Exceptional Photochemical Stability of the Co-C Bond of Alkynyl Cobalamins, Potential Antivitamins B₁₂ and Core Elements of B₁₂-Based Biological Vectors

Supporting Information

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**General.**

**Materials.** Aquocobalamin chloride ($\text{H}_2\text{OCl}*\text{Cl}$) *Roussel UCLAF*; water was purified using Epure, Barnstead Co; 2,4-difluorophenylethyne (97%), potassium phosphate monobasic $p$. $A.$ and potassium phosphate dibasic $p$. $A.$ were from Sigma-Aldrich; 3-propargyl alcohol *purum*, formic acid *purum*, triethylamine, *puriss*, dimethylsulfoxide (DMSO), *puriss*, were from *Fluka*; N-iodomorpholine and 3-iodoprop-2-yn-1-ol were synthesized according a modified procedure as described in$^1$; copper(I)iodide was from Merck, acetonitrile (MeCN) and methanol (MeOH) HPLC-grade were from *BDH prolabo*, dichloromethane (Acros) was distilled prior to use, tetrahydrofuran (THF) purum was from Merck, CD$_3$OD (99.80%)D was from Eurisotop; 1 g Sep - Pak - C18 Cartridges were from Waters Associates. LiChroprep RP-18 (25-40μm) was from *Merck*.

**Spectroscopy.** *UV/Vis Spectra*: Hitachi-U3000; $\lambda_{\text{max}}$ (log $\varepsilon$) in nm. *CD Spectra*: Jasco J715; $\lambda_{\text{max}}$ or $\lambda_{\text{min}}$ ($\Delta \varepsilon$) in nm. FT-IR-Spectra: Mattson 300 Galaxy Series; cm$^{-1}$. $^1$H and $^{13}$C- NMR spectra: Bruker AM 300 (for 1-iodopropyne) or 500 MHz Varian Unity Inova (for HOPryCbl), equipped with 5 mm triple-resonance probe with z-gradients; $\delta$(HDO) = 4.77 ppm; $\delta$(CD$_3$OD) = 3.31 ppm, $\delta$(DMSO-$d_6$) = 2.50 ppm; HSQC: 2k x 512 complex data points. SW1 11 ppm centered around the residual water signal, SW1-10-200 ppm. 64 scans per increment. HMBC: 2k x 512 data points (real). SW2 11 ppm centred on a residual water signal, SW1-10-200 ppm. 64 scans per increment. ROESY: 2k x 512 complex data points, 32 scans per t1 increment, mixing time 250 ms. *MALDI-MS*: Bruker Ultraflex MALDI-Tof, positive-ion mode, matrix: 2,5-dihydroxybencoic acid. *HPLC*: Dionex Ultimate 3000, variable wavelength detector; column: YMC-Triart –C18, 250x4.7 mm, S-5 μm, 12 nm, TATA12S05-2546WT; solvent composition: A: 10 mM aqueous K-phosphate pH 7, B= MeOH; 8% to 95%B 0-40 min, 95% B 40-44 min, 95% to 8%B 44-45 min

**Synthesis of Co$_\text{III}$-3-hydroxypropynylcobalamin (HOPryCbl)**

![Scheme S1. Synthesis of HOPryCbl from H$_2$OCbl by formate reduction and alkynylation with 3-iodo-propargyl alcohol.](image-url)
In a 5 ml round bottom flask, 100.0 mg of H$_2$OCbl*Cl (72.4 µmol) were dissolved in 2.5 ml of MeOH and the mixture was degassed with argon for 10 min. To this solution 50 µl formic acid (1.3 mmol) and 187 µl triethyl amine (1.3 mmol) were added under Ar and the solution was stirred for 30 min at RT. To the now brown solution 61.8 µl 3-iodoprop-2-yn-1-ol (300.0 µmol) were added under argon and the mixture was stirred for 24 hours. The reaction mixture was poured in 15 ml of ethyl acetate to precipitate the raw product. The mother liquor was removed and the red precipitate was dried under high vacuum for 2 hours. The raw product was purified by RP-18 column chromatography using MeOH/phosphate buffer pH 8 (10 mM), solvent system (5-50% MeOH, in 10% steps). H$_2$OCbl*Cl eluted first (at 30 % MeOH) followed by the red fraction of the product HOPryCbl (at 40 % MeOH). The product fraction was collected and the solvents were evaporated on rotary evaporator at room temperature. Raw red corrin HOPryCbl was dissolved in water (0.5 ml) and crystallized after addition of 5 ml of acetone. The crystals of HOPryCbl were first washed with (1:9) mixture of water: acetone, then with acetone. The sample of HOPryCbl was dried under high vacuum over night, to give 60.6 mg (43.7 µmol, 60.4% yield) of red crystalline solid, which was characterized as follows:

UV/Vis: (c = 4.03 x 10$^{-5}$ M in H$_2$O) $\lambda_{max}$ (log$\varepsilon$) = 262 (4.23), 280 (4.22), 287 (sh., 4.20), 350 (4.09), 368 (4.17), 401 (sh., 3.64), 444 (3.54), 489 (sh., 3.67), 523 (3.87), 550 (3.90) (Figure S1).

IR: (KBr) $\nu$ = 566.65 (m); 870.07 (m); 1018.97 (m); 1081.45 (s); 1146.45 (s); 1214.60 (s); 1350.52 (m); 1402.77 (s); 1497.62 (s); 1572.77 (s); 1666.05 (vs); 2134.87 (w); 2968.11 (m); 3367.92 (vs) cm$^{-1}$ (Figure S2).

$^1$H-NMR: (298 K, CD$_3$OD, c = 13.8 mM) $\delta$ = 0.46 (s, 3H, H$_3$C-1A); 1.12 (s, 3H, H$_3$C12B); 1.18 (m, 1H, H$_3$C-81); 1.26 (d, 3H, J = 6.4 Hz, H$_3$C-177); 1.34 (s, 3H, H$_3$C-17B); 1.34 (m, 1H, H$_3$C-82a); 1.40 (s, 3H, H$_3$C-2A); 1.44 (s, 3H, H$_3$C-12A); 1.71 (m, 1H, H$_3$C-82); 1.88 (s, 3H, H$_3$C-7A); 1.91 (m, 1H, H$_3$C-131); 1.93 (m, 2H, H$_3$C-31); 2.00 (m, 1H, H$_3$C-81); 2.06 (m, 1H, H$_3$C-131); 2.11 (m, 1H, H$_3$C-171); 2.13 (m, 1H, H$_3$C-71); 2.28 (m, 1H, H$_3$C-10N); 2.29 (s, 3H, H$_3$C-11N); 2.32 (m, 1H, H$_3$C-21); 2.41 (m, 2H, H$_3$C-32); 2.46 (m, 1H, H$_3$C-71); 2.54 (m, 1H, H$_3$C-51); 2.55 (m, 1H, H$_3$C-151); 2.55 (m, 2H, H$_3$C-132); 2.57 (m, 1H, H$_3$C-171); 2.59 (m, 1H, H$_3$C-181); 2.63 (m, 1H, H$_3$C-181); 2.63 (m, 1H, H$_3$C-21); 2.81 (m, 1H, H$_3$C-18); 2.87 (m, 1H, H$_3$C-175); 3.21 (d, 1H, J = 10.7, H$_3$C-13); 3.40 (dxd, 1H, J = 10.8, 5.2 Hz, H$_3$C-8); 3.67 (m, 1H, H$_3$C-81); 3.77 (dxd, 1H, J =12.6; 4.2 Hz; H$_3$C-5R); 3.90 (d, 1H, J = 5.3 Hz, H$_3$C-3L); 3.93 (m, 1H, H$_3$C-5R); 4.10 (m, 1H, H$_3$C-4R); 4.16-4.22 (m, 2H, H$_3$C-2R, H$_3$C-3); 4.34 (m, 1H, H$_3$C-176); 4.42 (d, 1H, J = 11.6 Hz, H$_3$C-19); 4.67 (m, 1H, H$_3$C-3R); 5.93 (s, 1H, H$_3$C-10); 6.25 (d, 1H, J = 3.2 Hz H$_3$C-1R); 6.60 (s, 1H, H$_3$C-4N); 7.17 (s, 1H, H$_3$C-2N); 7.21 (s, 1H, H$_3$C-7N) (Figure S3).

$^{13}$C-NMR: (298 K, CD$_3$OD, c = 13.8 mM) $\delta$ = 16.21 (C151); 16.21 (C51); 17.01 (C17B); 17.25 (C2A); 20.39 (C7A); 20.43 (C11N); 20.49 (C12A); 20.71 (C1A); 20.77 (C10N); 21.49 (C177); 27.41 (C81); 27.46 (C31); 29.21 (C131); 32.12 (C12B); 32.47 (C181); 32.60 (C171); 33.08 (C32); 33.40 (C172); 35.54 (C132); 36.35 (C32); 39.99 (C18); 43.32 (C21); 44.63 (C71); 46.65 (C175); 48.25 (C2); 49.06 (C12); 51.85 (C3L); 52.44 (C7); 55.09 (C13); 57.71 (C3); 57.72 (C8); 59.99 (C17); 62.47 (C5R); 70.76 (C2R); 73.71 (C176); 75.11 (C11L); 75.67 (C3R); 75.69 (C19); 83.22 (C4R); 86.56 (C1); 87.56 (C1R); 94.90 (C10); 100.30 (C2L); 105.02 (C15); 108.86 (C5); 112.03 (C7N); 118.60 (C4N); 131.90 (C9N); 134.15 (C6N); 138.99 (C8N); 143.50 (C2N); 165.15 (C5N); 165.59 (C6); 166.34 (C14); 173.68 (C9); 174.79 (C173); 175.33 (C72); 176.30 (C11); 176.58 (C182); 176.89 (C22); 177.21 (C83); 177.85 (C33); 177.97 (C133); 178.21 (C16); 179.60 (C4).
**MS:** MALDI-Tof pos, matrix 2,5-dihydroxybenzoic acid: m/z (%) = 1384.35 (39, [M+H]+), 1368.18 (29, [M-propynol]+K)+, 1351.44 (30 [M-(propynol)+Na]+), 1329.65 (100, [M-(propynol)]+), 1202.06 (64, [M+H2O-DMB-base]), 1184.30 (30, [M-propynol-DMB-base]+), 971.54 (35, [M-propynol-C14H18N2O7P]+);

**Figure S1:** UV/Vis spectrum of Coθ-3-hydroxypropynylcobalamin (c = 40.3µM) in H2O

**Table S1:** Position of C≡C stretch absorption in IR-spectra of alkynylcobalamins (in KBr)

| alkynylcobalamin  | wavenumber [cm\(^{-1}\)] |
|-------------------|---------------------------|
| PhEtyCbl          | 2117                      |
| F\(_2\)PhEtyCbl   | 2130                      |
| HOPryCbl          | 2135                      |
**Figure S2:** IR spectrum of Co\(_{\beta}\)-3-hydroxypropynylcobalamin dispersed in KBr.

**Figure S3:** 500 MHz \(^1\)H-NMR-spectrum of Co\(_{\beta}\)-3-hydroxypropynylcobalamin in CD\(_3\)OD (298 K, X marks residual solvent signal)
Crystal structure of Coβ-3-hydroxypropynyl-cobalamin

Crystals of Coβ-3-hydroxypropynyl-cobalamin (HOPryCbl) were grown from H₂O/acetone. Diffraction experiments were carried out with a Nonius Kappa CCD diffractometer at a temperature of 233 K. Diffraction data extended to a resolution of 0.91 Å. The asymmetric unit of the orthorhombic crystal contained B₁₂ molecule and well-ordered water molecules.

Indexing of diffraction images, intensity integration, and data scaling were performed with programs DENZO and SCALEPACK.² The crystal was orthorhombic (space group P2₁2₁2₁) with unit cell constants \( a = 16.2120(10) \, \text{Å}, b = 21.1070(10) \, \text{Å}, \) and \( c = 24.6140(10) \, \text{Å} \). The structure was solved by direct methods and refined against \( F² \)-values using the program SHELXL.³ Full matrix least-squares anisotropic refinement converged at \( R₁ = 0.0718 \) for all data. No absorption correction was applied to the data. The solvent region was modeled using 17 water molecules with anisotropic atomic displacement parameters (adp). H-Atom positions were calculated and refined as ‘riding’ on their respective non-H-atom. For methyl- and hydroxyl-groups the torsion angle around the C-C or C-O bond was also refined (omitted for water molecules). The isotropic adp for each H-atom was set to 1.5 times (for methyl- and hydroxyl-groups) and 1.2 times (for all other hydrogen atoms) the equivalent isotropic atomic displacement parameters of the adjacent non-H-atom. Data pertaining to diffraction data collection and structure refinement are summarized in Table S2.

**Figure S4.** Crystallographic model of Coβ-3-hydroxypropynylcobalamin (HOPryCbl) (small red balls mark positions of water molecules that are at H-bonding distances from the hydroxypropynyl ligand and from the nucleotide moiety). Left: Complete molecule of HOPryCbl. Right: Representation of the top moieties of the two slightly disordered structures of HOPryCbl, with H-bonded water molecules highlighted as small red balls.

Bond distances in the equatorial plane are 1.863(6) Å (Co-N1), 1.917(5) Å (Co-N2), 1.892(6) Å (Co-N3) and 1.887(6) Å (Co-N4). The Co-Cβ and Co-Nα-distances are 1.889(9) Å and 2.089(6) Å, resp.
**Table S2:** Crystallographic data of Coβ-3-hydroxyprop-1-ynyl-cobalamin

| Property                                      | Value                                                                 |
|-----------------------------------------------|----------------------------------------------------------------------|
| Molecular formula                             | C_{65}H_{91}CoN_{13}O_{15}P x 17 H_{2}O                               |
| Formula weight                                | 1690.68                                                              |
| Crystal system                                | Orthorhombic                                                         |
| Space group                                   | P2_12_12_1 (nr. 19)                                                  |
| Unit cell dimensions                          |                                                                      |
| \(a\) [Å]                                    | 16.2120(10) \(\alpha = 90^\circ\)                                   |
| \(b\) [Å]                                    | 21.1070(10) \(\beta = 90^\circ\)                                    |
| \(c\) [Å]                                    | 24.6140(10) \(\gamma = 90^\circ\)                                   |
| Volume [Å³]                                   | 8422.6(7)                                                            |
| \(Z\)                                         | 4                                                                   |
| Temperature [K]                               | 233(2)                                                              |
| Radiation                                     | 0.71073 Å                                                           |
| Density (calculated) [Mg m⁻³]                 | 1.333                                                               |
| Absorption coefficient [mm⁻¹]                 | 0.311                                                               |
| F (000)                                       | 3616                                                                |
| Crystal size [mm³]                            | 0.3 x 0.3 x 0.2                                                      |
| Theta range for data collection               | 1.50 to 22.99°                                                      |
| Index ranges                                  | -17\(\leq\)h\(\leq\)17, -23\(\leq\)k\(\leq\)23, -27\(\leq\)l\(\leq\)27 |
| Reflections collected                         | 20631                                                               |
| Independent reflections                       | 10383 [R(int) = 0.0453]                                              |
| Reflections [I>2sigma(I)]                     | 9335                                                                |
| Completeness to theta = 25.00°                | 94.5 %                                                              |
| Absorption correction                         | None                                                                |
| Refinement method                             | Full-matrix least-squares on F²                                      |
| Data / restraints / parameters                 | 10383 / 0 / 1017                                                     |
| Goodness-of-fit on F²                          | 1.065                                                               |
| Final R indices [I>2sigma(I)]                 | R1 = 0.0620, wR2 = 0.1598                                            |
| R indices (all data)                           | R1 = 0.0718, wR2 = 0.1677                                            |
| Absolute structure parameter                  | 0.017(8)                                                            |
| Largest diff. peak and hole [e.Å⁻³]            | 0.512 and -0.432                                                    |

CCDC1964021 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.
Figure S5. Stereo-pictures of the structure of Coβ-3-hydroxypropynylcobalamin (HOPryCbl), obtained by X-ray analysis, highlighting the derived small disorder of the position of the hydroxyl-group of the 3-hydroxypropynyl ligand in the two structures shown (color code of the corrin core: Co pink sphere; C red; N blue; color code of the axial ligands and the periphery: C green; N blue; O red; P turquoise).
Figure S6. Atom numbering used for 3-hydroxypropynylcobalamin
Hydrolysis of Coβ-3-hydroxypropynylcobalamin (HOPryCbl) in acidic aqueous solutions: 3 mg (2.17 µmol) of HOPryCbl were dissolved in 1 ml H2O dist. and 50 µl of the resulting solution were diluted with 2.5 ml 100 mM HCl pH 1. The sample was stored under air at RT in the dark. UV/Vis spectra were recorded at regular time intervals in the course of 200 min. After 200 min the UV/Vis spectrum was similar to that of aquocobalamin (see Figure S7).

Figure S7: Qualitative UV/Vis-spectroscopic kinetic analysis of the hydrolysis at pH 1 of Coβ-3-hydroxypropynylcobalamin in an aerated aqueous solution of HCl.

Figure S8: Time dependence of the absorbance at 550 nm of an aerated aqueous solution of Coβ-3-hydroxypropynylcobalamin in dilute HCl at pH 1 and room temperature.
Photolytic stability of Coβ-3-hydroxypropynylcobalamin:

In a UV/Vis cell a solution of Coβ-3-hydroxypropynylcobalamin in H$_2$O (ca. $3.5 \times 10^{-5}$ M) was exposed to daylight. UV/Vis spectra were recorded at regular intervals for 48 h.

![UV/Vis spectra of an aerated aqueous solution of Coβ-3-hydroxypropynylcobalamin before (black) and after (red) exposure to bright daylight at room temperature](image)

**Figure S9.** UV/Vis spectra of an aerated aqueous solution of Coβ-3-hydroxypropynylcobalamin before (black) and after (red) exposure to bright daylight at room temperature

Thermal stability of Coβ-3-hydroxypropynylcobalamin (HOPryCbl):

In polypropylene vials 10µl aliquots of a solution of 1mM HOPryCbl in DMSO were heated to 100°C. Samples, taken at five time points, were diluted with 190µl aq. 10mM K-phosphate buffer pH 7 and subjected to HPLC analysis. Significant decomposition of HOPryCbl could be detected, but not significant amounts of aquocobalamin.

![HPLC-chromatograms (detection at $\lambda=520$nm) of the thermolysis mixtures of HOPryCbl in DMSO at 100°C, sampled at the specified times between 0 and 24h. An arrow indicates the position where aquocobalamin would be observed.](image)

**Figure S10.** Left. HPLC-chromatograms (detection at $\lambda=520$nm) of the thermolysis mixtures of HOPryCbl in DMSO at 100°C, sampled at the specified times between 0 and 24h. An arrow indicates the position where aquocobalamin would be observed. Right. Table showing absolute and relative HPLC-detected areas of the HOPryCbl fraction as a function of the duration of the thermolysis reaction.

| Time  | area [mAU*min] | area% [%] |
|-------|----------------|-----------|
| t0    | 25.6           | 88.9      |
| 55min | 26.3           | 89.7      |
| 2h    | 25.9           | 90.0      |
| 20h   | 12.1           | 60.5      |
| 24h   | 7.5            | 46.5      |
Figure S11. Decay associated difference spectra following excitation of HOPrCyCbl at 540 nm.

Figure S12. Comparison of the DADS of HOPrCyCbl for the long-lived component following excitation at 408 nm and 540 nm.

Figure S13. Difference spectrum for the longest lived component of HOPrCyCbl in a range of solvents. All traces are near room temperature.
Figure S14. Dependence of the excited state lifetime of HOPryCbl on solvent polarity (upper plot) and viscosity (lower plot).

Figure S15. Surface plots for the transient spectra of HOPryCbl as a function of temperature following excitation at 540 nm.
**Figure S16.** Visible transient absorption of HOPryCbl in ethanol following 408 nm excitation.

**Figure S17.** Temperature dependence of the long-lived excited state of HOPryCbl in ethanol. Note that there is an increased absorption between 420 nm and 450 nm at higher temperatures, but the differences are not as pronounced as in water. Pump-scatter prevented characterization of the excited state absorption at shorter wavelengths.
**Figure S18.** F2PhEtyCbl transient absorption at 13°C following excitation at 550 nm. (a) Comparison of kinetic traces and the exponential fit at several key wavelengths as indicated. (b) SADS for the intermediates. The residual at long times (>> 1 ns) is zero except for the influence of pump scatter around 540 nm.

**Figure S19.** Comparison of the temperature dependent difference spectra for the long-lived excited state of PhEtyCbl and F2PhEtyCbl. The data were obtained using excitation centered at 540 to 550 nm in the αβ-band.
Figure S20. At low pH organometallic Cbls are protonated reversibly at their DMB-base, which decoordinates the DMB unit from the cobalt-center and generates a base-off form from the original base-on Cbl-form (see main text, Figure 1 for full formulae of the base-on Cbl-forms).

References

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