Supplementary Material (ESI) for Chemical Communications




**Supporting Information for**

# Vanadium-Vitamin $B_{12}$ Bioconjugates as Potential Therapeutics for Treating Diabetes

Riya Mukherjee, Edward G. Donnay, Michal A. Radomski, Catherine Miller, Duane A. Redfern, Arne Gericke, Derek S. Damron and Nicola E. Brasch

## Experimental Section

Hydroxycobalamin hydrochloride was purchased from AIDP Inc. (City of Industry, CA). The calculated number of moles of HOCbl•HCl used in the syntheses was corrected for the percentage of $H_2O$ (batch-dependent, typically 10-15%). $C_{18}$ silica gel resin for flash chromatography was purchased from Sorbent Technologies and $C_{18}$ solid phase extraction (SPE) columns were purchased from Varian. All other chemicals were purchased from Sigma-Aldrich, VWR or Fisher Scientific and were used as received. Water was purified using a Barnstead Nanopure Diamond water purification system.

For experiments conducted under anaerobic conditions, solutions were degassed on a Schlenk line using standard Schlenk techniques using three freeze-pump-thaw cycles and under argon, or by purging with argon for at least 12 hr. Air-free manipulations were carried out in an MBRAUN Labmaster 130(1250/78) glovebox.

$^1$H NMR spectra were recorded on a Varian Inova 500 MHz or a Bruker Avance 400 MHz spectrometer equipped with a 5 mm probe. Typically the HDO peak was saturated; saturation delays were 3-4 s. $^{51}$V NMR spectra were recorded on the Bruker instrument operating at 105.246 MHz with a pulse width of 8.25 μs, an acquisition time of 0.218 s, and a delay time of 0.5 s. Solutions for NMR measurements were prepared in deuterated solvents. $^1$H NMR spectra of ligands were internally referenced to TMS (0 ppm). $^1$H NMR spectra of Cbls were internally referenced to TSP (0 ppm). $^{51}$V NMR spectra were referenced using a sample of 0.0100 M $NaVO_3$ in 2.0 M NaOH (-541.2 ppm).

UV-visible spectra were recorded on a Cary 5000 spectrophotometer equipped with a thermostatted cell changer (25.0 ± 0.1 °C), operating with WinUV Bio software (version 3.00). GC-MS analyses were conducted on a Thermo-Electron GC-MS using the following method: splitless injection, initial temperature at 100$^o$C ramped to 300 $^o$C over 20 min, 300 $^o$C held for 20 min. ESI-MS were recorded on a ThermoFinnigan LCQDuo ion trap mass spectrometer at the mass spectrometry facility in the Department of Chemistry, Colorado State University. APCI-MS were recorded using a Bruker Esquire 3000 plus LC mass spectrometer.

pH measurements were recorded at room temperature on an Orion 440 pH meter using a Wilmad pH electrode calibrated using Fisher 7.00 and 4.00 standard buffers.

## General Procedures

To prevent decomposition, alkylcobalamins were synthesized and used under red light conditions and stored in the absence of light. Removal of solvents was achieved by rotary evaporation under reduced pressure (~5 mbar) and at temperatures < 35 $^o$C.

## Syntheses

**3-Benzyloxy-2-methylpyran-4-one (4).** Ligand **4** was synthesized according to a literature procedure.[1] Maltol (10.0 g, 79.4 mmol) and NaOH (7.94 mL, 11.0 M in $H_2O$) were added to $CH_3OH$ (80 mL) and the solution brought to reflux. Benzyl chloride (9.13 mL, 79.4 mmol) was added slowly. The reaction was refluxed for 18 hr and filtered. The filtrate was dried via rotary evaporation to yield a brown oil. $H_2O$ (32 mL) was added and the product extracted with $CH_2Cl_2$ (3 x 24 mL). The organic fraction was washed with NaOH (5% *wt*/*wt*, 2 x 32 mL) and $H_2O$ (1 x 32 mL). The $CH_2Cl_2$ was removed by rotary evaporation to yield a brown viscous oil. Yield: 14.2 g (82%). $^1$H NMR ($CDCl_3$): δ 7.59 (1H, d, O-C**H**), 7.34 (5H, m, $CH_2$-Ar**H**), 6.36 (1H, s, C**H**), 5.16 (2H, s, C**H$_2$**-Ar), 2.09 (3H, s, C**H$_3$**) ppm. APCI-MS$^+$, m/z: 217 ± 1 (calcd. for $[C_{13}O_3H_{12} + H]^+ = 217.1$).

**3-Benzyloxy-1-(3-chloropropyl)-2-methyl-1*H*-pyridin-4-one (5).** Ligand **5** was prepared by adapting a literature procedure.[1] Ligand **4** (12.1 g, 56.1 mmol), 3-chloropropylamine hydrochloride (10.9 g, 83.8 mmol), NaOH (47 mL, 2 M), and $H_2O$ (63 mL) were added to aqueous ethanol (95%, 100 mL). The solution was refluxed for ~18 hr, after which the solution was adjusted to pH ~1 using conc. HCl. The solvent was removed by rotary evaporation and transferred to a separating funnel using $H_2O$ (~80 mL). The resulting suspension was washed with diethyl ether (3 x 100 mL) to remove unreacted starting material (7.52 g). The aqueous fraction was adjusted to pH ~7 with NaOH (10 M) and the product extracted into $CH_2Cl_2$ (2 x 100 mL). The combined organic fractions were dried by rotary evaporation to yield a yellow oil. Addition of ethanol (~ 25 mL) and conc. HCl (4 mL) followed by removal of the solvent by rotary evaporation did not yield the expected solid HCl salt. A dark oil was instead obtained, which was used directly in the next step. Yield: 6.23 g (38%). $^1$H NMR ($CDCl_3$): δ 7.35 (5H, m, $CH_2$--Ar**H**), 7.23 (1H, d, N-C**H**), 6.42 (1H, d, C**H**), 5.24 (2H, s, C**H$_2$**-Ar), 3.95 (2H, t, C**H$_2$**-Cl), 3.50 (2H, t, C**H$_2$**-N), 2.10 (3H, s, C**H$_3$**), 2.07 (2H, m, $CH_2$-C**H$_2$**-$CH_2$) ppm. APCI-MS$^+$, m/z: 313 ± 1 (calcd for $[C_{16}O_2NH_{18}Cl + Na]^+ = 314.1$).

**1-(3-Chloropropyl)-3-hydroxy-2-methyl-1*H*-pyridin-4-one (6).** Ligand **6** was prepared by adapting a literature procedure.[1] To aqueous ethanol (83%, 120 mL) was added crude **5** (6.23 g, 19.9 mmol) and Pd/C (5%, 575 mg). The head space was purged with argon and the solution was hydrogenated (by bubbling $H_2$ through the reaction mixture) for 5 hr. The resulting solution was evaporated to yield a brown solid which was recrystallized from ethanol. Yield: 1.99 g (46%). $^1$H NMR (DMSO-*d*6): δ 10.53 (s-broad, O**H**), 8.23 (1H, d, N-C**H**), 7.33 (1H, s, C**H**), 4.45 (2H, t, C**H$_2$**-Cl), 3.73 (2H, t, C**H$_2$**-N), 2.54 (3H, s, C**H$_3$**), 2.24 (2H, m, $CH_2$-C**H$_2$**-$CH_2$) ppm. APCI-MS$^+$, m/z: 202 ± 1 (calcd for $[C_9H_{12}O_2NCl + H]^+ = 202.1$).

**3-(3-Hydroxy-2-methyl-1*H*-pyridin-4-one)propylcobalamin (1).** An anaerobic solution of HOCbl•HCl (501 mg, 0.326 mmol (corrected for 10% $H_2O$), in 10 mL degassed $H_2O$) was reduced to cob(I)alamin using $NaBH_4$ (73.6 mg, 1.94 mmol, in 5 mL anaerobic $H_2O$). Anaerobic acetone (1 mL) was added to decompose the excess borohydride and an anaerobic solution of ligand **6** (95.6 mg**,** 0.474 mmol, in 2 mL degassed $H_2O$, adjusted to pH ~ 9 with 2 M NaOH) added drop-wise with stirring. After 3 hr the solvent removed by rotary evaporation. The product was desalted on a $C_{18}$ SPE column and purified on a Sephadex-C25 column (see below). Yield: 0.288 g (58%); Purity: 95 ± 2 % by conversion of **1** to $(CN)_2Cbl^-$.[2] Examination of the aromatic region of the $^1$H NMR spectrum of Cbls is a useful way to determine the % of other Cbls in the product.[3] $^1$H NMR ($D_2O$, TES buffer, pD 7.4): 7.37 (1H, d), 7.18 (1H, s), 6.92 (1H,

s), 6.36 (1H, d), 6.26 (1H, d), 6.23 (1H, s), 6.00 (1H, s) ppm; purity (with respect to Cbl content) ≥ 98%. UV-Vis $\lambda_{max}$: 319, 339 (shoulder), 377, 434 and 523 nm, ES-MS$^+$, m/z: 1495.7 (calcd for [**1** + H]$^+$, [$C_9H_{12}O_2N$-Cbl + H]$^+$, $C_{71}H_{101}CoN_{14}O_{16}P$ = 1495.7); 1517.6 (calcd for **1** + Na]$^+$, $C_{71}H_{100}CoN_{14}O_{16}P$ = 1517.7); 748.5 (calcd for [**1** + 2H]$^{2+}$, $C_{71}H_{102}CoN_{14}O_{16}P$ = 748.4); 759.5 (calcd for [**1** + H + Na]$^{2+}$, $C_{71}H_{101}CoN_{14}O_{16}PNa$ = 759.4); 770.4 (calcd for [**1** + 2Na]$^{2+}$, $C_{71}H_{100}CoN_{14}O_{16}PNa_2$ = 770.3); 1351.9 (calcd for [Cbl + Na]$^+$, $C_{62}H_{88}CoN_{13}O_{14}PNa$ = 1351.5); 1329.7 (calcd for [Cbl + H]$^+$, $C_{62}H_{89}CoN_{13}O_{14}P$ = 1329.6); 665.5 (calcd for [Cbl + 2H]$^{2+}$, $C_{62}H_{90}CoN_{13}O_{14}P$ = 665.3); 687.7 (calcd for [Cbl + 2Na]$^{2+}$, $C_{62}H_{88}CoN_{13}O_{14}PNa_2$ = 687.3); 676.5 (calcd for [Cbl + H + Na]$^{2+}$, $C_{62}H_{89}CoN_{13}O_{14}PNa$ = 676.3). ES-MS$^-$, m/z: 1529.7 (calcd for [**1** + Cl]$^-$, $C_{71}H_{100}CoN_{14}O_{16}PCl$ = 1529.6); 1381.7 (calcd for [Cbl + Cl + $H_2O$]$^-$, $C_{62}H_{90}CoN_{13}O_{15}PCl$ = 1381.5); 1363.9 (calcd for [Cbl + Cl]$^-$, $C_{62}H_{88}CoN_{13}O_{14}PCl$ = 1363.5); 1328.9 (calcd for [Cbl - H]$^-$, $C_{62}H_{87}CoN_{13}O_{14}P$ = 1327.6).

*Desalting with a $C_{18}$ SPE Column.* The $C_{18}$ SPE cartridge (10 g, 60 mL barrel) was prepared by washing the column with $H_2O$ (~ 60 mL), $CH_3CN$ (~ 60 mL), and $H_2O$ (~ 120 mL). The crude Cbl product was redissolved in a minimal amount of $H_2O$ and loaded onto the column slowly (~30 min). The product was washed with $H_2O$ and eluted slowly (~ 45 min – 1 hr) using $CH_3OH$ (~50 mL), $CH_3CN$ (~50 mL), 1:1 $CH_3CN$:$H_2O$ (~50 mL), and 1:1 $CH_3OH$:$H_2O$ (~50 mL).

*Purification by Sephadex Ion Exchange Chromatography.* Preparation of a SP Sephadex C-25 ion exchange column (40 – 120 mm, $Na^+$ form, 30 x 3 cm) was accomplished by swelling the resin in $H_2O$, activating the column with NaCl (0.1 M, ~ 500 mL), and washing with $H_2O$ (~ 1 L) until the elutent tested chloride-free with $AgNO_3$ (~ 0.1 mL, 0.1% *wt*/*wt*). The product mixture was dissolved in a minimal amount of $H_2O$ and loaded on the column. A migrating red band was eluted with $H_2O$ (~ 1 L) over 45 min. Elution of the remaining aquacobalamin was accomplished with NaOH (0.5 M, ~ 500 mL) followed by washing with $H_2O$ (~ 2 L).

**Figure S1.** Aromatic region of the $^1$H NMR spectrum of **1** in aqueous buffer ($D_2O$, TES), pD =7.4 at 24 °C. Seven signals are observed at 7.37(d), 7.18, 6.92, 6.36(d), 6.26(d), 6.23 and 6.00 ppm.

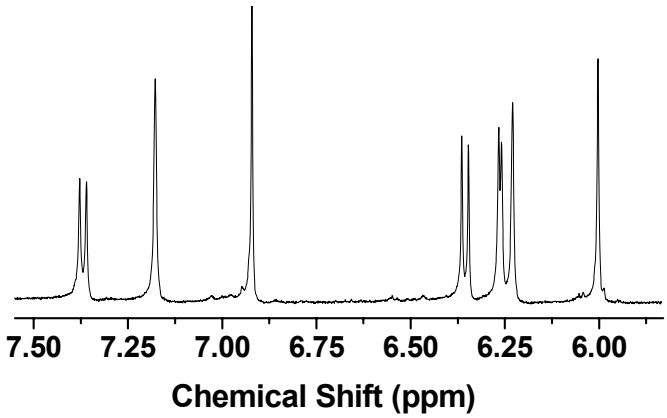

**Figure S2.** UV-Vis spectrum of **1** in aqueous buffer ($D_2O$, TES), pD =7.4 at 24 °C. $\lambda_{max}$ = 319, 339 (shoulder), 377, 434 and 523 nm.

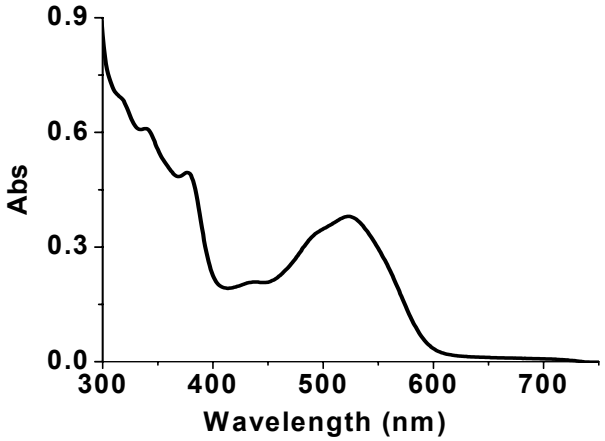


**Figure S3.** $^{51}V$ NMR spectrum of **1** ($6.4 \times 10^{-6}$ mol) with 1.0 equiv. of $NaVO_3$ in $D_2O$, pD = 8.9 at 24 °C. The broad peak at -506 ppm is attributed to $VO_2L$. Other peaks at -567 (probably $(H)V_4O_{13}^{5-/6-}$), -573 ($H_2V_2O_7^{2-}$) and -578 ppm ($V_4O_{12}^{4-}$)) are associated with V(V)(aq) species.[4,5] The peak at -553 ppm has, to our knowledge, not yet been assigned. It was, however, also present in an aqueous solution of $NaVO_3$ (0.0025 M, pD = 8.7) and is therefore also associated with a V(V)(aq) species.

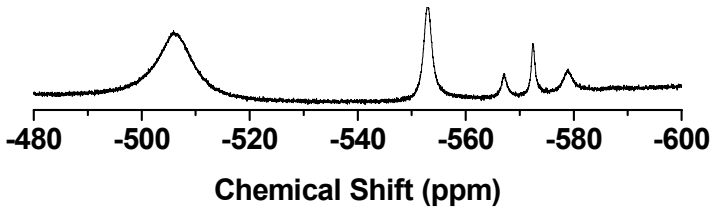


**Figure S4.** $^{51}V$ NMR spectrum of **1** ($4.2 \times 10^{-6}$ mol) and 3 equiv. of $NaVO_3$ in $D_2O$, pD = 8.7 at 24 °C. The broad peak at -506 ppm is attributed to $VO_2L$. The peak at -586 ppm can be assigned to $V_5O_{15}^{5-}$.[4,5] See caption to previous figure for assignments of the peaks at -567, -573, -578, and -555 ppm.

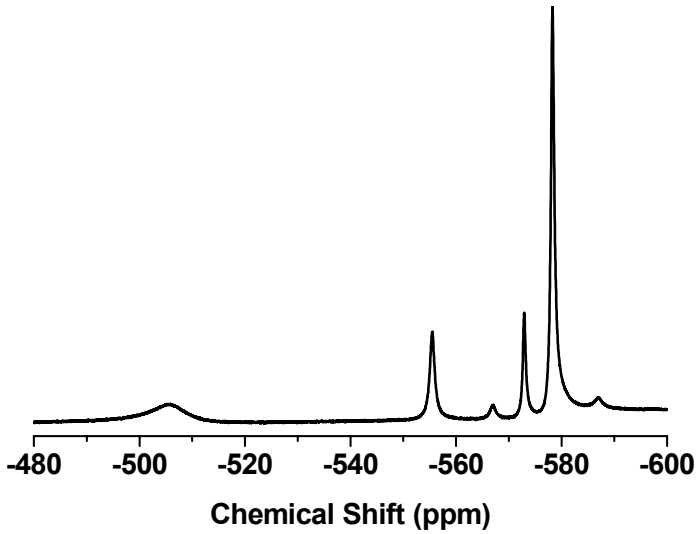

**Figure S5**. Aromatic region of the $^1$H NMR spectrum of **1** ($4.2 \times 10^{-6}$ mol) with 3.0 equiv. of $NaVO_3$ in $D_2O$, pD = 8.7 at 24 °C. Signals attributable to the protons of the Cbl macrocycle of **2**+**3** overlap at 7.17, 6.26 (d), 6.23 and 6.02 ppm. Peaks at 7.42(d), 6.92 and 6.50(d) are assigned to **2**. Peaks at 7.24(d), 6.94 and 6.20(d) are assigned to **3**.

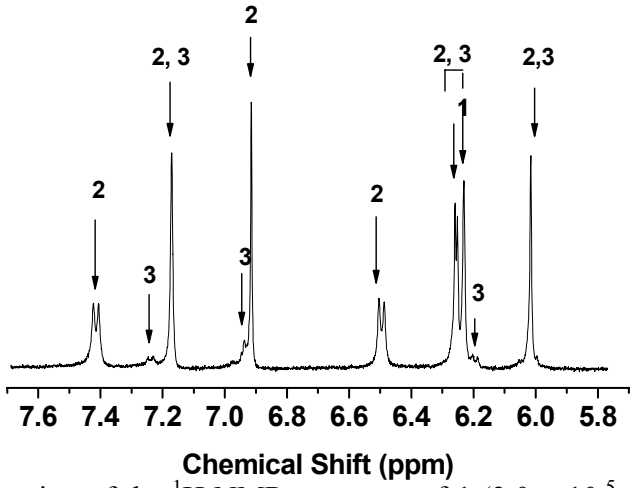


**Figure S6.** Aromatic region of the $^1$H NMR spectrum of **1** ($3.0 \times 10^{-5}$ mol) with 0.20 equiv. of $NaVO_3$ in $D_2O$, pD = 8.9 at 24 °C. Signals attributable to the protons of the Cbl macrocycle of **1**+**3** overlap at 7.18, 6.26 (d), 6.23 ppm. Peaks at 7.37(d), 6.92, 6.36(d) and 6.01 ppm are assigned to **1**. Peaks at 7.25 (d), 6.94, 6.21(d) and 6.02 ppm are assigned to **3**.Very small peaks attributable to **2** are observed at 7.42(d) and 6.50(d) ppm.

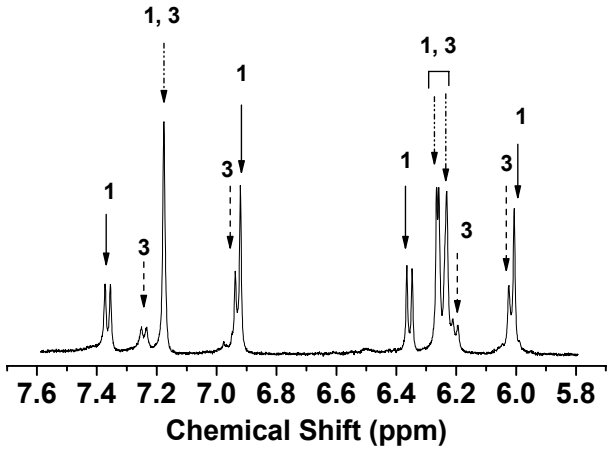


**Figure S7.** Aromatic region of the $^1$H NMR spectrum of **1** ($6.4 \times 10^{-6}$ mol) with 1.0 equiv. of $NaVO_3$ in $D_2O$, pD =7.4 at 24 °C. Ten signals are obtained at 7.42(d), 7.26(d), 7.17, 6.94, 6.92, 6.50(d), 6.37, 6.26(d), 6.23 and 6.01 ppm.

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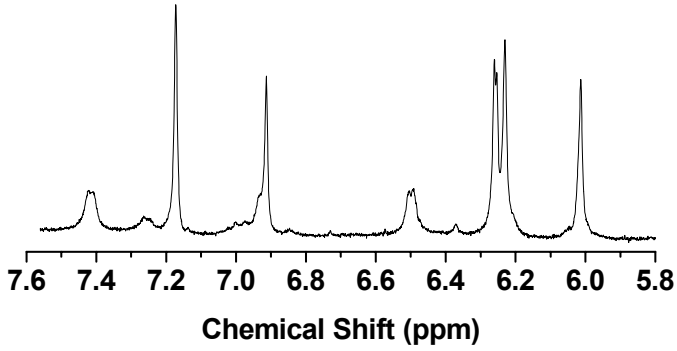
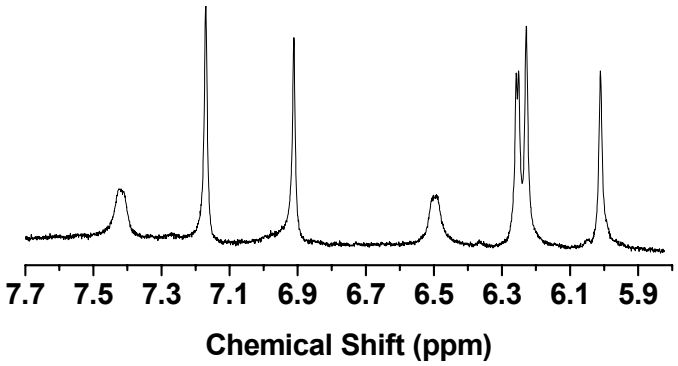


**Figure S8.** Aromatic region of the $^1$H NMR spectrum of **1** ($7.8 \times 10^{-6}$ mol) with 3.0 equiv. of $NaVO_3$ in $D_2O$, pD =7.4 at 24 °C. Seven main peaks are obtained at 7.42(d), 7.17, 6.91, 6.50(d), 6.26(d), 6.23 and 6.01 ppm. Three peaks are found with very small intensities at 7.26(d), 6.94 and 6.37 ppm.




**Attempted Separation of 2 and 3 by Chromatography.**

(a) Amberlite XAD-2 column: Upon the addition of 3.0 equiv. $NaVO_3$ to **1**, the *mono* species **2** is the predominate species formed, as shown by $^1$H NMR spectroscopy (Fig S3) Hence, a mixture of **2** and excess $NaVO_3$ was prepared by reacting **1** ($1.3 \times 10^{-5}$ mol) with 3.0 mol equiv. $NaVO_3$ ($3.9 \times 10^{-5}$ mol) and the product mixture desalted (to remove remaining excess vanadate) so as to obtain a pure sample of **2**. An Amberlite XAD-2 column (15 × 5 cm) was used and the product washed with water and eluted with 4:1 EtOH/$H_2O$. The solvent was evaporated from the eluted product by rotary evaporation at 35 ºC. The aromatic region of the $^1$H NMR spectrum of the product dissolved in $D_2O$ showed peaks corresponding to **1**, **2** and **3** in the ratio 4:1:3; hence the desired pure **2** was not obtained.

(b) Semipreparative HPLC experiments were carried out using an Alltech Alltima $C_{18}$ column (5μm, 100 Å, 10 mm × 300 mm) with a flow rate of 3mL/min under acidic and neutral conditions:

(i) A mobile phase consisting of acetic acid in $H_2O$ (0.1% v/v), pH = 3.5, **A**, and acetic acid in $CH_3CN$ (0.1% v/v), **B**, were used in the following method: 0-2 min, 83:17 A:B; 2-3 min, 83:17 to 77.5:22.5 A:B; 3-30 min, 77.5:22.5 A:B; 30-33 min 77.5:22.5 to 40:60 A:B; 33-35 min, 40:60 A:B; 35-36 min 40:60 to 83:17 A:B; 36-41 min, 83:17 A:B. All gradients were linear. A pure sample of the Cbl ligand, **1,** eluted at 11.2 min. A mixture of **2** and **3**, prepared by mixing equimolar amounts of **1** and $NaVO_3$, eluted as a single, broad peak from 10-15 min (note that Cbl standards eluted at well-separated times, each Cbl eluting within 1 min time period); hence the **2** and **3** could not be separated under these conditions.

(ii) A mobile phase consisting of phosphate buffer, pH = 7.4, **A**, and $CH_3CN$ (0.1% v/v), **B**, were used in the following method: 0-2 min, 83:17 A:B; 2-3 min 83:17 to 80.3:19.7 A:B; 3-30 min, 80.3:19.7 A:B; 30-33 min 80.3:19.7 to 40:60 A:B; 33-35 min, 40:60 A:B; 35-36 min 40:60 to 83:17 A:B; 36-41 min, 83:17 A:B. All gradients were linear. A pure sample of the Cbl ligand, **1,** eluted at 15.7 min. A mixture of **2** and **3**, prepared from an equimolar solution of **1** and $NaVO_3$ ($1.08 \times 10^{-3}$ mol), eluted as an extremely broad peak from 16-19 min (the Cbl standards eluted within 1 min); hence **2** and **3** could also not be separated under these conditions.

**Determination of the Diffusion Coefficient for complexes 2 and 3 by Pulsed-Field Gradient-Echo NMR Spectroscopy Measurements**

Determination of diffusion coefficients for complexes in solution can provide estimates of the molecular masses of these species because diffusion coefficients primarily depend upon the molecular mass and geometry of the complex. The Pulsed Field Gradient spin-echo NMR technique is a useful method to directly determine diffusion coefficients.[6-9] This technique is mainly used for liquids, where the anisotropic spin interactions are averaged by molecular motions and the NMR peaks are sharp. The decay of the echo intensity in the Stejskal-Tanner experiment[6-9] is described by

$$I = I_0 \exp[-(\gamma G)^2\delta^2(\Delta - \delta/3)D] \qquad (1)$$

where $I$ is the echo intensity for the spectral peak of interest, $I_0$ is the echo intensity at $\delta = 0$, $\gamma$ is the gyromagnetic ratio of the nuclei, $G$ is the amplitude of the two gradient pulses, $\delta$ is their duration, $\Delta$ is the interval between the gradient pulses, and $D$ is the diffusion coefficient. The experiment is performed at constant $(\Delta - \delta/3)$ by varying the amplitude of the gradient pulses G. From eq 1, it can be shown that the plot of $\ln_e(I/I_0)$ vs $[-(\gamma G)^2\delta^2(\Delta - \delta/3)]$ produces a straight line with slope $-D$. Hence, the diffusion coefficient for the molecule with a specific NMR resonance peak can be directly determined.

To validate our experimental design, we initially measured the diffusion coefficient for water. We obtained a value of $(2.58 \times 10^{-5}$ cm$^2$ s$^{-1}$, which is in excellent agreement with a literature value of $2.6 \times 10^{-5}$ cm$^2$ s$^{-1}$ for free water.[9] The peaks at $\delta = 7.42$ (d) and 7.24 (d) ppm in the $^1$H NMR spectrum of an equimolar ($4.2 \times 10^{-6}$ mol) solution of $NaVO_3$ and **1**, pD = 9.1 at 25°C, are assigned to complexes **2** and **3**, respectively (Fig. 1, main text). Figures S9 and S10 shows the plots of $\ln_e(I/I_0)$ vs $[-(\gamma G)^2\delta^2(\Delta - \delta/3)]$ for these two peaks, which give a straight line. The diffusion coefficients were calculated from the slope and found to be $(5.1 \pm 0.3) \times 10^{-6}$ cm$^2$ s$^{-1}$ and $(3.6 \pm 0.1) \times 10^{-6}$ cm$^2$ s$^{-1}$, respectively. Because the diffusion coefficients of molecules decrease with increasing size (molecular weight), it can be concluded that the molecular weight of complex **3** is significantly larger than that of complex **2**.[6] Note that the ratio of the molecular weights of complex **3** to **2** is 1.9, whereas the ratio of their diffusion coefficients is 1.4. This difference can be rationalized by consideration of not only the size but also the geometry of the hydrated complexes in solution, which also plays a key role in determining the magnitude of the measured diffusion coefficient.

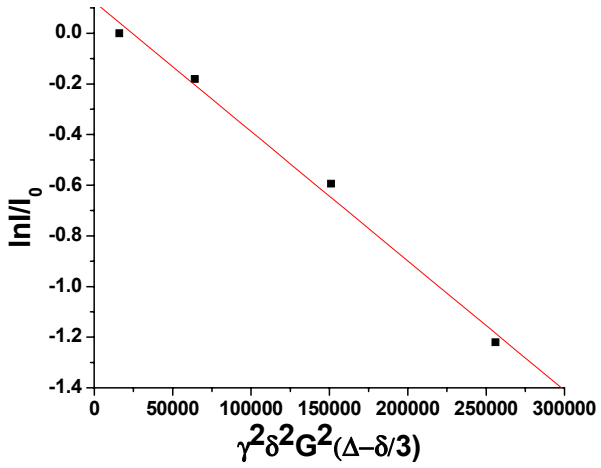



**Figure S9.** Plot of $\ln_e(I/I_0)$ vs $[-(\gamma G)^2\delta^2(\Delta - \delta/3)]$ for complex **2**.

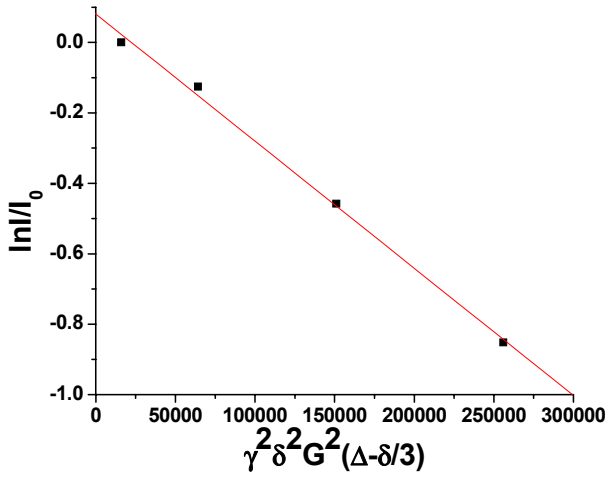



**Figure S10.** Plot of $\ln_e(I/I_0)$ vs $[-(\gamma G)^2\delta^2(\Delta - \delta/3)]$ for complex **3**.

**FTIR Spectroscopy experiments.**

The binding of $NaVO_3$ to **1** was studied by FTIR spectroscopy in $H_2O$ and $D_2O$ at pH (pD) 8.7 ± 0.2. Vibrational modes corresponding to the C=O and C=C stretches of the V(V) complexes of dmpp occur in the 1625-1450 $cm^{-1}$ region in the solid state.27 Upon the addition of increasing amounts (0.20-3.0 equiv.) of $NaVO_3$ to **1**, changes in the 1600-1500 $cm^{-1}$ region of the IR spectrum were clearly observable; however a detailed analysis of the spectral changes was not possible due to the low signal-to-noise ratio (**1** has limited solubility in aqueous solution; the concentration of **1** used approached its saturation limit) combined with significant overlap of the bands of interest with those of the solvent. Analysis was further hampered by the fact that the normal modes of vibrations of **1** are highly coupled and the expectation that the spectra contain contributions from at least three components (**1**-**3**). However, the observed spectral changes were consistent with the $^1H$ NMR spectroscopy data.

***In Vivo* Blood Glucose-Lowering Properties in the STZ-Rat Model for Type I Diabetes**

Investigation of the blood glucose-lowering ability of complex **1**, $NaVO_3$ and an equimolar solution of **1** + $NaVO_3$ were carried out in the STZ-rat model for Type I diabetes, with 3 different rats in each group. Adult, male, Sprague-Dawley rats (6 weeks old) were used for the study. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg) on day 0. Animals were maintained with free access to food and water following streptozotocin administration. Rats were administered a single tail vein injection (pH 7.0, 70 µl) of $H_2O$, 5.0 x $10^{-7}$ mol **1** in $H_2O$, 5.0 x $10^{-7}$ mol $NaVO_3$ in $H_2O$, or an equimolar (5.0 x $10^{-7}$ mol) solution of **1** + $NaVO_3$ in $H_2O$ on day 7, directly after measuring their blood glucose levels. The blood from all rats was collected from the tail vein (tail-nick procedure) and blood glucose levels were assessed using a glucometer.

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