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Design, Synthesis, and Characterization of Fluorescent Cobalamin Analogues with High Quantum Efficiencies

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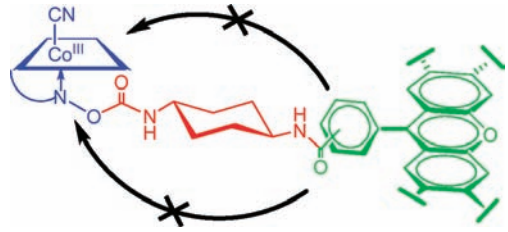
ABSTRACT



Cobalamin tethered to fluorescein or Rhodamine 6G has been synthesized and characterized. The fluorophore is conjugated to the ribose-5′-OH of cobalamin through a rigid linker to prevent the fluorophore from folding back through space and interacting with the corrin ring of cobalamin. This increases the fluorescence quantum yield. This new family of cobalamin analogues may be suitable for use as tumor markers to tag cancer cells for surgical resection.

Cancer cells exhibit an unusually high requirement for cobalamin (vitamin B_{12}) to support DNA synthesis prior to cell division.[1] Cobalamin analogues with a fluorophore attached at the β-axial ligand position of Co(III) or the ribose-5′-hydroxyl moiety of cobalamin have proved useful as probes of vitamin B_{12} trafficking. Furthermore, they show promise as visual indicators of tumor micromargins in murine xenograft models of human tumors.[2] While the nascent generation of fluorescent cobalamin conjugates was useful as a tool to explore the cellular biology of vitamin B_{12}, the earliest fluorescent cobalamin conjugates reported suffer from severe fluorescence quenching due to overlap of the electronic orbital of cobalamin and the excited state of the fluorophore.[3,4]

Herein, we report the design, synthesis, and characterization of a new generation of fluorescent cobalamin analogues that incorporate a rigid linker to thrust the fluorophore away from the corrin ring and enforce an orthogonal relationship between the electronic dipoles of the fluorophore and cobalamin.

The overarching goal of this work is to develop highly fluorescent molecular imaging agents that are targeted to rapidly dividing cancer cells by the cobalamin moiety and retain the ability to interact with the cobalamin transport protein, transcobalamin. Highly fluorescent cobalamin analogues that meet these criteria may be useful as intraoperative imaging agents to help surgeons delineate cancerous tissue from healthy tissue during surgical resection.

While some of the previous CobalaFluor fluorescent cobalamin analogues that were constructed by attaching the fluorophore directly to the Co(III) atom at the β-axial ligand

(1) (a) Hogenkamp, H. P. C.; Collins, D. A.; Grissom, C. B.; West, F. G. *Chemistry and Biochemistry of B12*; John Wiley and Sons: New York, 1999; pp 385. (b) Kunze, S.; Zobi, F.; Kurz, P.; Spingler, B.; Alberto, R. *Angew. Chem., Int. Ed.* **2004**, *43*, 5025. (c) Flodh, H. *Acta Radiol. Suppl.* **1968**, *284*, 1. (d) Hall, C. A. *J. Lab. Clin. Med.* **1984**, *103*, 70. (e) Collins, D. A.; Hogenkamp, H. P. C.; O'Connor, M. K.; Naylor, S.; Benson, L. M.; Hardman, R.; Thorson, L. M. *Mayo Clin. Proc.* **2000**, *75*, 568.

(2) Cannon, M. J. Ph. D. Thesis. University of Utah, Salt Lake City, UT, 2001.

(3) Horton, R. A. Ph. D. Thesis. University of Utah, Salt Lake City, UT, 2004.

(4) Smeltzer, C. C.; Cannon, M. J.; Pinson, P. R.; Munger Jr, J. D.; West, F. G.; Grissom, C. B. *Org. Lett.* **2001**, *3*, 799.

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position had respectable fluorescent quantum yields (ϕ_f) in the range of 0.01−0.1,[4] these compounds suffer from photochemical instability because of the low Co−C bond dissociation energy of ∼37 kcal/mol that falls well within the range of visible photons.[5] To solve this problem, we chose to activate the 5′-OH of the cobalamin α-ribofuranotide for conjugation to the fluorophore.

Chemical modification of cobalamin must not disrupt the binding interactions between the synthetic analogue and the proteins required for endocytosis and intracellular trafficking.[6] Modification of the ribose moiety of cobalamin is reported to be well tolerated by the plasma cobalamin binding protein, transcobalamin (TC) and the enteric cobalamin binding protein, intrinsic factor (IF).[7]

On the basis of preliminary experiments with *flexible* linkers of increasing length to separate the ribose-5′-hydroxyl group and the fluorophore, an increase in ϕ_f was observed as the distance separating the corrin ring and the fluorophore was increased, but the maximum increase in ϕ_f achieved was only 5-fold. This is likely due to the flexible linker allowing an intramolecular folding and π-stacking interaction between the corrin ring and the fluorophore.

To reduce the intramolecular dynamical movement that allows for fluorescence quenching, we incorporated a rigid linker shown in red (Figure 1) in the design of the synthetic analogues.

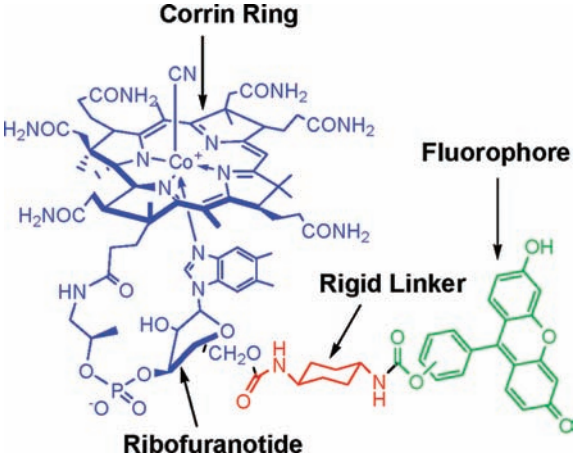


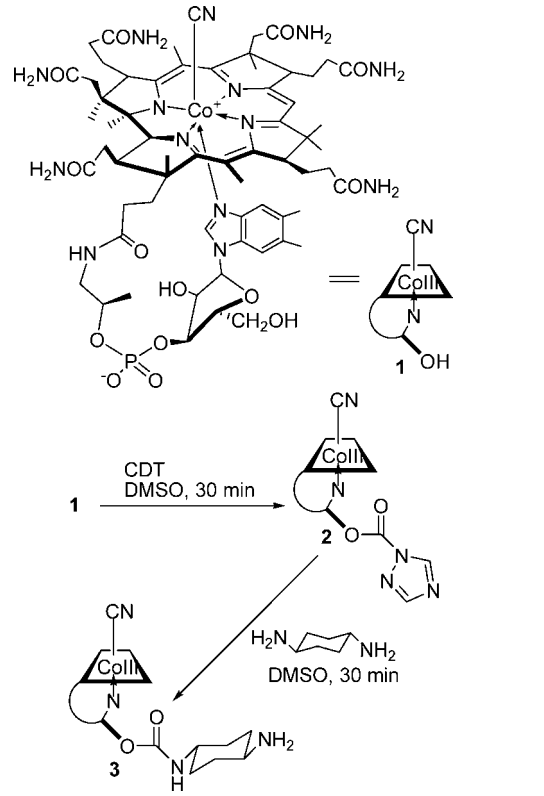
Figure 1. *trans*-1,4-Diaminocyclohexane was used as a rigid molecular scaffold to link cobalamin and the pendant fluorophore. The linker orients the fluorophore away from the corrin ring of cobalamin to minimize the intramolecular dynamical interaction, thereby increasing the overall fluorescence quantum yield of the fluorophore.

We have synthesized cobalamin-fluorescein **5** (Scheme 2) and cobalamin-Rhodamine 6G **7** (Scheme 3) conjugates as exemplars of rigidly tethered cobalamin-fluorophore conjugates.

(5) Martin, B. D.; Finke, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 585.

(6) (a) Fedosov, S.; Laursen, N. B.; Moestrup, S. K.; Nexo, E.; Petersen, T. E.; Jensen, E. O.; Berglund, L. *Biochemistry* **2004**, *42*, 15095. (b) Fedosov, S.; Dedosova, N. U.; Nexo, E.; Petersen, T. E. *J. Biol. Chem.* **2000**, *275*, 11791.

Scheme 1. Synthesis of the Cobalamin-*trans*-1,4-diaminocyclohexane Complex



The synthesis of the common precursor **2** is shown in Scheme 1. Derivatization of the ribose-5′-OH of cyanocobalamin with 1,1′-carbonyl-di-(1,2,4-triazole) (CDT) gives intermediate **2** in 75% yield. Nucleophilic attack by *trans*-1,4-diaminocyclohexane yields **3** in 60% yield. The use of CDT as an acylating agent for activation of the ribose-5′-OH is effective even in multigram preparations (Scheme 1). We also investigated the use of a less expensive acylating agent, 1,1′-carbonyldiimidazole (CDI); however, the results were less satisfactory with reaction yields of less than 5%.

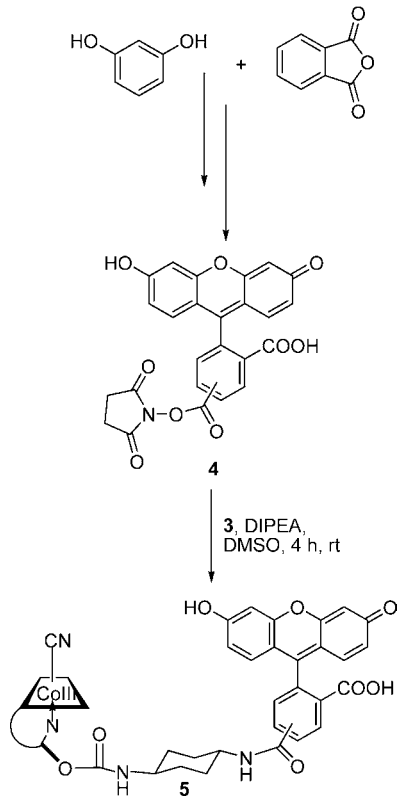
5(6)-Carboxyfluorescein was synthesized using a modified literature procedure.[8] A mixture of resorcinol and 1,2,4-benzenetricarboxylic acid was heated to reflux in concentrated methanesulfonic acid. The neat reaction conditions dramatically increase the rate of reaction and give 5(6)-carboxyfluorescein in 99% yield.

Reaction of 5(6)-carboxyfluorescein with *N*-hydroxysuccinimide and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) gave compound **4**.[9] The activated ester of fluorescein was used in subsequent reac-

(7) (a) Pathare, P. M.; Wilbur, D. S.; Heusser, S.; Quadros, E. V.; McLoughlin, P.; Morgan, A. C. *Bioconjugate Chem.* **1996**, *7*, 217. (b) Wuerges, J.; Garau, G.; Geremia, S.; Fedosov, S. N.; Petersen, T. E.; Randaccio, L. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 4386.

(8) This procedure yields an isomeric mixture of the 5- and 6-carboxyfluoresceins. Use of this mixture of isomers is common in the construction of fluorescein-based imaging agents. (a) Orndroff, W. R.; Hemmerm, A. *J. Am. Chem. Soc.* **1927**, *49*, 1272. (b) Sun, W.-C.; Gee, K. R.; Klaubert, D. H.; Haughland, R. P. *J. Org. Chem.* **1997**, *62*, 6469.

Scheme 2. Synthesis of **5**



Scheme 3. Synthesis of **7**

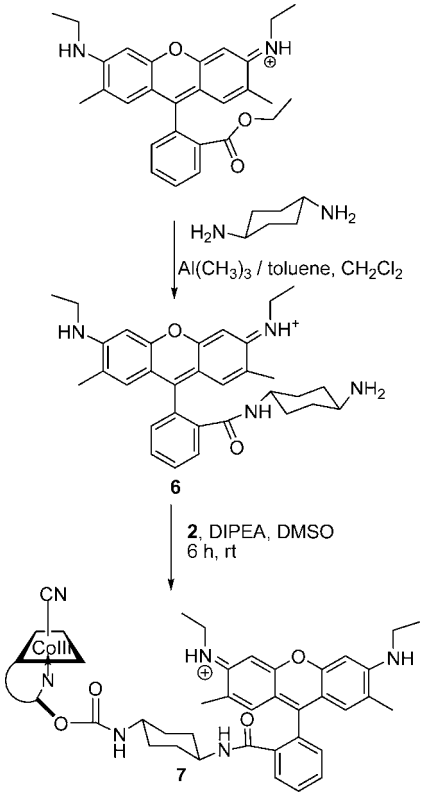


Table 1. Fluorescence Quantum Yield Values

compd	$\lambda_{max\ ex}$, nm	$\lambda_{max\ em}$, nm	ϕ_f
5	481	528	0.10 ± 0.01
7	530	554	0.20 ± 0.01

tions without further purification. The final coupling of cobalamin and fluorescein gives **5** with an overall yield of 22% after prep-HPLC purification. The reactions involving fluorescein were carried out in dim red light to reduce the adventitious photobleaching of the fluorophore.

Derivatization of Rhodamine 6G at the ester moiety by substitution of an amide bond for the ester was carried out by refluxing Rhodamine 6G with $Al(CH_3)_3$ with *trans*-1,4-diaminocyclohexane (Scheme 3).[10] Compound **6** was purified by prep-HPLC. The 1° NH_2 group of **6** is a good nucleophile for conjugation to biomolecules. Final coupling of **6** with **2** yields **7**.

The optical properties of **5** and **7** are reported in Table 1. Compound **5** exhibits an emission maximum at 528 nm (λ_{ex}

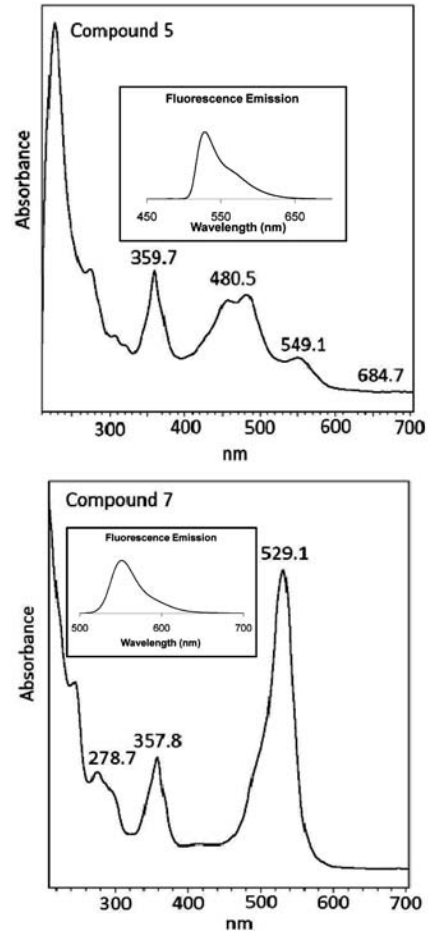


Figure 2. Absorption and fluorescence spectra of **5** and **7**. The 358 nm peak is characteristic of the corrin ring. The excitation wavelengths for **5** and **7** were 481 and 530 nm, respectively.

= 480 nm) and $\phi_f = 0.10$, whereas compound **7** exhibits an emission maximum at 554 nm ($\lambda_{ex} = 530$ nm) and $\phi_f = 0.20$. The quantum yields for both compounds were obtained by measuring the integrated fluorescence emission, optical density, and refractive index, and referencing these values to the reported values for fluorescein ($\phi_f = 0.92$) or Rhodamine 6G ($\phi_f = 0.95$) (Table 1; additional details are provided in Supporting Information). In contrast to the nascent generation of fluorescent cobalamin analogues, neither **5** nor **7** undergo adventitious photodegradation at the Co−C bond because the fluorophore is now attached to the ribose ring of cobalamin.[4]

We are investigating the binding kinetics and thermodynamics for the interaction of **5** and **7** with the cobalamin transport proteins TC and IF to determine their applicability as tumor imaging agents. The typical rates of dissociation for cobalamin from TC and IF are on the order of 10^{-7} s^{-1}. Experiments are also being carried out to assess the ability of each analogue to enter human cancer cells and to assess the fidelity of cellular accumulation relative to the naturally occurring cobalamins.

(9) Adamczyk, M.; Fishpaugh, J. R.; Heuser, K. J. *Bioconjugate Chem.* **1997**, *8*, 253.

(10) (a) Nguyen, T.; Francis, M. B. *Org. Lett.* **2003**, *5*, 3245. (b) Adamczyk, M.; Grote, J. *J. Bioorg. Med. Chem. Lett.* **2000**, *10*, 1539. (c) Adamczyk, M.; Grote, J. *Synth. Commun.* **2001**, *31*, 2681.

In summary, we have demonstrated the rational design and synthesis of two members of a new class of photostable fluorescent cobalamin analogues with fluorescein and Rhodamine 6G as the reporter groups. The use of a cyclohexane ring as a rigid linker orients the fluorophore away from the corrin ring of cobalamin to minimize through space intramolecular interactions, thereby increasing the overall fluorescence quantum yield relative to the analogous fluorophore tethered to cobalamin with a flexible linker. The cobalamin analogues reported herein may be suitable for use as intraoperative tumor markers to guide surgeons during tumor resection, ultimately improving the overall outcome of cancer surgery.

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Supporting Information Available: Full experimental procedures, and characterization data for compounds **1**−**7** and the method for determining the fluorescence quantum yields. This material is available free of charge via the Internet at http://pubs.acs.org.

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