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Supporting Information

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Nitroxylcob(III)alamin: Synthesis and X-ray Structural Characterization

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Experimental Section

General Procedures and Chemicals. Hydroxycobalamin hydrochloride (HOCbl•HCl; stated purity by manufacturer is = 96%; the aromatic region of the ^{1}H NMR spectrum shows it contains at least ~5% impurities[1]) was purchased from Fluka BioChemica. The percentage of water in HOCbl•HCl (•nH_2O) (batch-dependent; typically 10-15%), was taken into account when calculating the amount of HOCbl•HCl used in the syntheses, and was determined by converting HOCbl•HCl to dicyanocobalamin, $(CN)_2Cbl^-$ (0.10 M KCN, pH 10.0, $\varepsilon_{368} = 3.04 \times 10^4$ M^{-1} cm^{-1} [2]). 2-(N,N-Diethylamino)-diazenolate 2-oxide (DEA-NONOate, Na^+ salt, crystalline) and TES buffer (≥ 99%) were purchased from Sigma; the former was handled according to manufacturer's recommendations. All chemicals were used without further purification. Water was purified using a Barnstead Nanopure Diamond water purification system and/or HPLC grade water was used.

All solutions used for the synthesis and characterization of NOCbl were degassed by at least three freeze-pump-thaw cycles under argon using standard Schlenck techniques. Syntheses were carried out in a MBRAUN Labmaster 130(1250/78) glove box operating under an argon atmosphere. Measurements of pH were made in the glove box at room temperature with a Corning Model 445 pH meter equipped with a Mettler-Toledo Inlab 423 electrode. The electrode was filled with 3 M KCl/saturated AgCl solution, pH 7.0, and standardized with standard BDH buffer solutions at pH 4.01 and 6.98.

UV-visible spectra were recorded on a Cary 5000 spectrophotometer equipped with a thermostatted cell holder, operating with WinUV Bio software (version 3.00). ^{1}H NMR spectra were recorded on a Varian Inova 500 MHz spectrometer equipped with a 5 mm thermostatted (22.0 ± 0.5 °C) probe. Solutions were prepared in D_2O and TSP (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, Na^+ salt) used as an internal reference. J. Young NMR tubes (Wilmad, 535-JY-7) were utilized and samples were allowed to equilibrate for 30 min before recording of spectra commenced.

Synthesis of Nitroxylcobalamin. A freshly prepared anaerobic solution of DEA-NONOate (Na^+ salt, 25.1 mg, 2.5 equiv.) in NaOH (10 mM) was added quickly to an anaerobic solution of HOCbl•HCl (100.6 mg) dissolved in TES buffer (0.10 M, 1 mL, pH 7.4). The resultant pH was 8.9. The product solution was shaken gently to ensure complete mixing and the reaction left to proceed at room temperature for 3 h. Formation of the desired product was checked by UV-vis spectroscopy. The product was precipitated by drop-wise addition to cold acetone (20 mL, -20 °C), filtered and dried under vacuum (2×10^{-2} mbar) overnight at 25 °C. Both the synthesis and handling of the final product were carried out inside a glove box under an argon atmosphere. Yield (two independent syntheses): 85 and 84%. The purity assessed by ^{1}H NMR spectroscopy was 98 ± 2 % (see Figure S1). The percentage of non-cobalamin impurities in the product was determined by converting NOCbl to $(CN)_2Cbl^-$ (0.10 M KCN, pH 10.0, $\varepsilon_{368} = 3.04 \times 10^4$ M^{-1} cm^{-1} [2]), and found to be 5 ± 3%. Extinction coefficients were also determined for NOCbl (in H_2O, pH 7.5; $e_{259} = 19.9$ mM^{-1} cm^{-1}, $e_{314} = 14.4$ mM^{-1} cm^{-1} and $e_{478} = 6.91$ mM^{-1} cm^{-1}). The solid product is stable for at least 2 months in a desiccator, but decomposes if stored in the presence of atmospheric moisture. Exposure of 3 mL of a 50 μM solution of NOCbl to air resulted in a rapid conversion of NOCbl to NO_2Cbl. UV-visible spectroscopy showed that complete decomposition of NOCbl occurs in less than 5 min.

X-ray Diffraction Studies. HOCbl•HCl (100 mg) was reacted with DEA-NONOate (Na^+ salt, 32 mg) in TES buffer (1.00 mL, 0.10 M, pH 7.4) in a 10 mL glass vial under anaerobic conditions. The NOCbl product was precipitated by drop-wise addition to cold (-20 ^oC) anaerobic acetone, and anaerobic acetone added to the remaining product in the vial. Small orange crystals of NOCbl grew after 2 days in the vial. The crystals were stored in an argon atmosphere at room temperature until data collection.

A crystal of NOCbl (~0.1 x 0.1 x 0.3 mm) was mounted under paraffin oil in a nylon loop and flash frozen in liquid nitrogen. Diffraction experiments were carried out on beamline BL9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL). Data were collected at 100 K on a MarMosaic 325 CCD detector using X-rays produced by a 16 pole wiggler insertion device, with a wavelength of 0.82653 Å (15000 eV) from a liquid nitrogen cooled double Si(111) crystal monochromator. A data set was collected consisting of 120 1° images with a crystal to detector distance of 95 mm and an exposure time of 15 s. The data were processed with the program XDS[3] and scaled together with the program XSCALE.[3] Bijvoet pairs were not merged and no absorption correction was applied. A total of 39700 reflections were measured to a nominal resolution of 0.74 Å, resulting in a final unique dataset of 17159 reflections with a merging R-factor of 0.035.

The structure was solved by Patterson methods to locate the cobalt, phosphorus and sulfur atoms, then the lighter atoms located by difference Fourier synthesis, as implemented in the program SHELXS.[4] The structure was refined by full matrix least-squares methods using the program SHELXL[4] using data from 100 to 0.8 Å resolution (15872 unique reflections, 94.6% of the expected number of reflections in this range). All non-hydrogen atoms were refined with anisotropic thermal parameters and hydrogen atoms were added in idealized positions and refined in riding positions. A correction for the anomalous scattering from cobalt at 15000 eV was applied during refinement. Additional difference electron density peaks were modeled as water molecules with site occupancies of 1.0. The oxygen atom of the NO ligand was observed in three distinct positions, and the site occupancies of these atoms were refined. The final crystallographic R factor, R1 was 0.0964 for 13916 reflections with $F_o > 4\sigma_F$. Additional data collection and refinement statistics are given in Table S1.

Measurement of the Co(III) absorption spectrum. The Co(III) absorption edge for NOCbl was measured on SSRL beamline BL9-2 from the same frozen crystal used for X-ray diffraction data collection ("irradiated NOCbl"). The spectrum was collected in fluorescence mode between 7500 and 7900 eV using a Canberra/Eurisys Si drift detector with a total acquisition time of 440 s. The spectrum was normalized by dividing the sample fluorescence at each point by the sample fluorescence at the inflection point of the first EXAFS peak (7773 eV). A similar absorption spectrum was collected from a second freshly mounted crystal which had never been exposed to X-rays ("unirradiated NOCbl") and treated in an identical manner as the irradiated sample.

Determination of the Threshold Energy of the XAS Spectrum for NOCbl. The first derivative of the spectrum (see inset to Figure 2) was calculated using the program AUTOCHOOCH[5] which is based on the Kramers-Kronig transformation algorithm. The inflection point is taken as the minimum of this transform and is measured in electrons. The inflection point, or threshold energy of the absorption edge, is 7720.8 eV for the unirradiated NOCbl (corresponding to a dispersive component of the cobalt anomalous scatter (f') of -8.60 electrons) and 7720.6 eV for the irradiated sample. It has previously been suggested that the threshold energy is correlated with the oxidation state of the cobalt, and even though the energy of the NOCbl complex is only slightly lower than the threshold energy reported for other Co(III)Cbl systems where the measured values range from 7721.0 to 7723.5 eV,[6, 7] the position of the inflection point is apparently not a very reliable indicator of the oxidation state since the values obtained for Cbl(I) (7721.0 eV) and Cbl(II) (7722.0 eV) also fall within this range of energies.

Figure S1. ^{1}H NMR spectrum of the aromatic region of NOCbl (~1.04 x 10^{-2} M) re-dissolved in anaerobic TES buffer (0.010 M, pD 7.4) in D_2O: δ = 7.44, 7.19, 6.78, 6.35 and 6.26 ppm, in agreement with literature values.[8,9] Small signals (~2%) arising from impurities in the reactant HOCbl•HCl are present at 6.71, 6.17 and 6.06 ppm.

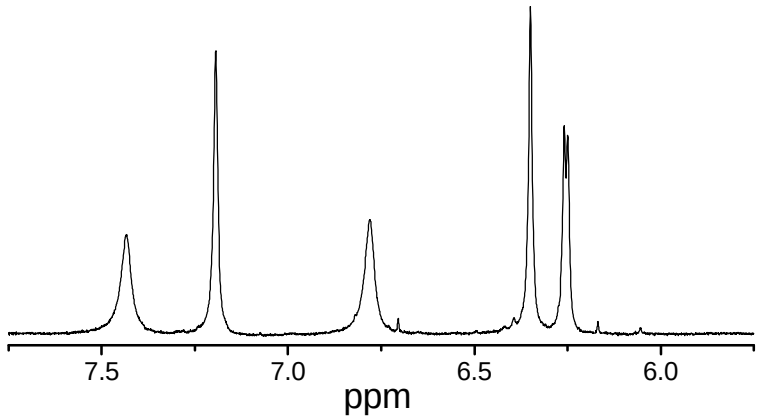
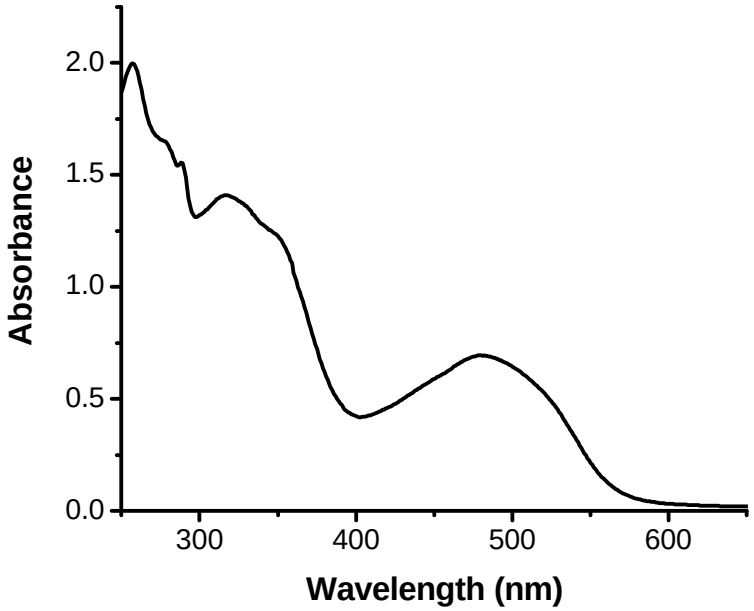


Figure S2. UV-visible spectrum of NOCbl (1.00 x 10^{-4} M) in anaerobic 0.10 M TES buffer, pH 7.4, 25 ^{o}C. Wavelength maxima occur at 257, 318, 350 (shoulder) and 479 nm.



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