ORGANIC LETTERS

2001
Vol. 3, No. 6
799−801

Synthesis and Characterization of Fluorescent Cobalamin (CobalaFluor) Derivatives for Imaging

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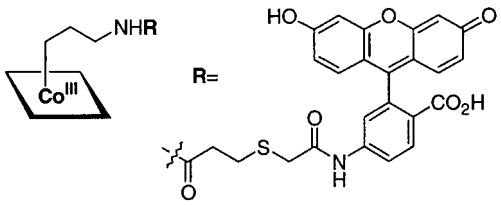
Received November 3, 2000

ABSTRACT



Fluorescent derivatives of cobalamin have been prepared by linking fluorophores to cobalamin through a propylamide spacer. Fluorescein, naphthofluorescein, and Oregon Green derivatives have been prepared in good yield by reaction of the fluorophore NHS-ester with β-(3-aminopropyl)cobalamin to form fluorescent cobalamin conjugates (CobalaFluors) that are potentially suitable for the in vitro and in vivo imaging of transcobalamin receptors on cancer cells.

Higher eukaryotes require cobalamin (vitamin B_{12}) as an essential cofactor for the methylation of uracil prior to DNA synthesis and cell replication. Cancer cells have an increased ability to transport and to sequester cobalamin in large excess over the amount required for normal cellular metabolism and cell replication.[1]

This observation has been used to target the delivery of chemotherapeutic agents[1c,2] and radionuclides[1c,1d,3] to cancer cells by conjugation of a drug or radionuclide to cobalamin, thereby enabling receptor-mediated endocytosis of the cobalamin−conjugate/transcobalamin complex.

Herein, we report the synthesis of novel cobalamin conjugates with pendant fluorophores. These fluorescent cobalamin conjugates (hereafter CobalaFluors) may be suitable for the imaging of cobalamin receptors via epi-fluorescence microscopy, flow cytometry, and intraoperative visualization.[4]

β-(3-Aminopropyl)cobalamin **1** has been selected as a common synthetic intermediate from which all fluorescent cobalamins can be prepared and isolated in good yield (Scheme 1).

Conjugation to cobalamin at the upper-axial (β) ligand position preserves recognition of cobalamin by the cobalamin transport protein, transcobalamin.[5]

The fluorescent β-(3-aminopropyl)cobalamin conjugates **2**, **3**, and **4** are stable at neutral pH, in common HPLC solvents, and in cell culture media. Desalting and purification

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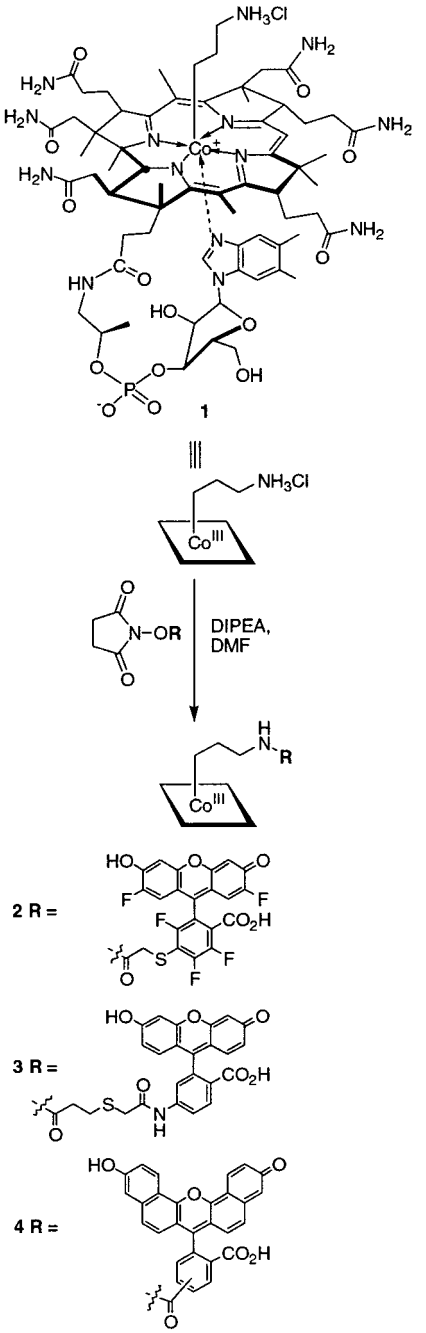
10.1021/ol006825v CCC: $20.00
Published on Web 02/17/2001

Scheme 1



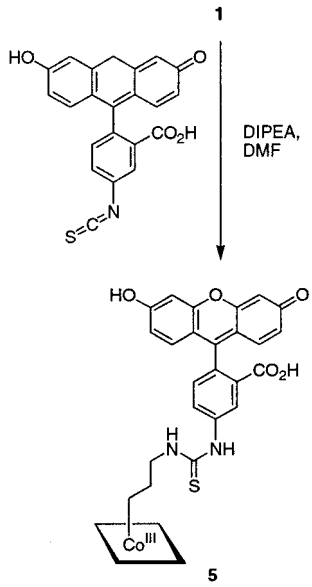
of CobalaFluors via adsorption chromatography[6] is a significant improvement over current methods of cobalamin purification that employ the tedious extraction of an aqueous CH_2Cl_2/phenol emulsion.[1a]

A modified literature procedure[7] was used to produce β-(3-aminopropyl)cobalamin **1** as the HCl salt in 80% yield from hydroxocobalamin and 3-aminopropyl chloride HCl with Zn dust as reductant. Adsorption of the crude reaction product mixture onto a Waters C-18 Sep-Pak cartridge containing C-18 adsorbant allows rapid desalting with H_2O, followed by elution of the crude β-(3-aminopropyl)cobalamin with MeOH. The red solid is 98% pure by analytical HPLC.

Fluorescent conjugates of Oregon Green, fluorescein, and naphthofluorescein were prepared according to Scheme 1 in which β-(3-aminopropyl)cobalamin is treated with the commercially available *N*-hydroxysuccinimide (NHS) esters to give the corresponding β-(3-amidopropyl)cobalamin conjugates **2**, **3**, and **4** in isolated yields of 73%, 60%, and 64%, respectively. CobalaFluor amides **2**, **3**, and **4** are stable in aqueous solution and they are sufficiently robust to withstand long-term storage at −20 °C as the dry powders. Solutions of β-alkylcobalamins should be handled in dim light to avoid photolysis of the Co−C bond.

Cobalamin derivative **5** was produced in 76% yield by the reaction of fluorescein isothiocyanate (FITC) with β-(3-aminopropyl)cobalamin according to Scheme 2. CobalaFluor

Scheme 2



5 is somewhat less stable than conjugates **2**, **3**, and **4** in aqueous solution and could not be purified by HPLC without partial decomposition.

Compounds **1**−**5** were characterized by positive-ion electrospray mass spectrometry, with observation of the characteristic M + H and M + Na^+ peaks and the cobalamin fragmentation peak at *m*/*z* 1329.5.

UV−vis spectra of compounds **1**−**5** were recorded before and after aerobic photolysis, with the absorption peak of hydroxocob(III)alamin appearing at 350 nm as the Co−C bond undergoes photohomolysis (Figure 1). No photobleaching of the fluorophore occurs under the mild conditions of photolysis reported herein. Therefore, all spectral changes are associated with cleavage of the Co−C bond.

The fluorescence quantum yield of CobalaFluors **2**, **3**, and **4** was determined by integrating the area under the emission

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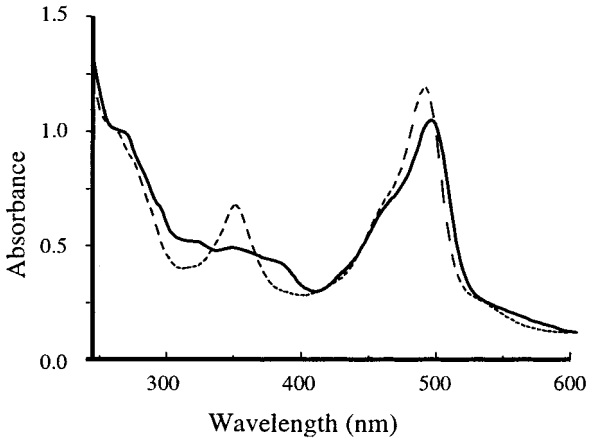

Figure 1. UV−Vis absorption spectra of **3** before (—) and after aerobic photolysis of the Co−C bond (- - -).

peak and correlating this value to the known fluorescence quantum yields of fluorescein and rhodamine 6G (Table 1).[8]

The fluorescence quantum yield of **5** was not determined because of the instability of the thiourea and its lesser suitability as an imaging agent. The value of ϕ_f for fluorescein conjugate **3** is 7-fold lower than the quantum yield for unconjugated fluorescein. An increase in the fluorescence intensity of CobalaFluors **2**, **3**, and **4** can be observed upon cleavage of the tether to cobalamin. Cob(III)alamin itself is not fluorescent and can quench the excited electronic state of pendant ligands. Unlike the cryptofluorescent and profluorescent cobalamin conjugates synthesized previously, the compounds reported herein exhibit less quenching of the fluorophore excited state by cobalamin.[9] The CobalaFluors are overtly fluorescent molecules that are suitable for the imaging of transcobalamin receptors. Fluorophores that emit green-to-red photons were selected to maximize the detection of CobalaFluors by a human eye or a CCD camera, respectively.

Table 1. Fluorescence Quantum Yields

cmpd	$\lambda_{max\ ex}$, nm	ϵ (M^{-1} cm^{-1})	$\lambda_{max\ em}$, nm	ϕ_f
2	510	7.9×10^4	525	0.16
3	494	7.1×10^4	514	0.13
4	604	4.2×10^4	668	0.013

Acknowledgment. The authors thank Joel S. Bentz, M.D., for suggestion of the name "CobalaFluor". This research is sponsored by NIH grant CA73003 to C.B.G and F.G.W.

Supporting Information Available: Experimental details for the synthesis of β-(3-aminopropyl)cobalamin and each CobalaFluor, along with absorption/emission spectra, are provided. A discussion of the method used to determine the fluorescence quantum yield of each CobalaFluor is included. This material is available free of charge via the Internet at http://pubs.acs.org.

OL006825V

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