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Syntheses of Fluorescent Vitamin B_{12}-Pt(II) Conjugates and their Pt(II) Release in a Spectroelectrochemical Assay

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§SCS Poster Prize Winner

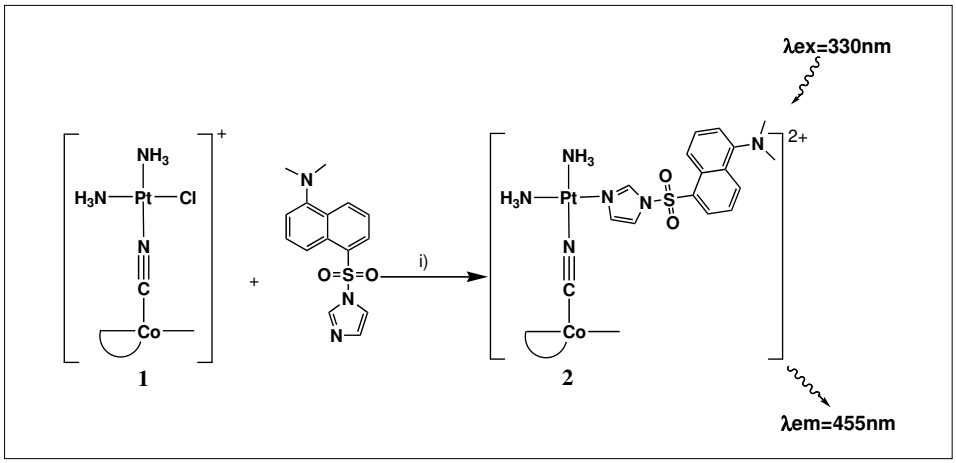
Abstract: The cisplatin adduct of vitamin B_{12}, *cis*-$[PtCl(NH_3)_2B_{12}]^+$ (**1**) reacts with dansyl-imidazole to form *cis*-$[Pt(dansyl\text{-}imidazole)(NH_3)_2B_{12}]$ (**2**). In a spectroelectrochemical assay, Co(III) in **2** was reduced to Co(II) under concomitant release of the Pt(II) complex. Since similar reduction occurs in the intracellular space, **2** can be used for studying cell uptake monitored by fluorescent microscopy. The insertion of a fluorescent marker into the platinum moiety will also facilitate the isolation of the platinum complex released from B_{12} and therefore to characterize its chemical authenticity.

Keywords: Cisplatin · Electrochemistry · Fluorescence · Vitamin B_{12}

1. Introduction

Fast proliferating cells such as cancer or microbial infections are high vitamin B_{12} consumers as compared to normal cellular metabolism.[1,2] This observation is the basis for the delivery of fluorescent, chemotherapeutic and radioactive agents to cancer cells by conjugation of a drug to cobalamin.[3]

We recently reported conjugates of vitamin B_{12} with rhenium, technetium and platinum complexes, binding to the cyanide of vitamin B_{12} and forming compounds of the general composition [{B_{12}Co}-CN-{M}].[4,5] Since cisplatin is a well established anticancer drug, we aim at site-specific uptake by conjugating vitamin B_{12} through the cyanide to Pt(II) complexes. We have extended this study and present herein the synthesis of novel cobalamin conjugates with the compound [{B_{12}Co}-CN-{Pt-fluorescent}] (**2**). This fluorescent cobalamin conjugate may be suitable for the imaging of cobalamin receptors *via* fluorescence microscopy, as well as for the characterization of the platinum moiety released from vitamin B_{12} after reduction of the cobalt center.



Scheme 1. Formation of the conjugate **2**. Conditions: i) H_2O/MeOH, 40 °C, 85 h.

2. Results and Discussion

2.1. *Synthesis and Characterization*

The cisplatin-vitamin B_{12} conjugate, *cis*-$[PtCl(NH_3)_2B_{12}]^+$ (**1**), reported by Mundwiler *et al.*, represents a good starting material for the metal-mediated introduction of further molecules. Due to the leaving group properties of chloride bound to Pt(II), it is expected that the chloride in **1** is replaced by an aromatic amine as previously observed for the reaction with 2'-dG.[4] The preparation of **2** follows a similar strategy. The reaction of **1** with four equivalents of dansyl-imidazole (Scheme 1) in a mixture of water/methanol yielded one single product with a retention time of 19.5 min as evident from HPLC.

NMR analysis of the product indicated the formation of a 1:1 adduct of **1** and dansyl-imidazole. The downfield part of the ^{1}H NMR spectrum shows the characteristic five signals of vitamin B_{12} and additional signals for dansyl-imidazole (Fig. 1). Furthermore, the ^{195}Pt NMR of **2** revealed a chemical shift at –2525 ppm, a region in

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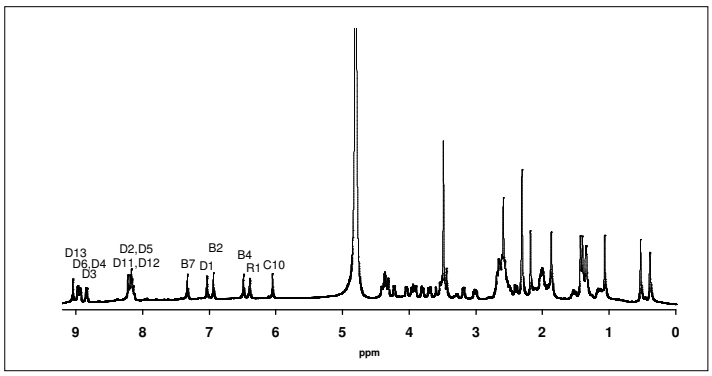


Fig. 1. ^{1}H NMR (500 MHz, D_2O, 300 K) of **2**. Signal legend; B = benzimidazole signals of the cobalamin unit; C= corrin of the cobalamin unit; D= dansyl imidazole ligand; R = ribose of the cobalamin unit.

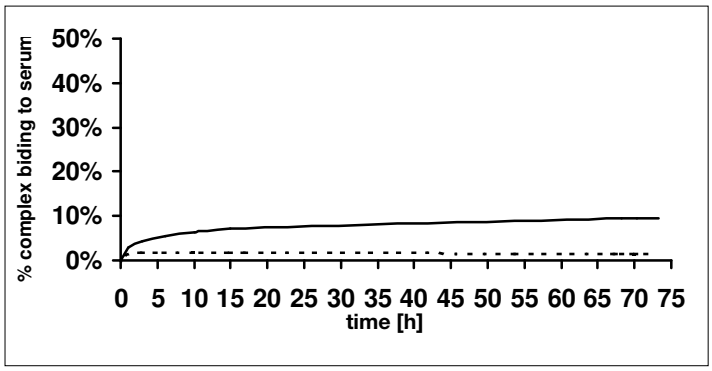
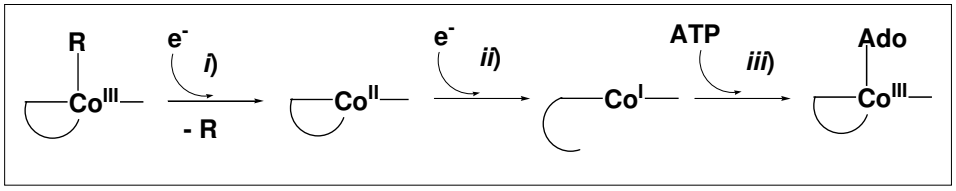


Fig. 2. Bovine serum albumin (BSA) binding stability (%) in phosphate buffer (pH 7.4) of **2** (___) and vitamin $\mathbf{B_{12}}$ (---)



Scheme 2. Corrinoid adenosylation assay. Reagents and conditions: *i*) Dihydroflavins or Fpr-FldA; *ii*) FldA-FMNH$^+$; *iii*)CobA[8]

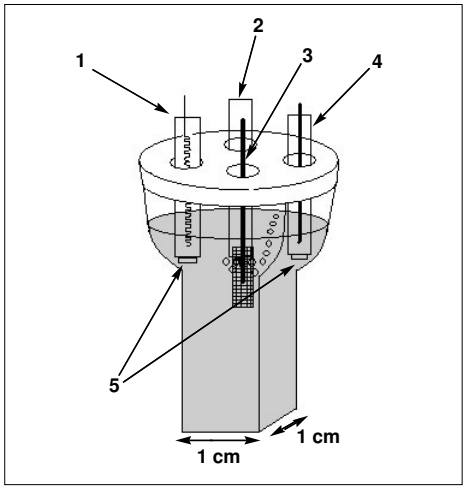


Fig. 3. Spectroelectrochemical cell for determination of the redox equilibria. 1) Counter electrode (Pt wire); 2) argon flow; 3) working electrode (Pt grid); 4) reference electrode Ag/AgCl; 5) fritted disk.

which Pt(II) bound to four nitrogen donors such as $[Pt(NH_3)_4]^{2+}$ is usually observed. The fluorescence properties of pure dansyl-imidazole show excitation at a wavelength λ_{ex} = 365 nm which leads to a strong fluorescence signal at a wavelength λ_{em} = 550 nm. In the case of **2** the fluorescence properties are retained and not quenched by the corrin ring system. A significant shift can be observed with excitation now at a wavelength λ_{ex} = 330 nm and a strong fluorescence signal at λ_{em} = 455 nm.

2.2. *Serum Stability Studies*

For application under physiological conditions, the stability of complex **2** in serum is crucial. Human serum proteins provide many coordinating groups in the protein side chains which can compete either for the cyanide in vitamin B_{12} or the dansyl-imidazole in the platinum complex, leading to the release of B_{12} from the intact Pt(II) complex in the former or the fluorescent marker in the latter case.[6] We investigated the stability of **2** under these aspects with two different HPLC systems in order to monitor decomposition (system 1) and serum protein binding (system 2). Since the conjugate **2** is more lipophilic than B_{12} we expect an increased binding to the protein due to lipophilic interaction of **2** with the protein without decomposition or cleavage of the Pt(II) complex (Fig. 2).

The conjugate **2** is stable in bovine serum albumin with respect to transmetallation of the platin complex to proteins and concomitant release of B_{12} or by direct competition with the cyanide leading to cleavage of the platinum core. We found lipophilic association of the conjugate **2** with the protein reaching a maximum of 10% after 72 h, native B_{12} associates to a lower rate and we found around 2% binding to protein after this period of time, whereas **1** binds around 20% to protein. The higher stability in serum of **2** *versus* **1** can be explained by the behavior in **1** which implies that the weakly bound single chloride ligand can be exchanged for potential coordinating sites from the protein.

2.3. *Electrochemical Properties*

The electrochemical properties of **2** are crucial with respect to the reducing processes taking place in the enzymatic intracellular adenosylation process. As was found in earlier studies, platination of the cyanide in vitamin B_{12} facilitates the reduction of the cobalt center from Co(III) to Co(II). We found a 86% reversible reduction wave at –530 mV for **2** (0.1 M Bu_4NPF_6 in MeOH; *vs*. E° (Fc/Fc$^+$) = 580 mV), which is at a significantly more positive potential than in native vitamin B_{12} (E = –670 mV in 0.1 M Bu_4NPF_6 in MeOH; *vs*. E° (Fc/Fc$^+$) = 580 mV). This is positive for the behavior of the compound in the cells since a lower potential makes the reduction of the cobalt center easier and the release of the platinum moiety might be expected.

In vivo, Co(III) in B_{12} is reduced in a multistep enzymatic assay to Co(I) and finally alkylated with ATP resulting in the formation of coenzyme B_{12}. A corresponding *in vitro* reducing system for the enzymatic conversion of cobalamin to adenosylcobalamin has been reported recently and is shown in Scheme 2.[7,8]

In order to mimic the *in vivo* corrinoid adenosylation process, a spectroelectrochemical assay has been set up using 0.2 M Tris-Cl buffer (pH = 8, 37 °C), 800 µM $MgCl_2$ and 50 µM of the corresponding cobalamin. These are the same conditions as used in the enzymatic *in vitro* experiments but now a continuous redox potential of –700 mV is applied instead of the Fpr-FldA proteins as reducing agents (Fig. 3).

Typical UV/Vis spectra for the stepwise reduction of aquo-cobalamin (a), vitamin B_{12} (b) and **2** (c) from Co(III) to Co(II) at a potential of –700 mV are shown in Fig. 4.

The upper axial ligand plays a crucial role in the reduction process as obvious from the different rates of conversion. For aqua-cobalamin HOCbl (a) the reduction is faster than for vitamin B_{12} (b). The same observation was made in previous studies using the Fpr-FldA system as enzymatic reducing agent. The half-life time for conversion is $t_{1/2}$ = 1.6 h for HOCbl and $t_{1/2}$ = 39 h for B_{12} under identical conditions. It should be noted that vitamin B_{12} is not a good substrate for this reduction process. For **2** we found a substantially lower half

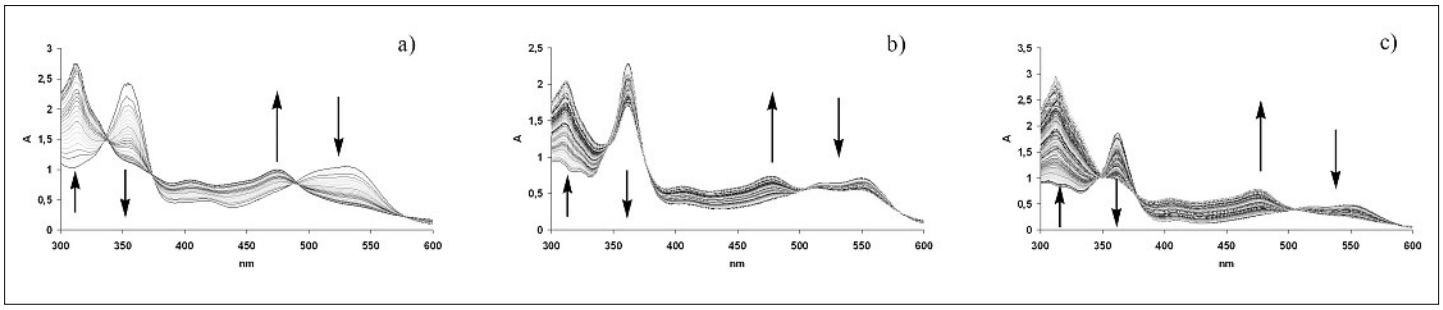


Fig. 4. Spectral changes associated with the conversion of cob(III)alamin to cob(II)alamin at E = –700 mV. a) UV/Vis spectrum of the solution of HOCbl every 10 min; b) UV/Vis spectrum of the solution of NCCbl every 15 min, the measurement was stopped after 24 h (before full conversion to Co(II)); c) UV/Vis spectrum of the solution of **2** every 10 min.

life time $t_{1/2}$ = 17 h for reduction. The half-life times for reduction follow the same trend as the corresponding $E^\circ_{1/2}$ values. Native vitamin B_{12} has the most negative potential, with $E_{1/2}$ = –820 mV (1 M KNO_3 in H_2O solution), as was observed by cyclic voltammetry measurement, compound **2** is in between, with $E_{1/2}$ = –700 mV (1 M KNO_3 in H_2O solution), and aqua-cobalamin is easiest to reduce under the same conditions, with $E_{1/2}$ = –40 mV. These similar trends are in agreement with an outer sphere electron transfer process. It should be noted here that the same trend was also observed in the fully enzymatic reduction process. Under electrochemical conditions, E = –700 mV and pH = 7.4, it is expected that the upper ligand of cobalamin is released during the reduction from Co(III) to Co(II).[9] Exposing the reduced form of either aquo-cobalamin, vitamin B_{12} or **2** to air led to reoxidation of Co(II) → Co(III). HPLC analysis revealed in all cases the formation of HOCbl as evident from Fig. 5. This underlines the hypothesis that the upper axial ligand is indeed released during (electrochemical or enzymatic) reduction, thus representing an important step in the use of B_{12} as a carrier for various bioactive substances.

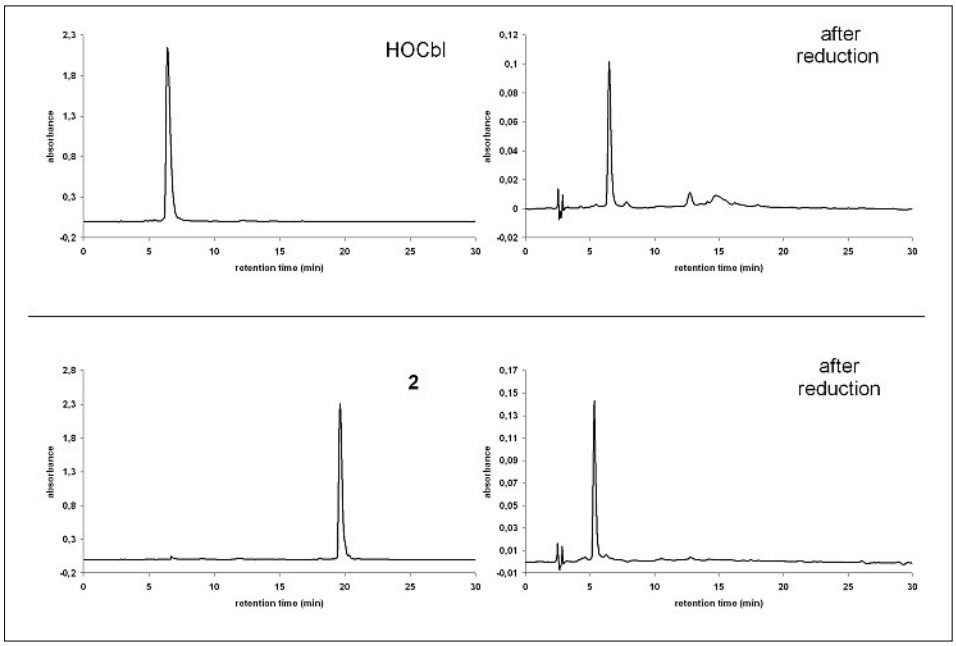


Fig. 5. HPLC traces of HOCbl and **2** before and after spectroelectrochemical assays and subsequent reoxidation in air

3. Conclusion

We have show that Cl^- in **1** can be substituted by good nucleophiles such as aromatic amines without cleaving the Pt(II) from B_{12}. This general approach allows the introduction of fluorescent labels in B_{12} as shown with dansyl-imidazole. Complex **2** is stable in buffer and in BSA and can be used for the study of the cellular uptake by fluorescent microscopy. Electrochemical reduction of Co(III) → Co(II) in **2** showed release of the Pt(II) complex together with the fluorescent marker. Since preliminary *in vitro* studies with an enzymatic assay and **2** showed qualitatively the same result, the Pt(II) complexes should also be released *in vivo*. The results presented in here will allow the isolation and characterization of the released Pt(II) complex.

4. Experimental Section

4.1. *General Procedures*

All chemicals were purchased from Fluka, Sigma, Strem and Acros. Chemicals were of reagent grade and used without further purification. Dansyl-imidazole[10] and *cis*-$[PtCl(NH_3)_2B_{12}]$[4] were synthesized according to published procedures. All reactions were performed under nitrogen or argon atmosphere.

HPLC analyses were performed on a Merck-Hitachi L-7000 system equipped with a diode array UV/Vis spectrometer, the following HPLC columns, solvents systems and gradients were used: *Columns*: Macherey Nagel C-18ec RP columns (5 µm particle size, 100 Å pore size, 250×3 mm); Column 2: Bio-Sil® SEC columns (5 µm particle size, 250 Å pore size, 300×7.8 mm). *Solvent System*: Solvent system 1: 0.1% trifluoroacetic acid in water (A); MeOH (B); Solvent system 2: 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.01 M NaN_3, 0.15 M NaCl, pH = 6.8 (A). *Gradients*: Gradient 1: 0–5 min (75% A), 5–30 min (75%A → 0%A); Gradient 2: 0–15 min (100% A).

Preparative HPLC purifications were performed on a Varian Prostar system equipped with two Prostar 215 pumps and a Prostar 320 UV/Vis detector, using Macherey Nagel Nucleosil C-18ec RP columns (7 µm particle size, 100 Å pore size, 250×20 mm and 250×40 mm).

IR spectra were recorded on a Bio-Rad FTS-45 spectrometer with the samples in compressed KBr pills.

NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer. The chemical shifts are reported relative to residual solvent protons as a reference. The chemical shifts of ^{195}Pt NMR spectra are relative to K_2PtCl_4 at –1626.4 ppm. Peak assignments of cobalamin derivatives were determined by interpolation of the ^{1}H COESY and C-H correlation spectra.

Cyclic voltammetry (CV) was carried out on a Metrohm 757VA Computrace system with a glassy carbon working electrode, a glassy carbon counter electrode, and a Ag^+/AgCl reference electrode. All

compounds were measured as a solution (1 mM) in tetrabutylammonium hexafluorophosphate in methanol (0.1 M) and in potassium nitrate in water (1 M) , the sweep rate for CV being 0.03 Vs^{-1}.

Fluorescence emission spectra were recorded in water on a Perkin Elmer LS-50B.

4.2. *Synthesis of cis-[Pt(NH$_3$)$_2$(Dansyl-imidazole)B$_{12}$]$^{2+}$ (2)*

A solution of dansyl-imidazole (51 mg, 0.169 mmol) in 5ml of MeOH was added to a solution of *cis*-[PtCl(NH$_3$)$_2$B$_{12}$]$^+$ (70 mg, 0.042 mmol) in 5 ml of H_2O and allowed to stir at 40 ºC for 85 h. The course of the reaction was monitored by HPLC measurements (column 1; solvent system 1; gradient 1; flow: 0.5 ml/min). The solvent was evaporated and the crude product purified by preparative HPLC (solvent system 1; gradient 1; flow: 40 ml/min) to give **2** as a red powder (37 mg, 45%). IR (KBr, cm^{-1}) νCN: 2196. ^{195}Pt NMR (107 MHz, D_2O, 293 K) δ: –2525 ppm. ESI-MS: 1884.6 $[M]^+$, 1867.6 $[M\text{-}NH_3]^+$.

4.3. *Binding of Vitamin B$_{12}$ and 2 to Bovine Serum Albumin*

Bovine serum albumin (13 mg, 0.2 μmol) was dissolved in phosphate buffer (pH 7.4, 0.1 M, 1 ml). Aliquots of the freshly dissolved cobalamin derivatives (0.2 μmol) were added and the solutions stirred at RT. Binding to the albumin was measured by HPLC with detection at 360 nm (column 2; solvent system 2; gradient 2; flow: 1 ml/min), as well as rate of decomposition after 48 h (column 1; solvent system 1; gradient 1; flow: 0.5 ml/min). The following amounts of protein-bound cobalamin were found: vitamin B_{12}: no binding after six days; **2**: 4.2% after 4 h, 8.5% after 24 h, 9.6% after 74 h.

4.4. *Spectroelectrochemical Studies*

Reduction of cobalamin derivatives from Co(III) to Co(II) was monitored spectrophotometrically using a Varian Cary 50 spectrometer equipped with a temperature-regulated cuvette holder. The assay mixture (final volume, 5 ml) contained cobalamin (100 μM), $MgCl_2$ (800 μM) and tris-Cl buffer (pH = 8 at 37 ºC, 0.2 M). All experiments were carried out at 37 ºC. The cell was as represented in Fig. 3 with a platinum net working electrode and a platinum wire countered electrode. Controlled potential coulometry was carried out with a potentiostat AMEL-549.

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