (**3**) are also shifted up-field (by 0.08 and 0.24 ppm) compared to AdoCbi.[28]

Partial synthesis of coenzyme B_{12} (2) from Co_β-5'-deoxyadenosyl-cobyric acid (AdoCby, 3).

Coenzyme B_{12} (AdoCbl, **2**) was prepared in aqueous solution by activation of the carboxylic acid function of AdoCby (**3**) with 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide (EDC*HCl) and *N*-hydroxybenzotriazole (HOBt). Thus, AdoCby (**3**, 1.0 mg, 0.83 µmol), B_{12}-nucleotide (**5**, 0.50 mg, 1.16 µmol) and HOBt (1.6 µmol) were dissolved in 750 µl water at 0°C. EDC*HCl (8.72 µmol, dissolved in 250 µl of water) was added. The reaction was monitored via HPLC (see Supporting Information, **Figure S9**).The stirred reaction mixture was first kept for 2 h at 0°C, then for additional 22 h at RT, till full conversion to coenzyme B_{12} (**2**). After workup and crystallization from water/acetone, 1.1 mg (85% yield) of coenzyme B_{12} (AdoCbl, **2**) were obtained.

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The spectra of here prepared AdoCbl (**2**) confirmed its identity. Hence, its UV/Vis spectrum in H_2O corresponded to the published spectrum,[21] with absorption maxima at 523, 488 and 430 nm (see Supp Info **Figure S10**). Likewise, a CD-spectrum of AdoCbl (**2**) in H_2O (see Supp. Info **Figure S11**) matched the reported CD-data of AdoCbl (**2**).[32] A fully assigned ^{1}H-NMR spectrum of AdoCbl (**2**) (see Supp. Info **Figure S12**) was consistent with the known structure and showed chemical shifts very similar as published.[30] An ESI-MS spectrum of the here prepared AdoCbl (**2**) (see Supp. Info **Figure S13**) displayed the signals of a sodium adduct $[C_{72}H_{100}CoN_{18}NaO_{17}P]^+$ at m/z= 1602.4 (as base-peak) and of the proton adduct $[C_{72}H_{101}CoN_{18}O_{17}P]^+$ at m/z= 1579.5.

Partial synthesis of vitamin B_{12} (CNCbl, 1) from CN,H_2O-cobyric acid (CN,H_2OCby, 4).

Crystalline CN,H_2O-cobyric acid (**4**, 1.0 mg, 1.02 µmol), B_{12}-nucleotide (**5**, 0.65 mg, 1.43 µmol) and HOBt (0.30 mg, 2.24 µmol) were dissolved in 900 µl water at 0°C. EDC*HCl (13.86 µmol, dissolved in 100 µl of water) was added. Crystalline CN,H_2OCby (**4**) represents the Co_α-cyano form according to its x-ray crystal structure. [11] However, Co_α-cyano and the Co_β-cyano forms are soon present in solution in almost a 1:1-mixture (see ^{1}H-NMR analysis in D_2O, Supporting Information, Figure S1). Only the latter would allow for coordination of the B_{12}-nucleotide (**5**) at the α-face, proposed to be helpful for the actual (intramolecular) coupling step. Direct monitoring of the reaction mixture by HPLC (see Supporting Information, **Figure S14**) also indicated rapid equilibration of Co_α-cyano and the Co_β-cyano forms of CN,H_2O-Coby (**4**). In the event, the stirred reaction mixture was first kept for 2 h at 0°C, then for 5 h more at RT, till full conversion was analyzed, and nearly quantitative formation of vitamin B_{12} (**1**). Vitamin B_{12} (**1**) was crystallized from water/acetone and was obtained with 92% yield.

The UV/Vis- and CD-spectra of the here prepared CNCbl (**1**) matched the known spectra [32, 33] (see Supp. Info **Figures S15 and S16**). The 500 MHz ^{1}H-NMR spectrum of the here prepared vitamin B_{12} (**1**) (see Supp. Info **Figure S17**) and the published spectrum matched with chemical shift differences less than 0.1 ppm.[34] An ESI-MS spectrum of CNCbl (**1**) (see Supp. Info **Figure S18**) showed the sodium adduct $[C_{63}H_{88}CoN_{14}NaO_{14}P]^+$ at m/z 1377.7 as base-peak, as well as signals of the proton adduct $[C_{63}H_{89}CoN_{14}O_{14}P]^+$ at m/z 1355.5.

Investigations on the coordination properties of AdoCby (3) and CN,H_2OCby (4) towards N-methyl-imidazole (MeIm): The synthetic work carried out here counted on a coordination of the DMB-nitrogen of the B_{12}-nucleotide loop (**5**) to the cobalt center, as setting the stage for an efficient intramolecular amidation-step of the activated *f*-carboxylic side chain (see **Figure 5**). This strategy followed the essential structural component of the mechanism laid out in the B_{12}-reconstitution experiment, so beautifully designed by Eschenmoser and Bartels.[19] In order to judge the relevance of this process for our synthetic work, the tendency of DMB to undergo cobalt-coordination in CN,H_2OCby (**4**) and AdoCby (**3**) was to be tested. In fact, N-methyl-imidazole (MeIm) was used here as a surrogate of 5,6-dimethylbenzimidazole (DMB), as poor solubility of DMB in aqueous solution inhibited its practical use in the spectral studies. Hence, the coordination behavior of N-methyl-imidazole to the cobalt center of CN,H_2OCby (**4**) and AdoCby (**3**) was studied at room temperature.

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UV/Vis-spectra of AdoCby (**3**) and of CN,H_2OCby (**4**) were recorded in buffered aqueous solution (pH=7) and MeIm was added to increasing concentrations. Due to the presence of both coordination isomers of CN,H_2OCby (**4**) in aqueous solution, axial MeIm coordination is presumed to occur with similar strengths at the α- or the β-side of the Co-center. Binding of MeIm to CN,H_2OCby (**4**) led to a shift of the absorbance maxima to longer wavelengths (see Supp. Info **Figure S19**) and to a final spectrum resembling the one of CNCbl (**1**). Based on the absorbance change at 550 nm an effective average binding constant of MeIm was derived as $K_{on\ (MeIm/CNCby)} = 1.2 \cdot 10^4\ M^{-1}$. The here analyzed coordination of MeIm to CN,H_2OCby (**4**) could be compared to intramolecular coordination of the DMB-base in CNCbl (**1**), which exhibits a concentration independent $K'_{on} = 2.9 \cdot 10^5$ at 25°C.[6,34]

Binding of MeIm to Co_β-5'-deoxyadenosylcobyric acid (AdoCby, **3**) was studied similarly. It was weaker and much higher concentrations of MeIm were needed, in order to see a significant effect in UV/Vis-spectra (see Supporting Info **Figure S20**). Based on the absorbance changes a rough binding constant $K_{on\ (MeIm\ /\ AdoCby)} = 0.8\ M^{-1}$ was estimated at room temperature. This value, when compared with the concentration independent K_{on} in AdoCbl (**2**) of 14.3 (at 25°C),[35] again indicated a high effective molarity of the DMB-base of about 10M for intramolecular coordination to the Co(III)-center. Furthermore, imidazole binding to AdoCby (**3**) was indicated to be about $1.5 \cdot 10^4$ fold weaker than to CN,H_2OCby (**4**), reflecting the thermodynamic trans-effect of the CN-ligand vs. the adenosyl group.

Clearly, the intramolecular coordination of the DMB-base in AdoCbl (**2**) and in CNCbl (**1**) is remarkably more effective at low overall concentrations, than binding of (external) MeIm to the Co(III)-centers of AdoCby (**3**) and CN,H_2OCby, respectively. In previous studies in ethylene glycol, MeIm was found to coordinate with methylcobinamide about 2.5 times better than 1,5,6-trimethylbenzimidazole.[36] Likewise, taking into account the different bases (MeIm or DMB), the direct comparison of these two values is very qualitative, but it points to a high effective molarity (of the order of 10 M) of the DMB-base in CNCbl (**1**) and in AdoCbl (**2**). In the situation, where amide formation was considered, the terminal amino group of the coordinated B_{12}-nucleotide (**5**) would be selected with, approximately, the same magnitude over the one in the free B_{12}-nucleotide **5**. This argument would suggest the intramolecular path from CN,H_2O-Cby (**4**) or AdoCby (**3**) to CNCbl (**1**) or AdoCbl (**2**) to be faster by up to 10^4 times at the (sub)mM concentrations of the reaction partners in our synthetic experiments.

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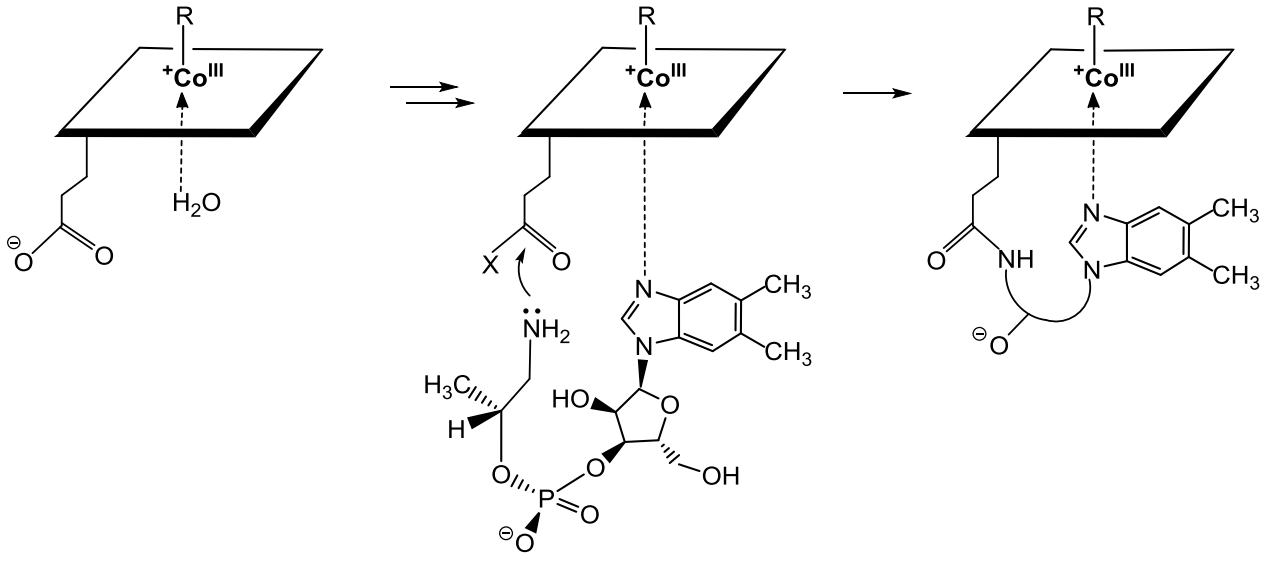

Figure 5. Coordination of the B_{12}-nucleotide moiety **5** at the lower face of the corrin-bound Co(III)-center of AdoCby (**3**) or of CN,H_2OCby (**4**) prepares the B_{12}-nucleotide moiety for efficient intramolecular amide bond formation to the *f*-side chain of the activated cobyric acid derivatives AdoCby (**3**) or CN,H_2OCby (**4**).

Conclusions

AdoCby (**3**) is a key common intermediate in the biosynthesis of adenosylcobamides, such as coenzyme B_{12} (**2**),[8, 9] as well as in diverse pathways of 'B_{12}-salvaging'.[37] Here, a reliable partial synthesis of Co_β-5'-deoxyadenosylcobyric acid (AdoCby, **3**) from CN,H_2O-cobyric acid (CN,H_2OCby, **4**) is reported, as well as the first extensive spectroscopic characterization of AdoCby (**3**). These studies have provided a spectroscopic data set consistent with the expected structural similarity of AdoCby (**3**) with the previously characterized analogue Co_β-5'-deoxyadenosylcobinamide (AdoCbi),[28] and likely to be useful in future biological studies.

The 'complete corrinoids' vitamin B_{12} (CNCbl, **1**) and coenzyme B_{12} (AdoCbl, **2**) could be obtained in a single step, high yield partial synthesis by coupling the B_{12}-nucleotide moiety (**5**) to the corresponding cobyric acid derivatives, CN,H_2OCby (**4**) or AdoCby (**3**), respectively, using EDC*HCl and HOBt in aqueous solution. The same reagents were used in DMF in the recent synthesis of vitamin B_{12} mimics with a peptide backbone.[38] In our experiment, crystalline CNCbl (**1**) was obtained in a yield of 92%. The corresponding assembly of AdoCbl (**2**) took place with a yield of about 85%. Pre-coordination of the B_{12}-nucleotide moiety **5** to the 'lower' α-face of the Co-center is a suggested crucial element in obtaining a higher effective reaction rate in the amide-forming coupling step of the partial synthesis of CNCbl (**1**) and AdoCbl (**2**) (and, thus, helpful for obtaining a good yield of these cobalamins). This conclusion is congruent with qualitative coordination experiments, also done, using 1-methylimidazole (Me-Im). From these we deduce pre-coordination of the DMB unit of the B_{12}-nucleotide moiety **5** to the cobalt-center of CN,H_2OCby (**4**) or AdoCby (**3**) to be relevant under the conditions of our synthesis experiments, increasing the 'effective' concentration of its terminal amino group and accelerating the amide-bond forming step.

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The strategy of the pre-coordination of the B_{12}-nucleotide moiety **5**, considered here, has similarly been employed recently in the last step of the total synthesis of 5'-deoxyadenosyl-rhodibalamin (AdoRhbl), the rhodium homologue of coenzyme B_{12} (AdoCbl, **2**), from 5'-deoxyadenosyl-rhodibyric acid (AdoRhby, the Rh-analogue of AdoCby, **3**).[39] An analogous synthesis strategy may also be helpful in the preparation of other transition metal analogues of the cobalamins,[40] i.e. of 'metbalamins' carrying other metals than (the cobalamin's) cobalt. Some of such structural similars of vitamin B_{12} (**1**) or of coenzyme B_{12} (**2**) [39] are in the focal point of current B_{12}-research [41] and may represent effective 'antivitamins B_{12}'.[41, 42]

The biosynthetic completion of AdoCbl (**2**) from AdoCby (**3**) depends upon several enzyme catalyzed steps [8,9] and does not involve direct attachment of the complete B_{12}-nucleotide **5** to AdoCby (**3**). However, the central biosynthetic relevance of the adenosylated cobalt-corrin AdoCby (**3**) as organometallic precursor of AdoCbl (**2**) is reflected by its apparently obligatory intermediacy in the two studied branches of B_{12}-biosynthesis. In this sense, the here reported partial synthesis of AdoCbl (**2**) from AdoCby (**3**) may be classified as having a biomimetic element. Furthermore, as the 'intact' B_{12}-nucleotide (*R*-2-amino-1-methylethyl)-3'-(α-ribazolyl)-diphosphate (**5**) has proven to be a surprisingly effective structural complement of the cobyrinic acid moiety in Bartels' and Eschenmoser's successful studies of the self-assembly of vitamin B_{12} (CNCbl, **1**),[19] our experiments suggest that a correspondingly selective formation of the organometallic cofactor coenzyme B_{12} (AdoCbl, **2**) might represent an interesting alternative for the direct formation of this metabolically active 'complete' cobalamin.

Experimental Section

Solvents:

Water (H_2O; purified over reversed Osmose); ddH_2O (Millipore MiliQ Academic, HPLC gradient grade); methanol (MeOH; BDH prolabo, >99.9%, HPLC gradient grade); acetone (Fluka, puriss p.a); acetonitrile (MeCN; BDH prolabo, >99.9%, HPLC gradient grade); diethyl ether (distilled); tert-butyl methyl ether; toluene (Fluka, >99.7%); D_2O (Euriso-top, 99.96%D).

Reagents:

Acetic acid (AcOH; AnalaR Normpure, 100%), hydrochloric acid (12 mol/l; AnalaR Normpure, 35%); benzoic acid crystalline (practical lab); sodium hydrogencarbonate ($NaHCO_3$; practical lab); sodium borohydride ($NaBH_4$; Fluka, purum p.a.); potassium cyanide (KCN); dipotassium hydrogenphosphate (K_2HPO_4; Fluka, p.a); potassium dihydrogenphosphate (KH_2PO_4; Roth, p.a.); iodine (Sigma Aldrich, puriss p.a.); triphenylphosphine (Sigma Aldrich, 99%); N-methylimidazole (MeIm) (Fluka p.a. >99%, GC); *N*-hydroxybenzotriazole (HOBt) hydrate (12% water; Sigma Aldrich >97%); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC*HCl; Fluka, purum p.a.); vitamin B_{12} (CNCbl, crystalline; Hoffmann La Roche, **1**); B_{12}-nucleotide (*R*-2-amino-1-methylethyl)-3'-(α-ribazolyl)-diphosphate, **5**) was obtained from partial hydrazinolysis of CNCbl (**1**),[19, 43] dicyano-cobyric acid by hydrolysis of CNCbl (**1**);[44, 45] 5'-iodo-5'-deoxyadenosine was prepared as reported [46].

Spectroscopy:

UV/Vis spectra: Agilent Technologies Cary 60; $\lambda_{max}(\log\varepsilon)$ in nm, sh signifies shoulder. CD-spectra: JASCO J715 spectrophotometer; $\lambda_{max/}$ λ_{min} and λ_0 in nm, $\Delta\varepsilon$ (molar circular dichroism) in [l mol^{-1} m^{-1}],

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sh signifies shoulder. ^{1}H- and ^{13}C-NMR spectra: Varian Unity Inova 500 spectrometer; ^{1}H-NMR (500 MHz), coupling constant J in Hz, multiplicities abbreviated as s, d, t, q, m (singlet, doublet, triplet, quartet, multiplet). ^{13}C-NMR signal assignments from ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC spectra. For atom numbering, see Supp. Information **Figure S6**.[47] ESI-MS: Finigan LCQ classic, positive ion mode; data reported as: m/z (relative intensity in % in reference to the base peak); samples were desalted and injected with MeOH.

HPLC:

Hitachi Pump, type L-2130; Diode Array Detector L2450 L-P19596-1/07 (detection at λ=520 nm); injection: manual loading of the injection loop (~70 µl); stationary phase: Phenomenex HyperClone ODS (C18); column parameters: 250 x 4.60 (5 µm; # 522787-11); mobile phase: potassium phosphate buffer 10mM pH=7; solvent composition: Potassium phosphate buffer 10mM pH=7/MeOH: 0-25 min (5%-90% MeOH), 25-33 min (90%-95% MeOH), 33-35 min (95% MeOH), 35 min-40min (95%-5% MeOH).

*Preparation of CN,H_2O-cobyric acid (**4**):*

Crystalline cyano-aquo-cobyric acid (CN,H_2OCby, **4**) was prepared from dicyanocobyric acid following a published procedure.[44, 45] CN,H_2OCby (**4**) was identified by its UV/Vis, ESI-MS and ^{1}H-NMR spectra, as well as by comparison by HPLC with a sample provided by Prof. Ray Bonett (Queen Mary College, London). In D_2O a roughly 1:1 mixture of the Co_α-CN and Co_β-CN isomers of (CN,H_2OCby, **4**) was soon present, as seen in ^{1}H-NMR spectra (see Supp. Info **Figure S1**)

UV/VIS (c = $4.0\cdot10^{-5}$ M, H_2O): λ_{max}(log(ε)) = 526 (3.82), 495 (3.85), 353 (4.36), 273 (3.98). ESI-MS: 980.4 (16, $[M+Na]^+$, $C_{47}H_{67}CoN_{11}NaO_8$); 960.5 (16.9), 959.5 (66.0) 958.5 (100, $[M+H]^+$, $C_{47}H_{68}CoN_{11}O_8^+$).

*Preparation of Co*β*-5'deoxyadenosylcobyric acid (AdoCby, **3**):*

In a round bottom flask fitted with a UV/Vis cell, crystalline CN,H_2OCby (**4**, 2.00 mg, 2.1 µmol) was dissolved in 500 µl MeOH and 500 µl 10 mM phosphate buffer pH=7. The solution was purged with argon and degassed using freeze-pump-thaw cycling. $NaBH_4$ (10 mg, 130 equiv.) was added under argon and the apparatus was purged with argon again. After half an hour the reaction mixture had turned brown, and 10 mg of $NaBH_4$ were again added. After 1 h, benzoic acid (25.0 mg, 100 equiv.) was added, followed by another portion of 10 mg $NaBH_4$. After 4 hours a UV/Vis spectrum indicated full conversion to a Co(I)-species (see Supp. Info **Figure S2**). All further operations were carried out in the dark. 5'-iodo-5'-deoxyadenosine (1.0 mg, 1.2 equiv.) was added under argon and the reaction mixture was stirred for half an hour, when a UV/Vis-analysis indicated conversion to AdoCby (**3**). After addition of 5 ml phosphate buffer pH=5 (50 mM) the reaction mixture was desalted over a Sep-Pak column. The mixture was purified over a RP column using phosphate buffer pH=7 (10mM) with increasing amounts of MeCN. At 2% MeCN unidentified cobyric acid derivatives were eluted as small red bands. At 7% MeCN AdoCby (**3**) and CN,H_2O-Cby (**4**) were separated as two bands and were eluted with 10% MeCN. Fractions containing AdoCby (**3**) were identified by HPLC, and were combined. Solvents were removed on a rotary evaporator. The orange residue was dissolved in 100µl MeOH and precipitated by dropwise addition to diethyl ether. The precipitate was washed with acetone and dried (HV, overnight), and 1.85 mg (74% yield) of Co_β-5'-deoxyadenosyl-cobyric acid (AdoCby, **3**) were obtained as an orange powder.

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UV/Vis (c= $2.5 \cdot 10^{-5}$ M, H_2O): λ_{max}(log(ε)) = 457 (3.85), 378 (3.80), 303 (4.24), 262 (4.44). CD (c= $1.25 \cdot 10^{-4}$ M, H_2O): $\lambda_{max/min}$ (Δε): 564 (1.1), 500 (-1.2), 432 (-1.8), 401 (0.4), 378 (-0.5), 330 (5.6), 302 (1.3, sh), 282 (-1.1), 240 (-3.4). λ_0: 525, 408, 391, 360, 291, 223. ^{1}H-NMR (500 MHz, 298 K, D_2O, c=$7.0 \cdot 10^{-3}$ M): δ = 0.35 (*t*, *J* = 9.5 Hz, 1H, H_AC5RL), 0.50 (*d*, *J* = 8Hz, 1H, H_BC5RL), 0.80 (*s*, 3H, H_3C1A), 0.84 (*s*, 3H, H_3C12B), 1.12 (*s*, 3H, H_3C17B), 1.41 (*s*, 3H, H_3C2A), 1.58 (*s*, 3H, H_3C12A), 1.78 (*s*, 3H, H_3C7A), 1.80 (*m*, 1H, H_2C81), 1.93 (*m*, 1H, HC4RL), 2.05 (*m*, 1H, H_2C31), 2.21 (*m*, 1H, H_2C81), 2.09 (*dd*, *J* = 3.7 Hz, *J* = 15 Hz, 2H, H_2C172), 2.18 (*m*, 2H, H_2C131), 2.22 (*m*, 2H, H_2C171), 2.34 (*m*, 2H, H_2C132), 2.47 (*m*, 2H, H_2C181), 2.48 (*m*, 2H, H_2C82), 2.54 (*m*, 2H, H_2C32), 2.60 (*d*, J=13Hz, 2H, H_2C21), 2.77 (*ddd*, *J* = 4.4 Hz, *J* = 2.2 Hz, *J* = 10.0 Hz, 1H, HC18), 3.39 (*dd*, *J* = 3.7Hz, *J* = 4.8 Hz, 1H, HC13), 3.71 (d, 1H; HC3RL), 3.75 (*m*, 1H, HC8), 4.20 (*d*, J=8.8 Hz, 1H, HC3), 4.39 (*dd*, *J* = 4.4 Hz, *J* = 4.8 Hz, 1H, HC2RL), 4.62 (*d*, *J* = 10.3 Hz, 1H, HC19), 5.59 (*d*, *J* = 4.4 Hz, 1H, HC1RL), 6.71 (*s*, 1H, HC10), 8.01 (*s*, 1H, HC8L), 8.20 (*s*, 1H, HC2L). ESI-MS: m/z = 1204.5 (29, $[M+Na]^+$, $C_{55}H_{76}CoN_{15}NaO_{11}^+$); 1184.4 (24.6), 1183.4 (68.0), 1182.4 (100, $[M+H]^+$, $C_{55}H_{77}CoN_{15}O_{11}^+$); 932.6 (7, $[M+H-(adenosyl)]^+$, $C_{45}H_{65}CoN_{10}O_8^+$),

*Preparation of coenzyme B_{12} (**2**) from Co_6-adenosyl-cobyric acid (AdoCby, **3**):*

All operations were carried out in the dark. In a 10 ml round bottom flask, 1.0 mg of AdoCby (0.83 µmol, **3**), B_{12}-nucleotide (**5**, 0.50 mg, 1.4 equiv) and HOBt (0.25 mg, 1.6 µmol) were dissolved in 750 µl H_2O and cooled in an ice bath. The round bottom flask was closed with a septum and the solvent purged with argon. With a syringe EDC*HCl (1.70 mg, 10.5 equiv.), dissolved in 250 µl ice-cold ddH_2O, was added. The reaction mixture was stirred at 0°C for 2 hours and subsequently at RT. After 8 h, 1.70 mg (10.5 equiv.) of EDC*HCl, dissolved in 50 µl ddH_2O, were added. The reaction mixture was stirred at RT overnight. The conversion was monitored via HPLC. After full conversion, the reaction mixture was purified on a RP-column using phosphate buffer pH=7 10mM with increasing gradient of MeCN. The red fractions of coenzyme B_{12} (**2**) were eluted with 10% MeCN, were combined and the solvents were removed using a rotary evaporator. The red residue was dissolved in 200 µl of MeOH and precipitated by the dropwise addition to a solution of 2ml tert-butyl methyl ether. To complete the precipitation the solution was stored at 5 ± 3°C overnight. Then solvents were carefully removed and the precipitate was washed 3 times with acetone. The red solid was dried under high vacuum for 24 hours. 1.2 mg of coenzyme B_{12} (**2**, 91%) were obtained as a red powder. For crystallization, the red solid was dissolved in 400 µl of MeOH, transferred in equal portions into two small tubes, where solvents were removed and the residue was again dried under vacuum. The red residues were dissolved in 20 µl H_2O and 10 µl of acetone. Then the tubes were closed with aluminum foil, into which a small hole was made. The small tubes were put into vial, which contained about 4 ml acetone. The crystallization batches were stored at 5 ± 3 °C for 14 days and 10 µl portions of acetone were added once every two days. When crystals had separated, the mother liquor was removed and the crystals were washed with acetone and dried under high vacuum for 24 hours. Some seed crystals were taken aside and the remaining AdoCbl (**2**) was dissolved in water, transferred into a vial and evaporated again on the rotary evaporator. The residue was dissolved in 100 µl H_2O and acetone (300 µl) was added dropwise until the point of turbidity. In addition, the separated seed crystals were added and the mixture was left at 5 ± 3 °C for 5 days. The red crystals of coenzyme B_{12} (AdoCbl, **2**) formed were separated from the mother liquor and dried under high vacuum for 24 hours (yield: 1.10 mg, 85%).

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UV/Vis (c = $2.96 \cdot 10^{-5}$ M, H_2O): $\lambda_{max}(\log(\varepsilon))$ = 523 (3.90), 488 (3.82), 430 (3.64), 375 (4.00), 339 (4.1), 316 (4.11). CD (c = $8.9 \cdot 10^{-5}$ M, H_2O): $\lambda_{max/min}(\Delta\varepsilon)$: 554 (-2.3), 481 (2.7), 429 (-0.7), 386 (1.3), 359 (-2.5), 333 (0.25), 323.5 (-0.1), 299 (1.3); λ_0 at 515, 449, 406, 375, 337, 328, 321, 246. ^{1}H-NMR: (500 MHz, 298 K, D_2O, c = $1.7 \cdot 10^{-3}$ M): δ = 0.44 (*s*, 3H, H_3C1A), 0.55 (*t*, *J* = 8.8 Hz, 1H, H_AC5RL), 0.84 (*s*, 3H, H_3C12A), 0.88 (*m*, 2H, H_2C82), 1.17 (*d*, *J* = 6.6 Hz, 3H, H_3C177), 1.30 (*s*, 3H, H_3C12B), 1.33 (*s*, 3H, H_3C17B), 1.34 (*s*, 3H, H_3C17B), 1.50 (*d*, *J* = 8.8 Hz, H_BC5RL), 1.69 (*s*, 3H, H_3C7A), 1.71 (*m*, 1H, H_2C71), 1.71 (*m*, 1H, H_2C82), 1.73 (*m*, 2H, H_2C81) , 1.77 (*m*, 2H, H_2C172), 1.97 (*m*, 1H, H_2C31), 2.06 (*m*, 1H, H_2C31), 2.08 (*m*, 1H, H_2C171), 2.18 (*s*, 6H, H_3C11N, H_3C10N), 2.19 (*m*, 1H, H_2C71), 2.2 (*m*, 2H, H_2C132), 2.38 (*m*, 2H, H_2C21), 2.45 (*m*, 1H, H_2C171), 2.5 (*m*, 2H, H_2C32), 2.54 (*m*, 2H, H_2C132), 2.62 (*m*, 2H, H_2C181), 2.87 (*d*, *J* = 10.3 Hz, CH13), 3.12 (dd, J= 7.3 Hz, J= 10.7 Hz, 1H, CH18), 3.20 (m, 2H, $CH_2$175), 3.27 (m, 1H, HC89), 3.55 (*m*, 2H, H_2C175), 3.68 (*m*, 1H, HC3RL), 3.72 (*m*, 1H, HC3R), 4.07 (*m*, 1H, HC4R), 4.10 (*m*, 1H, HC3), 4.2 (*m*, 1H, HC2R), 4.24 (*d*, *J* = 10.3 Hz, HC19), 4.33 (*ddd*, *J* = 2.2 Hz, *J* = 11Hz, 1H, HC176), 4.51 (*dd*, *J* = 3.7 Hz, *J* = 4.8 Hz, HC2RL), 5.54 (*d*, *J* = 3.7 Hz, HC1RL), 5.91 (*s*, 1H, HC10), 6.21 (*s*, 1H, HC4N), 6.22 (*d*, *J* = 2.93 Hz, HC1R), 6.91 (*s*, 1H, HC2N), 7.13 (*s*, 1H, HC7N), 7.98 (*s*, 1H, HC8L), 8.19 (*s*, 1H, HC2L); signals according to MTBE: 3.25 (*s*, CH_3-O), 1.19 (*s*, $(CH_3)_3$C-O). ESI-MS: m/z = 1617.5 (9, $[M+K]^+$, $C_{72}H_{100}CoKN_{18}O_{17}P^+$); 1604.4 (12.62), 1603.4 (46.61), 1602.4 (100, $[M+Na]^+$, $C_{72}H_{100}CoN_{18}NaO_{17}P^+$); 1581.5 (5.4), 1580.5 (10.0), 1579.5 (11, $[M+H]^+$, $C_{72}H_{101}CoN_{18}O_{17}P^+$); 1351.6 (10, $[M+Na\text{-}(adenosyl)]^+$, $C_{62}H_{88}CoN_{13}NaO_{14}P^+$); 1239.3 (7, $[M\text{-}(\alpha\text{-ribazole phosphate})]^+$ $C_{58}H_{84}CoN_{16}O_{11}{}^+$); 812.8 (30, $[M+2Na]^{2+}$, $C_{72}H_{100}CoN_{18}Na_2O_{17}P^{2+}$); 687.9 (12.7, $[M\text{-}(adenosyl)+2Na]^{2+}$, $C_{62}H_{88}CoN_{13}Na_2O_{14}P^{2+}$).

*Preparation of vitamin B_{12} (CNCbl, **1**) from CN,H_2O-cobyric acid (**4**):*

In a 5 ml round bottom flask, crystalline CN,H_2O-cobyric acid (**4**, 1.0 mg, 1.02 µmol), HOBt (0.30 mg, 2.2 equiv.) and B_{12}-nucleotide (**5**, 0.65 mg, 1.4 equiv.) were dissolved in 900 µl H_2O. The flask was closed with a septum and the solvent purged with argon. With a syringe a solution of EDC*HCl (2.10 mg, 10.5 equiv.) in 100 µl ice-cold H_2O was slowly added. The reaction mixture was cooled and stirred for 2 hours in an ice bath, which was then removed. The reaction progress was monitored via HPLC and full conversion was observed after 7 hours (see Supp Info **Figure S14**). The reaction mixture was purified via RP18-column chromatography using potassium phosphate buffer pH=7 10 mM with an increasing content of MeCN. At 10% MeCN vitamin B_{12} (**1**) eluted as red band. The red CNCbl (**1**) fractions were collected, solvents removed on the rotary evaporator and desalted via RP18 Sep-Pak cartridge. A red methanolic eluate was obtained that was dried on the rotary evaporator. The residue was dissolved in 100 µl H_2O, transferred into a vial for crystallization at room temperature and acetone was added dropwise until turbidity (about 300 µl). After 4 hours the vial with crystallization batch was put in a refrigerator at 5°C overnight. On the next day, the supernatant was removed and the crystals were washed with acetone three times. The red crystals of vitamin B_{12} (CNCbl, **1**) were dried under high vacuum for 24 hours (yield: 1.30 mg, 92%).

UV/Vis (c = $2.5 \cdot 10^{-5}$ M, H_2O): $\lambda_{max}(\log(\varepsilon))$ = 549 (3.99), 521 (3.94), 408 (3.62), 360 (4.51), 322 (3.95), 305 (4.02), 277 (4.25). CD (c = $1.45 \cdot 10^{-4}$ M, H_2O): $\lambda_{max/min}(\Delta\varepsilon)$ = 548 (-0.7), 527 (-0.4), 483 (-1.5), 433 (4.8), 362 (-6.1), 333 (-1.0), 326 (-1.3), 316 (-0.4), 309 (-0.7), 277 (1.8), 251 (-3.8); λ_0 at 463, 382, 302, 261, 242. ^{1}H-NMR (500 MHz, 298 K, D_2O, $2.1 \cdot 10^{-3}$ M): δ = 0.42 (*s*, 3H, H_3C1A), 0.99 (*m*, 1H, H_2C81), 1.16 (*s*, 3H, H_3C12A), 1.22 (*d*, *J* = 6.6 Hz, 3H, H_3C177), 1.35 (*s*, 3H, H_3C17B), 1.37 (*s*, 3H, H_3C2A), 1.41

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(*s*, 3H, H_3C12B), 1.78 (*m*, 1H, H_2C81), 1.80 (*m*, 1H, H_2C171), 1.83 (*s*, 3H, H_3C7A), 1.95 (*m*, 2H, H_2C31), 1.98 (*m*, 2H, H_2C131), 2.07 (*m*, 2H, H_2C81), 2.16 (*m*, 1H, H_2C172), 2.20 (*m*,1H, H_2C71) 2.22 (*s*, 3H, H_3C11N), 2.22 (*s*, 3H, H_3C10N), 2.37 (*m*, 2H, H_2C21), 2.50 (*s*, 3H, H_3C51), 2.51 (*m*, 1H, H_2C171), 2.54 (*s*, 3H, H_3C151), 2.58-2.5 (*m*, 1H, H_2C32), 2.6 (*m*,1H, H_2C71), 2.6 (*m*, 2H, H_2C132), 2.65 (*m*, 1H, H_2C172), 2.65 (*m*, 1H, H_2C181), 2.71 (*m*, 1H, H_2C181), 2.72 (*d*, *J* = 12.5 Hz, 1H, HC18), 2.92 (*dd*, *J* = 14 Hz, *J* = 8,0Hz, H_2C157), 3.3 (*d*, *J* = 10.3 Hz, 1H, HC13), 3.39 (*dd*, *J* = 5.14 Hz, *J* = 11.5 Hz, 1H, HC8), 3.57 (*d*, *J* = 14,7 Hz, 1H, H_2C157), 3.72 (*dd*, *J* = 4.4 Hz, *J* = 8.4 Hz, 2H, HC5R), 3.89 (*m*, 1H, HC5R), 4.03 (*d*, *J* = 8.8 Hz, 1H, HC4R), 4.05 (*d*, *J* = 11.0 Hz, 1H, HC19), 4.15 (*d*, *J* = 8.8 Hz , 1H, HC3), 4.24-4.29 (*m*, 1H, HC2R), 4.24-4.29 (*m*, 2H, H_2C176), 6.05 (*s*, 1H, HC10), 6.31 (*d*, *J* = 2.9Hz, HC1R), 6.47 (*s*, 1H, HC4N), 7.05 (*s*, 1H, HC2N), 7.24 (*s*, 1H, HC7N). ESI-MS: m/z = 1393.5 (13, $[M+K]^+$, $C_{63}H_{88}CoKN_{14}O_{14}P^+$); 1377.7 (80, $[M+Na]^+$, $C_{63}H_{88}CoN_{14}NaO_{14}P^+$); 1357.5 (36.6), 1356.5 (74.0), 1355.5 (100, $[M+H]^+$, $C_{63}H_{89}CoN_{14}O_{14}P^+$).

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

[1] These two authors contributed equally. The experiments were carried out by F. J. W. and F. G. under the supervision of B.K., and the manuscript was written by F. J. W., F. G. and B.K.

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