Supporting Information

Contents

Material and Methods:

Vitamin B12 (B12) was a generous gift from DSM Nutritional Products (Basel/ Switzerland). All other chemicals were obtained from Aldrich, Sigma or Fluka (Buchs, CH). The chemicals were of reagent grade and used without further purification.

Deuterated solvents were obtained from Armar Chemicals (Döttingen, Switzerland).
Chromafix C18ec for solid phase extraction (SPE) was obtained from Macherey Nagel. In general the compound was dissolved in little water, transferred to the adsorbent, washed with water and eluted with MeOH.

The pH measurements were performed with a Metrohm 827 pH lab.

HPLC solvents were 0.1 % aqueous trifluoroacetic acid (A) and methanol (B). HPLC analyses were performed on a Merck-Hitachi L-7000 system equipped with a diode array UV-Vis spectrometer and Macherey Nagel Nucleosil C-18ec RP columns (5 µm particle size, 100 Å pore size, 250 x 3 mm. Flow rate: 0.5 ml min^{-1}). Preparative HPLC separations were performed on a Varian Prostar system equipped with two Prostar 215 pumps, a Prostar 320 UV-vis detector and Macherey Nagel Nucleosil C-18ec RP columns (7 µm particle size, 100 Å pore size, 250 x 40 mm. Flow rate: 40 ml min^{-1}). The following gradient was used for HPLC and preparative HPLC measurements: 25 % B for 5 min, then in 25 min to 100 % B, then 100 % B for 10 min. UPLC solvents were 0.1% aqueous formic acid (C) and acetonitrile (D). UPLC measurements were performed on an Acquity™ Ultra performance LC and ACQUITY UPLC® BEH C18 column (1.7 µm 2.1×50 mm; flow rate: 0.5 ml min^{-1}). The UPLC gradient was: 7 % D for 0.1 min, then in 2.9 min to 17 % D, then in 1 min to 100 % D, and finally 100% D for 1 min.

Size exclusion chromatography was performed on an ÄKTAdesign system with a Superdex peptide column 10/300 GL from GE Healthcare.

A Britton-Robinson buffer contains 0.04 M H_3BO_3, 0.04 M H_3PO_4, and 0.04 M CH_3COOH and can be easily adjusted by adding either HCl or NaOH.

The total "cobalt concentration" ($[Co]_{total}$) of a compound was determined from the absorbance of the γ-band at 367-368 nm ($[\varepsilon_\gamma$ (367-368 nm) = 3.04 x 10^4 M^{-1} $cm^{-1}]$[S1] after conversion of the corresponding corrinoid to the dicyano-compound **1-CN**.

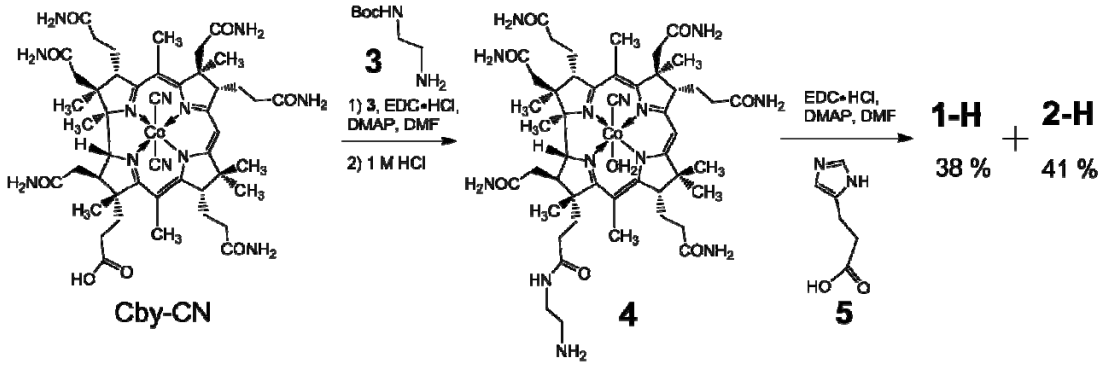
UV-Vis spectra were recorded on a Cary 50 spectrometer using quartz cells with a path length of 1 cm. The pH dependant UV-Vis titration experiments were performed as follows. An aqueous solution of **1-1** ([**1-1**] = 87 µM, 0.2 M KCl) was titrated stepwise at 20 °C with an HCl solution (32%). The UV-Vis absorption spectra and the corresponding pH values were measured after each step.

The inflection point (x_0) was obtained from the analysis of a Boltzmann function: $y = A_2 + (A_1 - A_2) / (1 + \exp((x - x_0) / dx))$ fitting the absorption at 551 nm (compound **1-1**).

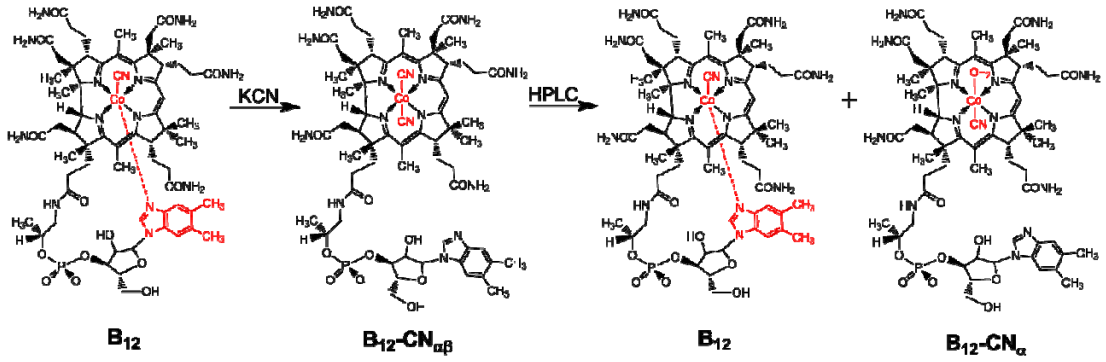
NMR spectra were recorded on a Bruker *AV-500* spectrometer (Karlsruhe, Germany). The chemical shifts are given in ppm relative to the signal from the deuterated solvent. The data processing was carried out with ACD/SpecManager (*Advanced Chemistry Development*).

Mass spectra were recorded either in the positive or negative mode on an Esquire HCT from Bruker (Bremen, Germany).

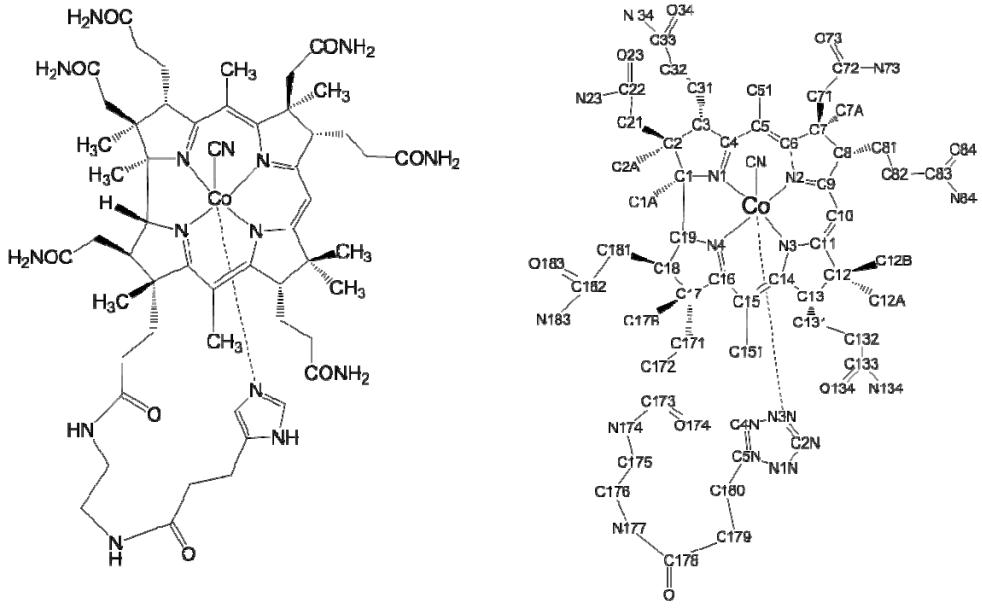
Scheme S1: Synthesis of compound **1-H** and **2-H** (charges and CF_3COO^- counterions have been omitted).



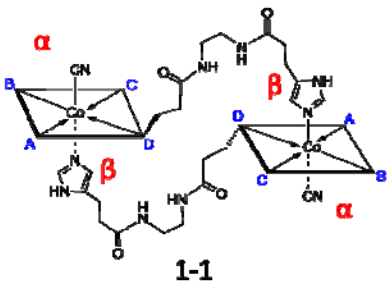
Scheme S2: Synthesis of compound **B12-CN$_\alpha$** (charges have been omitted).



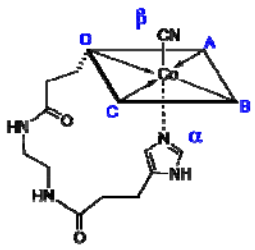
Scheme S3: Atom labelling of compound **2-M** (the labelling of the two subunits in compound **1-1** is the same).



Experimental Procedures:



1-1



2-M

Compound **1-1** and **2-M**: Dicyanocyano cobyric acid (Cby-CN, 9.8 mg, 10 µmol) was dissolved in dry DMF (2 mL) and cooled to 0 °C before DMAP (3.0 mg, 2.5 µmol) and EDC·HCl (9.0 mg, 45 µmol) was added. After 5 min, N-*Boc*-ethylenediamine (**3**, 16 mg, 0.10 mmol) was added and the reaction was allowed to warm up to room temperature. After 6 hours, the solvent was removed under reduced pressure and it was precipitated with acetone. The precipitate was dissolved in aqueous HCl (3 mL, 1 M) and it was stirred for 3 h. The solution was lyophilized to yield crude **4**. The latter was dissolved in DMF (1 mL) and it was cooled to 0°C, after which DMAP (1.0 mg, 8 µmol) and deamino histidine (**5**, 4.5 mg, 15 µmol) were added. After 10 min, EDC·HCl (3.0 mg, 15 µmol) was added. The solution was allowed to warm up to room temperature. It was stirred for 10 h and the solvent was removed under reduced pressure. The residue was washed with acetone and further purified with preparative HPLC to afford **1-H** (5.2 mg, 3.8 µmol, yield: 38 %) and **2-H** (5.6 mg, 4.1 µmol, yield: 41 %) as TFA salts. **1-1** and **2-M** are derived from **1-H** and **2-H** in water at pH 8.1 and pH 6.9, respectively.

UPLC-UV-vis: R_t = 0.9 min (**1-H**) and R_t = 1.7 min (**2-H**)

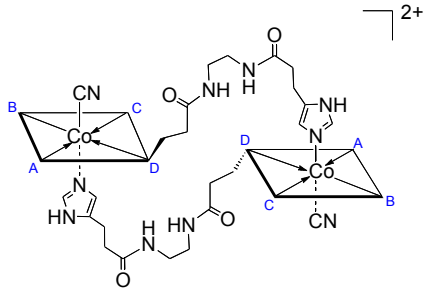
^{1}H-NMR of **1-1**, **2-M**, **1-H** and **2-H**: see table S1.

ESI-MS: m/z (%): Compound **1-1**: 1122.6 (100) $[M/2]^+$, Compound **2-M**: 1122.6 (100) $[M]^+$

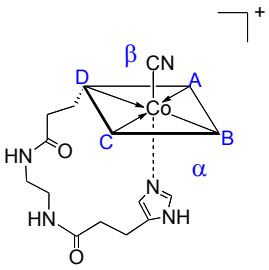
UV-vis spectrum of **1-1** (c = 87 µM, 0.2 M KCl, pH = 8.1): 551 nm (4.31), 521 nm (4.28), 411 nm (3.84), 322 nm (4.21), 303 nm (4.27), 277 nm (4.37).

UV-vis spectrum of **2-M** (c = 24 µM, 0.2 M KCl, pH = 6.9): 555 nm (3.96), 524 nm (3.95), 411 nm (3.67), 362 nm (4.49), 322 nm (3.94), 306 nm (4.00), 278 nm (4.10).

Cation Exchange Experiment:



1-1



2-M

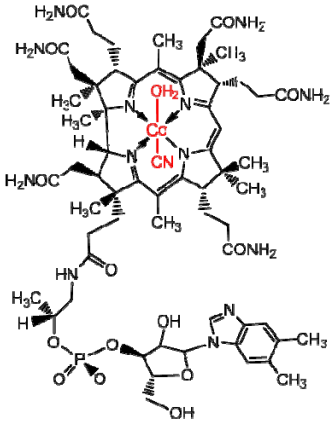
A cation exchange experiment was performed in order to further support the existence of the double positive charged dimer **1-1**.

An aqueous mixture (0.5 mL, pH 8, Britton Robinson Buffer) of monomer **2-M** ($([Co]_{total}$ = 40 µM; positive charged) and dimer **1-1** ($[Co]_{total}$ = 20 µM; double positive charged) was loaded on a CM Sephadex® C25 column (1.2 cm × 8 cm).

The mixture was separated into two well-defined bands using a Britton Robinson Buffer (pH 8). We observed that the faster running compound was the majority of the mixture (single positive charged **2-M**) and the slower one was the minority (double positive charged **1-1**).

Size Exclusion Experiment:

A mixture of **1-1** ($n(Co_{total}) = 10^{-9}$ mol) and **2-M** ($n(Co_{total}) = 2 \times 10^{-9}$ mol) was separated with size exclusion chromatography using a Superdex peptide column 10/300 GL from GE Healthcare (V_t = 24 mL, V_0 = 7 mL; solvent: ammonium acetate (10 mM), pH 7.4; flow rate: 0.3 mL/min; detection wavelength: 220 nm). Two partially overlapping peaks were observed at 15.5 mL (minor product, **1-1**) and 17.4 mL (major product, **2-M**).



B_{12}-CN_α

Compound **B12-CN_α**: B12 (27 mg, 20 µmol) was dissolved in water (5 ml), before KCN (13 mg, 200 µmol) was added. After observation of a colour change from red to violet, the solution was injected directly into the preparative HPLC to yield **B12-CN_α** (17 mg, 12.5 µmol, 62.7 %) (The solvent waste was collected into a bottle containing solid NaOH to neutralize the acidic solution and to absorb potential HCN as cyanide; an HCN-detector from Dräger was used.)

HPLC-UV-vis: R_t = 11 min

^{1}H-NMR of **B12-CN_α**: see table S1 (The chemical shifts of the protons of the corrin ring are consistent with those of Cbi-CN_α, but differ significantly from those of Cbi-CN_β)[S3]

ESI-MS: m/z (%): Compound **B12-CN_α**: 1355.6 (100) $[M-H_2O+H]^+$

UV-vis spectrum of **B12-CN_α** (c = 35 µM, pH = 4.5): 528 nm (3.88), 496 nm (3.92), 407 nm (3.67), 354 nm (4.49), 321 nm (4.43), 276 nm (4.22).

Table S1: Comparison of NMR chemical shift values for **1-1**, **2-M**, **1-H**, **2-H**, α-aqua-β-cyano-cobinamide (**Cbi-CN$_\beta$**) and β-aqua-α-cyano-cobinamide (**Cbi-CN$_\alpha$**):

	δ ^{1}H[ppm]						
	2-M	**2-H** [a]	**Cbi-CN$_\beta$**	**1-1**	**1-H** [a]	**Cbi-CN$_\alpha$**	**B12-CN$_\alpha$**
	α-imidazole β-cyano	α-aqua β-cyano	α-aqua β-cyano	α-cyano β-imidazole	α-cyano β-aqua	α-cyano β-aqua	α-cyano β-aqua
C1							
C1A	0.69	1.37	1.38	1.54	1.71	1.73	1.67
C2							
C2A	1.50	1.61	1.62	1.54	1.64	1.64	1.56
C3	4.11	4.11	4.11	4.11	3.99	4.00	3.98
C4							
C5							
C6							
C7							
C7A	1.81	1.82	1.82	1.67	1.74	1.74	1.76
C8	3.47	3.68	3.68	3.67	3.68	3.69	3.69
C9							
C10	6.10	6.53	6.52	6.03	6.46	6.45	6.48
C11							
C12							
C12A	1.50	1.41	1.42	1.54	1.35	1.37	1.32
C12B	1.21	1.17	1.18	1.21	1.35	1.35	1.35
C13	3.33	3.48	3.48	3.51	3.57	3.57	3.54
C14							
C15							
C16							
C17							
C17B	1.45	1.61	1.61	1.38	1.58	1.58	1.59
C18	2.71	3.09	3.10	3.40	3.11	3.13	3.07
C19	4.09	4.26	4.25	3.21	4.08	4.08	4.08
C21	2.34/2.47	2.39/2.49	2.37/2.47	1.51/2.05	2.23/2.34	2.24/2.35	2.22/2.33
C31	2.05/2.24			2.18			
C32	2.59			2.59			
C51	2.40	2.37	2.38	2.42	2.42	2.44	2.41
C71	2.26/2.61	2.23/2.57	2.23/2.56	2.71/2.88	2.74	2.74	2.75
C81	1.37/2.17			2.00/2.45			
C82	1.37/2.56			2.20/2.38			
C131	1.96			1.96/2.19			
C132	2.64			2.37			
C151	2.50	2.45	2.47	2.52	2.41	2.41	2.40
C171	2.44/2.50			2.35/2.50			
C172	1.82/2.02			2.11/2.82			
C175	3.18/3.37[b]			3.21/3.40[b]			
C176	3.29[b]			3.34[b]			
C177							
C181	2.74/2.85			2.66			
C3P							
C179	2.15/2.30			2.06/2.42			
C180	2.70			2.68/2.83			
C2N	6.65	8.62		7.05	8.53		
C4N	5.84	7.26		5.35	7.17		

Assignment by ^{1}H-NMR, ^{13}C-NMR, ^{1}H, ^{13}C-HSQC correlation, ROESY and comparison with B12.
a. Compound **1-H** and **2-H** are partially signed for comparison with **Cbi-CN$_\beta$** and **Cbi-CN$_\alpha$**.
b. The protons at C175 and C176 positions were not assigned specifically.

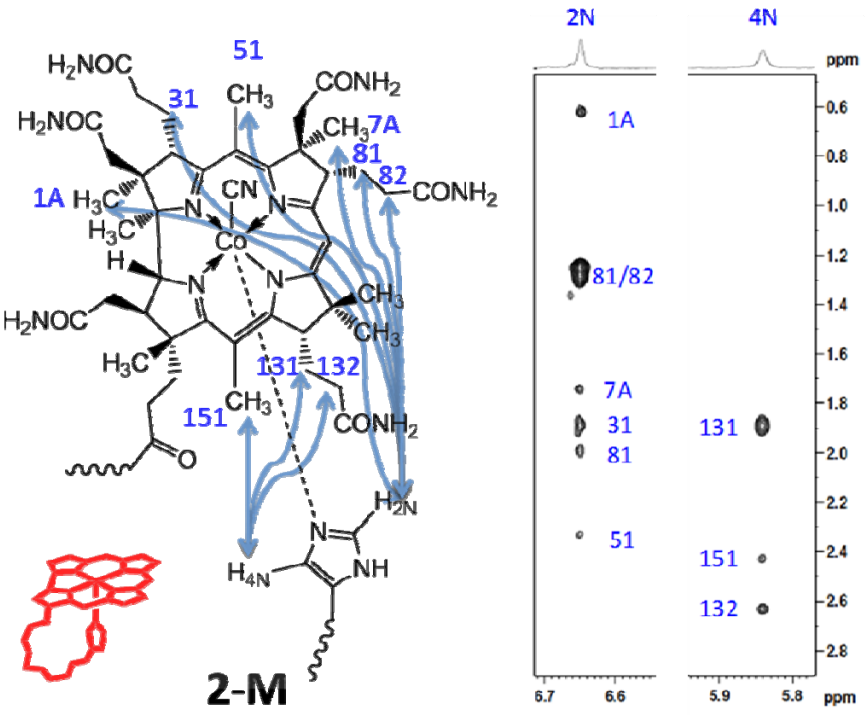


Figure S1. ROESY spectra (500 MHz, 300 K) of **2-M**. NOEs between the imidazole moiety (H_{2N}, H_{4N}) and hydrogen atoms at the periphery of the corrin ring of **2-M** are indicated.

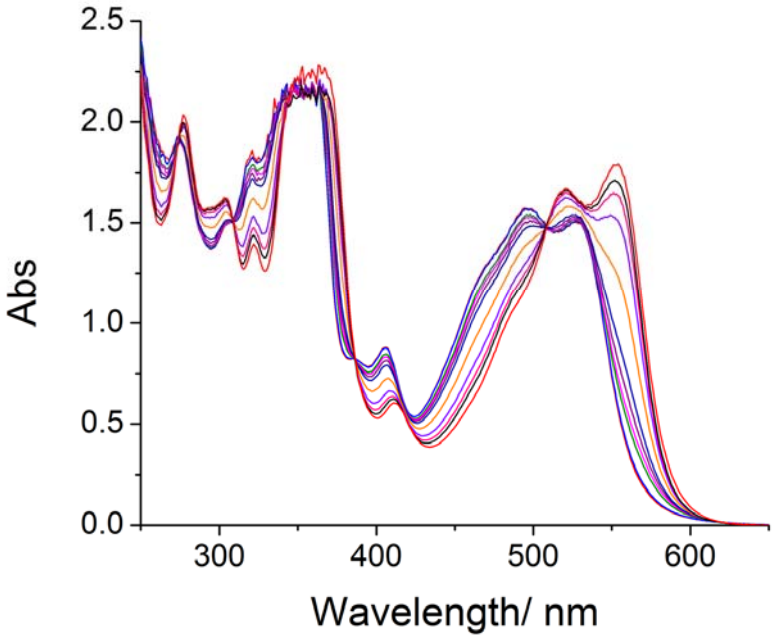


Figure S2: Spectrophotometric pH titration of a solution (c_{KCl} = 0.2 M) of compound **1-1** (87 µM) from pH 8 to 2.

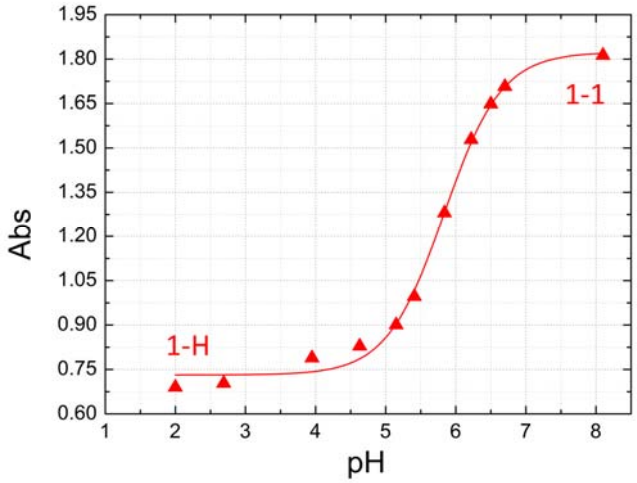


Figure S3: Changes in absorbance for the conversions of **1-H** to **1-1** ([**1-1**] = 87 μM; 551 nm) at increasing pH values.

Calculation of the equilibrium constants K_2

For the determination of the dissociation constant K_2 ($K_2 = [1]^2/[1\text{-}1]$), a series of solution with different total cobalt concentrations ($[Co]_{total}$) was prepared starting from **1-H** (in a Britton-Robinson buffer at pH 8): 174 μM, 49.3 μM, 35.1 μM, 17.4 μM, 9.79 μM and 4.44 μM.

It was analyzed as follows and the results are summarized in Table S2:

$$2[1-1] + [1] = [\mathrm{Co}]$$

$$[1] = \frac{A_{1-1(551)} - A_{x(551)}}{A_{1-1(551)} - A_{1(551)}} \times [\mathrm{Co}]$$

$$[1-1] = \frac{A_{x(551)} - A_{1(551)}}{A_{1-1(551)} - A_{1(551)}} \times [\mathrm{Co}]/2$$

$$K_2 = \frac{[1]^2}{[1-1]} = \frac{(A_{1-1(551)} - A_{x(551)})^2}{(A_{x(551)} - A_{1(551)})(A_{1-1(551)} - A_{1(551)})} \times 2[\mathrm{Co}]$$

Table S2: Calculation of the equilibrium constant K_2 at different total cobalt concentrations:

$[Co]_{total}$	17.4 μM	9.79 μM	4.44 μM
$A_{1\text{-}1(551)}$	0.174	0.0979	0.0444
$A_{1(551)}$	0.0677	0.0388	0.0175
$A_{x(551)}$	0.155	0.0836	0.0367
K_2 / M	1.35×10^{-6}	1.51×10^{-6}	1.02×10^{-6}

The K_2 values were calculated from solutions with low total cobalt concentrations (17.4 μM, 9.79 μM and 4.44 μM), for which equlibria between **1-1** and **1** was explicitly observed. The average value of K_2 is 1.29×10^{-6} M.

$A_{1\text{-}1(551)}$ and $A_{1(551)}$ are the absorbance at 551 nm of pure **1-1** and **1** at the indicated total cobalt concentration and have been calculated with the corresponding extinction coefficients. The extinction coefficient of **1-1** is ε (551 nm) = 2.00×10^{4} M^{-1} cm^{-1} and has been derived from the spectrum of **1-1** (87 μM). It is reasonable to assume that the extinction coefficient of **1** is the same as for **1-H** at a wavelength above 250 nm. The imidazole base in either the protonated or deprotonated form has no UV/Vis absorbance above 250 nm and does not affect the π to π* transitions of the corrin ring. The extinction coefficient of **1** is ε (551 nm) = 3.96×10^{3} M^{-1} cm^{-1}.

$A_{x(551)}$ represents the actual absorbance at 551 nm at the indicated concentration.

References:

[S1] J. Hill, J. Pratt, in *The Chemistry of Vitamin B12. Part I. the Valency and Spectrum of the Coenzyme*, J Chem Soc (London), **1964**, p.46.

[S2] G. Müller, O. Müller, *Z. Naturforsch.* **1966**, *21b*, 1159.

[S3] K. Zhou, F. Zelder, *Eur. J. Inorg. Chem.* **2011**, 53-57.