Supporting Information to Accompany

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

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Experimental

General Methods. Chemicals were purchased from Aldrich, Inc. unless otherwise specified. Hydroxocobalamin was purchased from Sigma, Inc. The Oregon Green™ N-hydroxysuccinimidyl ester, fluorescein-5-isothiocyanate, fluorescein-5-EX N-hydroxysuccinimidyl ester, and naphthofluorescein 5-(and 6)-carboxy N-succinimidyl esters were purchased from Molecular Probes, Inc. Synthetic reactions were monitored by HPLC using a Waters Delta 600 system equipped with a Waters 2487 dual wavelength absorbance detector. Positive-ion electrospray mass specrometric analysis of each cobalamin in 50% aqueous acetonitrile was carried out on a Micromass Quattro II instrument. The absorption spectra were recorded with an HP8452 diode-array UV-visible spectrophotometer. Fluorescence emission spectra were recorded with a Fluorolog 3 SPEX double-grating fluorimeter with the cell compartment thermostatted to 25°C. Refractive index values were determined with a refractometer from Milton Roy, Inc. Compounds with a Co-C bond were manipulated and stored in dim red light provided by cold cathode neon discharge.

Synthesis of β-(3-aminopropyl)cobalamin 1. Hydroxocobalamin (200 mg, 0.144 mmole) was dissolved in 10 mL deionized H_2O and 3-aminopropylchloride HCl (37 mg, 0.288 mmole) was added to the solution followed by NH_4Cl (77 mg, 1.44 mmole). The solution was deaerated by bubbling with $N_{2(g)}$ for 20 min. Zinc dust (94 mg, 1.44 mmole) was added in one portion. The reaction was stirred under nitrogen for 5 h, at which time all starting material had been consumed, as indicated by HPLC analysis. The reaction was filtered with Whatman #42 filter paper and the reaction mixture was applied to a Waters C-18 Sep-Pak cartridge (10 g C-18 sorbent) that had been prepared by washing with 60 mL MeOH, followed by 100 mL H_2O. Salts were eluted from the Sep-Pak with 100 mL H_2O and the product **1** was eluted with 20 mL MeOH. The alcohol was removed by rotary evaporation and the resulting aqueous solution of **1** was dried by lyophylization. The red solid was dissolved in methanol (2 mL) and added to rapidly stirring 1:1 dichloromethane/diethyl ether (100 mL). The product was filtered on a medium frit and triturated with 3×5 mL each of dichloromethane, acetone, and finally diethyl ether. Compound **1** was dried over P_2O_5 *in vacuo*. β-(3-aminopropyl)cobalamin: 165 mg, 80% yield. Analytical HPLC: 98% pure, retention time = 10.0 m; ES^+ MS: (1:1 H_2O/CH_3CN) M+H=1387.5, (calc. $C_{65}H_{97}CoN_{14}O_{14}P$=1387.64); $M+Na^+$ = 1409.5, (calc. $C_{65}H_{96}CoN_{14}O_{14}PNa$ =1409.62)

Analytical HPLC method for 1. The HPLC flow rate was 2.0 mL/min. Column: Waters C-18, 15 micron, 3.9 mm X 100. Solutions: A: 50 mM H_3PO_4 brought to a pH of 3.0 with concentrated NH_4OH B: 9:1 CH_3CN/H_2O Method: 0-2 min: Isocratic elution of A, 2-10 min: ramp to 7:3 A/B, 10-15 min: ramp to 5:95 A/B, 15-25 min: ramp to 95:5 A/B.

Synthesis of 2. Oregon Green™ N-hydroxysuccinimidyl ester (Molecular Probes P/N O-6139, 16 mg, 0.026 mmole) was added to a slurry of β-(3-aminopropyl)cobalamin (**1**, 29 mg, 0.021 mmole), DMF (0.4 mL) and N,N-diisopropylethylamine (DIPEA) (8 μL, 0.05 mmole). The reaction was stirred for 1.5 h, after which it was judged complete by HPLC analysis. MeOH (0.5 mL) was added to the reaction mixture and the solution was added to rapidly stirring 1:1 dichloromethane/diethyl ether (50 mL). The red solid was filtered on a medium frit and triturated with 2×3 mL each of dichloromethane, acetone, and finally diethyl ether. The compound **2** was dried over P_2O_5 *in vacuo*: 29 mg, 73% yield.
The compound was purified by semi-preparative HPLC (see method below): 11 mg of **2** obtained.
Analytical HPLC: 91% pure, retention time = 12.0 min; ES^+ MS: (1:1 H_2O/CH_3CN) M+H=1881.5 (calc. $C_{87}H_{104}CoF_5N_{14}O_{20}PS$=1881.63), $M+Na^+$=1903.4 (calc. $C_{65}H_{96}CoN_{14}O_{14}PNa$=1903.61)

Synthesis of 3. Fluorescein-5-EX N-hydroxysuccinimidyl ester (Mol. Probes P/N F-6130, 14 mg, 0.024 mmole) was added to a slurry of β-(3-aminopropyl)cobalamin (**1**, 28 mg, 0.020 mmole), DMF (0.4 mL) and N,N-diisopropylethylamine (9 μL, 0.05 mmole). The reaction was stirred for 2 h, after which it was judged complete by HPLC analysis. MeOH (0.5 mL) was added to the reaction mixture and the solution was added to rapidly stirring 1:1 dichloromethane/diethyl ether (50 mL). The red solid was filtered on a medium frit and triturated with 2×3 mL each of dichloromethane, acetone, and finally diethyl ether. The compound **3** was dried over P_2O_5 *in vacuo*: 23 mg, 60% yield. The compound was purified by semi-preparative HPLC (see method below): 18 mg of **3** obtained. Analytical HPLC: 89% pure, retention time = 12.1 min; ES^+ MS: (1:1 H_2O/CH_3CN) M+H=1862.4 (calc. $C_{90}H_{114}CoN_{15}O_{21}PS$=1862.71), $M+Na^+$=1884.4 (calc. $C_{90}H_{113}CoN_{15}NaO_{21}PS$=1884.69)

Synthesis of 4. 5-(and-6)-Carboxynaphthofluorescein (Mol. Probes P/N C-653, 33 mg, 0.058 mmole) was added to a slurry of β-(3-aminopropyl)cobalamin (**1**, 61 mg, 0.044 mmole), DMF (1.0 mL) and N,N-diisopropylethylamine (19 μL, 0.11 mmole). The reaction was stirred for 75 min., after which it was judged complete by HPLC analysis. MeOH (0.5 mL) was added to the reaction mixture and the solution was added to rapidly stirring 1:1 dichloromethane/diethyl ether (100 mL). The red solid was filtered on a medium frit and triturated with 2×10 mL each of dichloromethane, acetone, and finally diethyl ether. The compound **4** was dried over P_2O_5 *in vacuo*: 52 mg, 64% yield. The compound was purified by semi-preparative HPLC (see method below): 21 mg of **4** obtained. Analytical HPLC: 97% pure, retention time = 17.4 min; ES^+ MS: (1:1 H_2O/CH_3CN) M+H=1845.2 (calc. $C_{94}H_{111}CoN_{14}O_{20}P$=1845.72), $M+Na^+$=1867.4 (calc. $C_{65}H_{96}CoN_{14}O_{14}PNa$=1867.70)

Semi-Preparative HPLC method for 2-4. The compound was dissolved in a minimum of DMSO and injected on the HPLC and eluted with the following method: The HPLC flow rate was 40 mL/min. Column: Waters C-18, 15 micron, 25 mm × 100. Solutions: A: 50 mM H_3PO_4 brought to a pH of 3.0 with concentrated NH_4OH. B: 9:1 CH_3CN/H_2O. Method: 0-2 min: Isocratic elution of A, 2-6.8 min: ramp to 7:3 A/B, 6.8-10.3 min: ramp to 5:95 A/B 10.3-17.1 min: ramp to 95:5 A/B. The pure compound was collected in 18×150 mm test tubes and pure fractions were pooled. All CH_3CN was removed by rotary evaporation and the compound in H_2O was applied to a Waters C-18 Sep-Pak cartridge (820 mg C-18 adsorbent) that had been prepared by washing with 10 mL MeOH, followed by 20 mL H_2O. Salts were eluted from the Sep-Pak with 10 mL H_2O and the product was eluted with 5 mL MeOH. The alcohol was removed by rotary evaporation and the resulting aqueous solution was dried by lyophylization.

Synthesis of 5. Fluorescein-5-isothiocyanate (Mol. Probes P/N F-1906, 35 mg, 0.090 mmole) was added to a slurry of β-(3-aminopropyl)cobalamin (**1**, 104 mg, 0.0750 mmole), DMF (1.0 mL) and N,N-diisopropylethylamine (33 μL, 0.19 mmole). The reaction was stirred for 110 min. when it

contained 55% **5** and 8% **1** by HPLC analysis. MeOH (0.5 mL) was added to the reaction mixture and the solution was added to rapidly stirring 1:1 dichloromethane/diethyl ether (100 mL). The red solid was filtered on a medium frit and triturated with 2×5 mL each of dichloromethane, acetone, and finally diethyl ether. The compound **5** was dried over P_2O_5 *in vacuo*: 101 mg, 76% yield. The compound was purified by Semi-Preparative HPLC (see method below): 23 mg of **5** obtained. Analytical HPLC: 75%, retention time = 12.6 min; ES^+ MS: (1:1 H_2O/CH_3CN) M+H=1776.6 (calc. $C_{86}H_{108}CoN_{15}O_{19}PS$=1776.67), $M+Na^+$=1798.4 (calc. $C_{86}H_{107}CoN_{15}O_{19}PS$=1798.66)

Semi-Preparative HPLC method for 5. The HPLC flow rate was 40 mL/min. Column: Waters C-18, 15 micron, 25×100 mm. Solutions: A: 100 mM triethylamine brought to a pH of 7.0 with glacial acetic acid. B: 9:1 CH_3CN/H_2O. Method: 0-2 min: Isocratic elution of A, 2-10 min: ramp to 7:3 A/B, 10-15 min: ramp to 5:95 A/B 15-25 min: ramp to 95:5 A/B. The pure compound was collected in 18×150 mm test tubes and pure fractions were pooled. All CH_3CN was removed by rotary evaporation and the compound in H_2O was applied to a Waters C-18 Sep-Pak cartridge (820 mg C-18 sorbant) that had been prepared by washing with 10 mL MeOH, followed by 20 mL H_2O. Salts were eluted from the Sep-Pak with 10 mL H_2O and the product was eluted with 5 mL MeOH. The alcohol was removed by rotary evaporation and the resulting aqueous solution was dried by lyophylization.

Analytical HPLC method for 2-5. The HPLC flow rate was 2.0 mL/min. Column: Waters C-18, 15 micron, 3.9×100 mm. Solutions: A: 100 mM triethylamine brought to a pH of 7.0 with glacial acetic acid. B: 9:1 CH_3CN/H_2O. Method: 0-2 min: Isocratic elution of A, 2-20 min: ramp to 4.5:5.5 A/B, 20-25 min: ramp to 95:5 A/B.

Spectral Analysis. All spectra were recorded at 25°C. Rhodamine 6G and fluorescein dyes were used as references in determining the fluorescence quantum yield of compounds **2, 3,** and **4.**[1-3] Rhodamine 6G (1 mg/mL) was dissolved in ethanol and sodium fluorescein (1 mg/mL) was dissolved in 0.025 M sodium carbonate buffer at pH 9.5. Fluorescent cobalamins **2, 3,** and **4** were dissolved in 0.025 M sodium carbonate buffer (pH 9.5) at a final concentration of 1 mg/ml. All solutions were equilibrated at 25°C for 12 h. The stock solution of each cobalamin analog was diluted to make five additional solutions for the determination of the maximum absorbance wavelength values and the corresponding molar absorptivity of each compound. The refractive index value of compounds **2, 3,** and **4** was required to determine the relative fluorescence quantum efficiency.[1,3,4] Refractive index measurements were determined with 1×10^{-6} M solutions of the reference and sample compounds.

Fluorescence emission spectra of reference and sample compounds (1×10^{-6} M) were collected 90° to the angle of excitation, with the excitation and emission slit widths set to 1.6 mm. Wavelength scanning was carried out in increments of 1.0 nm with a 0.1 second integration time. The fluorimeter software (GRAMS/32) corrects raw spectra for fluctuations in the excitation source and integrates the intensity signal according to defined integration limits. Corrected spectra were employed in all quantum efficiency (ϕ) calculations.

Fluorescence Quantum Yield Calculation. The procedure for determining the fluorescence quantum yield follows established techniques,[1,2,4] and indicates the efficiency of photon energy transfer for compounds **2, 3,** and **4** relative to rhodamine 6G and fluorescein. The quantum yield is determined according to Equation 1:

$$\phi_s = \phi_r \left(\frac{D_s}{D_r} \right) \left(\frac{\eta_r^2}{\eta_s^2} \right) \left[\frac{\left(1 - 10^{-OD_r}\right)}{\left(1 - 10^{-OD_s}\right)} \right] \qquad (1)$$

where subscripts s and r refer to the sample and reference, respectively. The integrated area of the corrected emission peak in arbitrary units is given as D, η is the measured refractive index, and OD is the optical density at the excitation wavelength in arbitrary units. This method typically provides an estimate of the fluorescence quantum yield that is accurate to within 10% of the exact value.[1]

Two standards were utilized to determine the fluorescence quantum yields: rhodamine 6G[1] (ϕ = 0.95) and fluorescein[3] (ϕ = 0.92). To verify the suitability of this method, two internal controls were employed. First, rhodamine 6G was used as the sample compound and fluorescein was the reference compound. In the converse experiment, fluorescein was employed as the sample compound and rhodamine 6G was the reference compound. For each calculation, accepted standard values for ϕ_s were used, and the experimental values of ϕ determined for both reference compounds within experimental error.

Excitation and emission spectra for compounds **2, 3,** and **4** are shown in Figures 1A-C. The quantum yield values for the fluorescent cobalamin analogs are summarized in Table 1 along with the physical constants used in the calculation. Triplicate analyses were performed for each compound, and values from Table 1 indicate ϕ of individual cobalamin analogs are reproducible to within 0.02 when using either rhodamine 6G or fluorescein as the reference material.

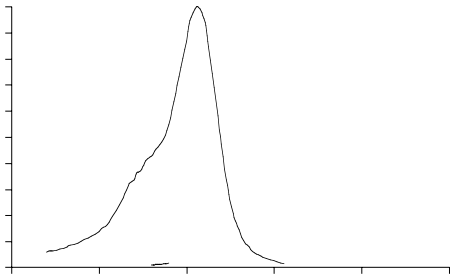
Table 1. Physical constants and quantum yield values

Compound	λ_{max}	ε ($M^{-1}cm^{-1}$)	OD	D	η	$\phi_{literature}$	$\phi_{exp.}$ [c]	ϕ_{exp} [d]
Fluorescein[a]	490 nm	8.0×10^4	0.0089	1746194	1.3339	0.92		0.99
Rhodamine 6G[b]	530 nm	10.0×10^4	0.023	40491731	1.3061	0.95	0.97	
2	510 nm	7.9×10^4	0.026	6995843	1.3337		0.15	0.16
3	494 nm	7.1×10^4	0.027	8561673	1.3345		0.12	0.14
4	604 nm	4.2×10^4	0.0086	215660	1.3339		0.012	0.013

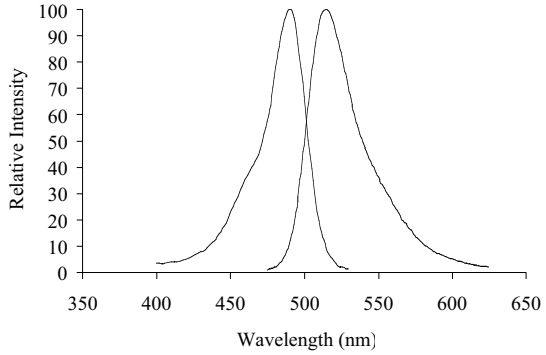
[a] See Reference 4
[b] See Reference 1
[c] Fluorescein reference material
[d] Rhodamine 6G reference material

The fluorescence quantum yield of compounds **2, 3,** and **4** are of a lower magnitude than either standard. The lower quantum efficiencies suggest the bioconjugates have some cryptofluorescent character, but are still overtly fluorescent. Similar behavior has been observed with other fluorescent cobalamin analogs.[5]

A



B



C

Figure 1. (A) Excitation and emission spectra of **2**, (B) excitation and emission spectra of **3**, (C) excitation and emission spectra of **4**.

References

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