Vanadium–vitamin B_{12} bioconjugates as potential therapeutics for treating diabetes†

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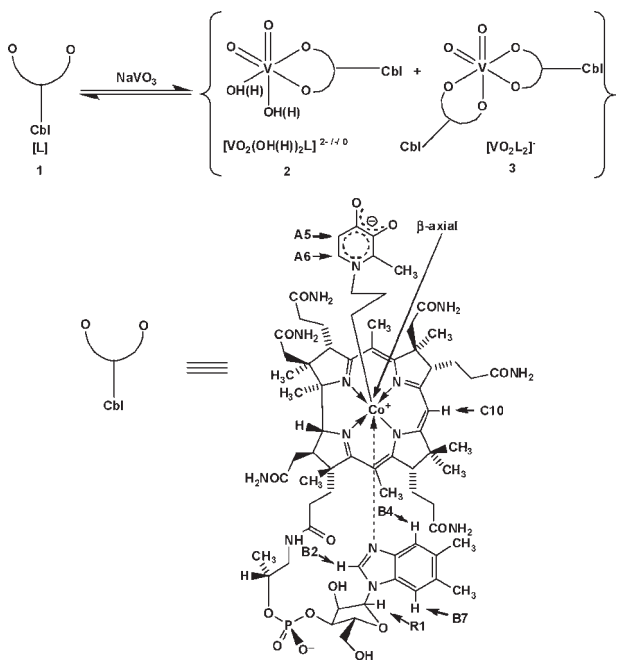
The synthesis and blood glucose lowering properties of the first vanadium–vitamin B_{12} bioconjugates are reported.

The development of vanadate (V(V)) and vanadyl (V(IV)) therapeutics as oral insulin substitutes or for co-administration with insulin for treating diabetes is an active research field.[1,2] Insulin regulates carbohydrate and lipid metabolism. Diabetes mellitus is characterized by an absolute or relative lack of insulin and/or insulin resistance, leading to hyperglycemia and serious secondary complications.[2] Vanadium complexes not only lower blood glucose levels, but also alleviate most of the symptoms attributable to this disease.[1,2] However, toxicity was a serious problem in stage I clinical trials of inorganic vanadium salts.[3] Poor intestinal absorption ($<5\%$[1]) necessitated large doses, resulting in gastrointestinal distress, dehydration and weight loss. Tissue accumulation also occurs, the consequences of which are under investigation.[1,3] Over the past decade many V(IV) and V(V) complexes with organic chelating ligands have therefore been evaluated in animal and cell models, with the aim of improving absorption and tissue uptake.[1,2] This includes porphyrin complexes,[4] complexes incorporating established antioxidants and hypoglycemic agents,[2] and vanadium-containing capsules and hydrogels.[5,6] The most promising vanadium complexes in terms of efficacy (dose-related response) are up to one order of magnitude better than inorganic vanadium salts.[7,8] Indeed, Phase I clinical trials were recently completed for a V(IV)–ethyl maltol complex.[2]

We report the synthesis and characterization of novel B_{12} conjugates of vanadium, complexes **2** and **3** (Scheme 1), potentially orally active therapeutics for the treatment of diabetes. The absorption and cellular uptake of imaging agents and drugs (including insulin) has been shown to be significantly improved by conjugation to cobalamins (Cbls = vitamin B_{12} derivatives).[9–15] 3-Hydroxy-2-methyl-1-propyl-1*H*-pyridin-4-one was used to link the Cbl and vanadium(V) center *via* the β-axial site of Cbl. Binding the drug or imaging agent to the β-axial site of the Cbl molecule has minimal effect on the binding of Cbl to B_{12} transport proteins.[16] The binding of the closely related ligand 3-hydroxy-1,2-dimethyl-1*H*-pyridin-4-one (dmpp) to aqueous V(IV) and V(V) to form mono- or bis-dmpp complexes is well characterized.[17–24] Two bidentate dmpp ligands bind strongly to V(IV) (log K_1 = 12.18, log K_2 = 10.65[17]) and V(V) (log K_1 = 10.48, log K_2 = 5.25[18,19]) centers. V(V) complexes are reduced to V(IV) inside cells[2] and V(IV)(dmpp)$_2$ has promising insulin-enhancing properties.[21,25] 3-Hydroxy-4-pyridinones have also been used in Fe and Al overload chelation therapy and for administering Ga- and In-based radiopharmaceuticals.[20]

The alkylcobalamin 3-(3-hydroxy-2-methyl-1*H*-pyridin-4-one)-propylcobalamin (**1**) was synthesized by reacting cob(I)alamin with 1-(3-chloropropyl)-3-hydroxy-2-methyl-1*H*-pyridin-4-one using standard reductive alkylation procedures.[26] Complex **1** (58% yield) was purified by ion exchange chromatography and found to be 95 ± 2% pure. The percentage of other Cbls in the product was determined to be ≤2% by ^{1}H NMR spectroscopy (Fig. S1, ESI†). Seven signals are observed in the aromatic region



Scheme 1 Structures of **1** and the corresponding mono- (**2**) and bis- (**3**) ligated vanadium–B_{12} conjugates.

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† Electronic supplementary information (ESI) available: Synthesis, purification, ^{1}H NMR and UV-Vis spectra for **1**, ^{1}H and ^{51}V NMR data at varying ratios of **1** to $NaVO_3$, experimental details on the attempted purification of **2** and **3**, FTIR experiments and the determination of diffusion coefficients. See DOI: 10.1039/b806598e

of the ^{1}H NMR spectrum of **1** at 7.37(d), 7.18, 6.92, 6.36(d), 6.26(d), 6.23 and 6.00 ppm (pD 7.4), attributable to the A5 (7.37) and A6 (6.36) protons of the hydroxypyridinone ring and the B2, B4, B7, R1 and C10 protons of the Cbl macrocycle (see Scheme 1 for labeling). Complex **1** was also characterized by electrospray mass spectrometry (+ve and −ve modes; 1495.7 (calcd for $[\mathbf{1} + H]^+$, $[C_9H_{12}O_2N\text{–Cbl} + H]^+ = 1495.7$; peaks also observed for $[\mathbf{1} + Na]^+$, $[\mathbf{1} + 2(H/Na)]^{2+}$ and $[\mathbf{1} + Cl]^-$) and by UV-visible spectroscopy (λ_{max} = 319, 339(shoulder), 377, 434 and 523 nm, Fig. S2, ESI†). The presence of the light-sensitive Co–C bond was confirmed by exposing an aqueous solution of **1** to light; **1** decomposes cleanly to give aquacobalamin (λ_{max} at 350, 411 and 523 nm[27]), with isosbestic points at 332, 368, 457, 535 and 603 nm.

The binding of sodium metavanadate ($NaVO_3$) to complex **1** was investigated by NMR spectroscopy. Fig. 1 gives the ^{1}H NMR spectrum obtained upon reacting 1.0 mol equiv. $NaVO_3$ with **1** (pD = 9.1). The A5 and A6 proton signals of the hydroxypyridinone ring of complex **1** shift significantly. Two new species are formed, labeled **2** and **3**, in agreement with previous studies which show that mono- ($VO_2(OH/H)_2L$, L = the Cbl ligand, **1**) and bis-ligated (VO_2L_2) complexes are formed upon the binding of V(v) to dmpp.[18,19] The order or rate of addition of the reactants had no effect on the products formed. The proposed structures of species **2** and **3** are given in Scheme 1.[18] The composition and structures of the mono-ligated (**2**) and the bis-ligated (**3**) complexes were assigned on the basis of MS and NMR measurements as follows: (a) ES-MS (−ve mode) of a solution of **1** and 1.0 equiv. $NaVO_3$ gave a peak with a maximum intensity at 1593.4 attributable to a $[VO_2(OH)L]^-$ adduct (peak splitting pattern in excellent agreement with a simulation for $C_{71}H_{100}CoN_{14}O_{19}PV$, with a peak maximum at 1593.6). (b) A new broad resonance was observed at −506 ppm in the ^{51}V NMR spectrum (Fig. S3 and S4, ESI†), which narrowed in line width (from ~900 to 400 Hz) upon increasing the temperature from 24 to 65 °C. A similar broad resonance (−502 ppm, pH 7.5) was observed for $VO_2(OH/H)_2$(dmpp).[18] (c) If indeed **3** is ligated by two Cbl ligands, the ratio of **2** : **3** is expected to increase when higher equiv. of $NaVO_3$ are added to **1**. ^{1}H NMR spectroscopy measurements confirmed that this is indeed the case. With 3.0 mol equiv. of $NaVO_3$, the amount of VO_2L_2 (**3**) is almost negligible (Fig. S5, ESI†), whereas with 0.20 equiv. $NaVO_3$, **3** is the predominant vanadium–B_{12} complex in solution (Fig. S6, ESI†). (d) Although ES-MS evidence for the formation of **3** could not be obtained, presumably due to its size and hence lower volatility, measurements of the diffusion coefficients of **2** and **3** using pulsed-field gradient-echo NMR spectroscopy methods showed that the molecular weight of **3** is clearly much larger than **2** (diffusion coefficients of **2** and **3** were found to be $(5.1 \pm 0.3) \times 10^{-6}$ and $(3.6 \pm 0.1) \times 10^{-6}$ cm^2 s^{-1}, respectively, consistent with the proposed structures). Finally, (e) although a ^{51}V NMR spectroscopy resonance for **3** (**1** + 0.20 equiv. $NaVO_3$) was not observed even after collecting data for 24 h (24 or 65 °C), this can be rationalized given that **3** is more asymmetric and tumbles much slower in solution compared with **2** due to its larger size, resulting in more efficient quadrupolar relaxation and hence a larger line width.[18] It therefore seems likely that this peak was too broad to be observed. Note that under these conditions (**1** + 0.20 equiv. $NaVO_3$), a weak peak for **2** was observed, as expected, since a small amount of **2** was observed by ^{1}H NMR spectroscopy (ESI, Fig. S6†).

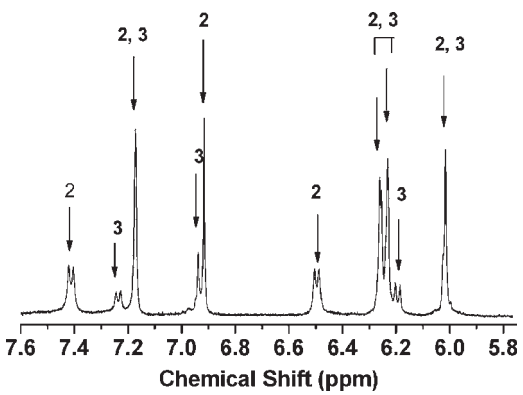

Fig. 1 Aromatic region of the ^{1}H NMR spectrum of an equimolar (6.4×10^{-6} mol) solution of **1** and $NaVO_3$ in D_2O, pD = 9.1 at 24 °C. Peaks at 7.42(d, A5), 6.92 and 6.50(d, A6) are assigned to **2** (Scheme 1). Peaks at 7.24(d, A5), 6.94 and 6.20(d, A6) are assigned to **3**. Signals attributable to the Cbl macrocycle of **2** and **3** overlap at 7.17, 6.26(d), 6.23 and 6.02 ppm.

Negligible spectral changes were observed by UV-Vis spectroscopy upon the addition of 0.2–3.0 equiv. $NaVO_3$ to **1** (pH 7.4, 25 °C). This is expected, because (a) the π–π* transitions within the corrin ring dominate UV-Vis spectra of Cbls[28] and (b) the structural differences between **1**–**3** are far removed from the corrin ring ($\geq$ 8 bond lengths away). The binding of $NaVO_3$ to **1** was also studied by FTIR spectroscopy in H_2O and D_2O at pH (pD) 8.7 ± 0.2. Although a detailed analysis of the data was not possible, the observed spectral changes were consistent with the ^{1}H NMR spectroscopy data (see ESI†).

Attempts to purify **2** and **3** were unsuccessful. Passing a solution of predominately **2** (**1** + 3.0 equiv. $NaVO_3$) through an Amberlite XAD-2 column to separate **2** from excess vanadate resulted in a mixture of **1**–**3**. C_{18} reverse-phase HPLC has been used routinely to separate Cbls;[29] however, only a single, broad product peak was observed in HPLC chromatograms of mixtures of **1**–**3** under either acidic or neutral isocratic conditions. In hindsight our failure to obtain pure **2** and/or **3** using standard chromatography techniques is not unexpected, given the considerable literature precedence for rapid exchange of ligands for V(v) complexes.[30,31]

The products of the reaction between **1** and 1.0 or 3.0 equiv. $NaVO_3$ were also studied at pD 7.4, and once again the mono- and bis-V(v) species were observed (Fig. S7 and S8, ESI†). Note, however, that under these pD conditions, the A5 and A6 proton signals of the vanadium-bound complexes are broader. This can be attributed to partial reduction of the V(v) to V(iv) by the ligand, which is more favorable at lower pH conditions.[18] Indeed, weak signals attributable to V(iv) complexes were observed for these solutions by EPR spectroscopy. The analysis of these spectra will be addressed in a follow-up study. No noticeable differences were observed in the ^{51}V NMR spectra of these solutions compared with those at pD 9.0.

Finally, preliminary experiments on the blood glucose-lowering ability of a single injection of an equimolar **1** + $NaVO_3$ solution

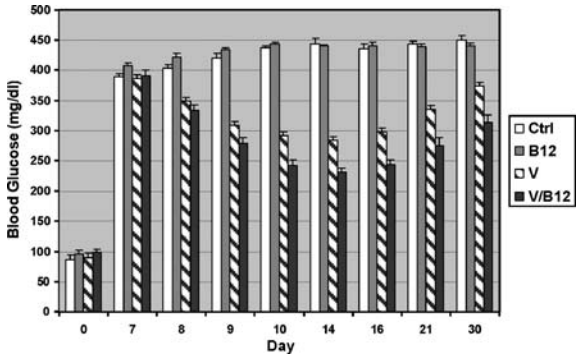

Fig. 2 Blood glucose levels for STZ-rats administered a single tail vein injection (pH 7.0, 70 μl) of H_2O (= control, Ctrl), 5.0×10^{-7} mol **1** in H_2O (B_{12}), 5.0×10^{-7} mol $NaVO_3$ in H_2O (V), or an equimolar (5.0×10^{-7} mol) solution of **1** + $NaVO_3$ in H_2O (V/B_{12}) on day 7, directly after measuring their blood glucose levels. The rats were injected with STZ (55 mg kg^{-1}) on day 0. The mean values represent independent observations from 3 different animals in each group; errors are ±1 standard deviation.

(V/B_{12}) *versus* $NaVO_3$ (V) were carried out using the streptozotocin (STZ) rat model for Type 1 diabetes. Elevated blood glucose levels were confirmed one week following intraperitoneal injection of STZ (55 mg kg^{-1}; levels rose from 94 ± 7 (day 0) to 394 ± 11 mg dl^{-1} (day 7), Fig. 2). Importantly, from day 8 onwards, statistical analysis (student's *t*-test) showed that the V/B_{12} conjugate mixture lowered glucose levels further than $NaVO_3$ alone ($p < 0.05$). Complex **1** did not significantly reduce blood glucose levels in the absence of $NaVO_3$.

To summarize, we have synthesized novel vanadate conjugates of 3-(3-hydroxy-2-methyl-1*H*-pyridin-4-one)propylcobalamin, the first vanadium–vitamin B_{12} bioconjugates with potential as insulinomimetics. The conjugates were characterized by ^1H and ^{51}V NMR spectroscopy, mass spectrometry and FTIR spectroscopy, and diffusion coefficients were determined using pulsed-field gradient-echo NMR spectroscopy methods. Future studies include further testing of these complexes and the preparation of other vanadium–vitamin B_{12} bioconjugates.

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