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Facile synthesis of monofunctional pentamethine carbocyanine fluorophores

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ABSTRACT

A high-yield route to symmetric, conjugatable pentamethine carbocyanine dyes with far-red/near infrared (NIR) emission between 650 and 700 nm is reported. The dyes are prepared via condensation of indolium or benz[*e*]indolium inner salts with an alkyl carboxylic acid derivatized malonaldehyde dianil or alternatively in a one-pot reaction without isolation of the malonaldehyde intermediate. The fluorophores are water-soluble, have bright fluorescence emission, are easily prepared in good yield, and are promising candidates for use in a variety of biochemical and in vivo imaging applications.

1. Introduction

The use of conjugatable fluorescent dyes for bioimaging applications is ubiquitous [1]. Of these fluorescent reporters, far-red and near infrared (NIR) dyes that have both absorption and emission wavelengths between 600 and 1000 nm are ideal. These long-wavelength fluorophores minimize autofluorescence interference from tissue and have minimal overlap with biological chromophores such as hemoglobin [2]. NIR fluorophores are receiving widespread attention for use as fluorescent tags and as components of fluorogenic probes for in vivo imaging [3,4]. For example, NIR dyes conjugated to peptides or nanoparticles have been applied successfully to in vivo imaging of tumors [3–5], myocardial infraction [6] and inflammation [7]. Carbocyanine fluorophores have excellent optical properties, including tunable NIR emission, high extinction coefficients, and good fluorescence quantum yields [8]. Since the 1980s, a variety of carboxylic acid derivatized carbocyanines have been prepared to meet the increasing demand for their use in bioconjugation and imaging applications [9,10]. However, their widespread use is hindered by the high cost and limited availability of large quantities for many of these fluorescent labels. Most monofunctional carbocyanine dyes are asymmetric with the carboxylic acid functional group attached to one of the quaternary nitrogen atoms of the indolium or benz[*e*]indolium moieties. During the synthesis of these asymmetric dyes, undesired symmetric dyes are also formed (Scheme 1A). These symmetric dye byproducts are often difficult to separate from the desired monofunctional dyes and contribute to decreased synthetic yields of the intended asymmetric product, often significantly lower than 10% [11,12]. Therefore, a simple, high-yield synthetic route to monofunctional carbocyanine labels would allow for their expanded use in a variety of biochemical and in vivo imaging settings. One strategy to circumvent the disadvantages of asymmetric carbocyanine dye synthesis is to prepare symmetric heptamethine carbocyanines with fluorescence emission above 750 nm via either nucleophilic or Suzuki reactions with chloro-substituted cyclohexene cyanine dyes, which normally result in high conversion yields [13–15]. However, these procedures have not been demonstrated for the analogous pentamethine carbocyanine dyes with fluorescence emission between 650 and 700 nm. In this work, our focus is the development of new straightforward routes to symmetric, monofunctional pentamethine carbocyanine fluorophores, on which very few studies have been conducted [16].

2. Experimental

2.1. General materials and methods

Unless noted, all chemicals were purchased from Aldrich or TCI and were used as received. The indole and benz[*e*]indole precursors (2,3,3-trimethyl-3*H*-indole-5-sulfonic acid and 1,1,2-trimethyl-1*H*-benz[*e*]indole-7-sulfonic acid, respectively) were prepared and

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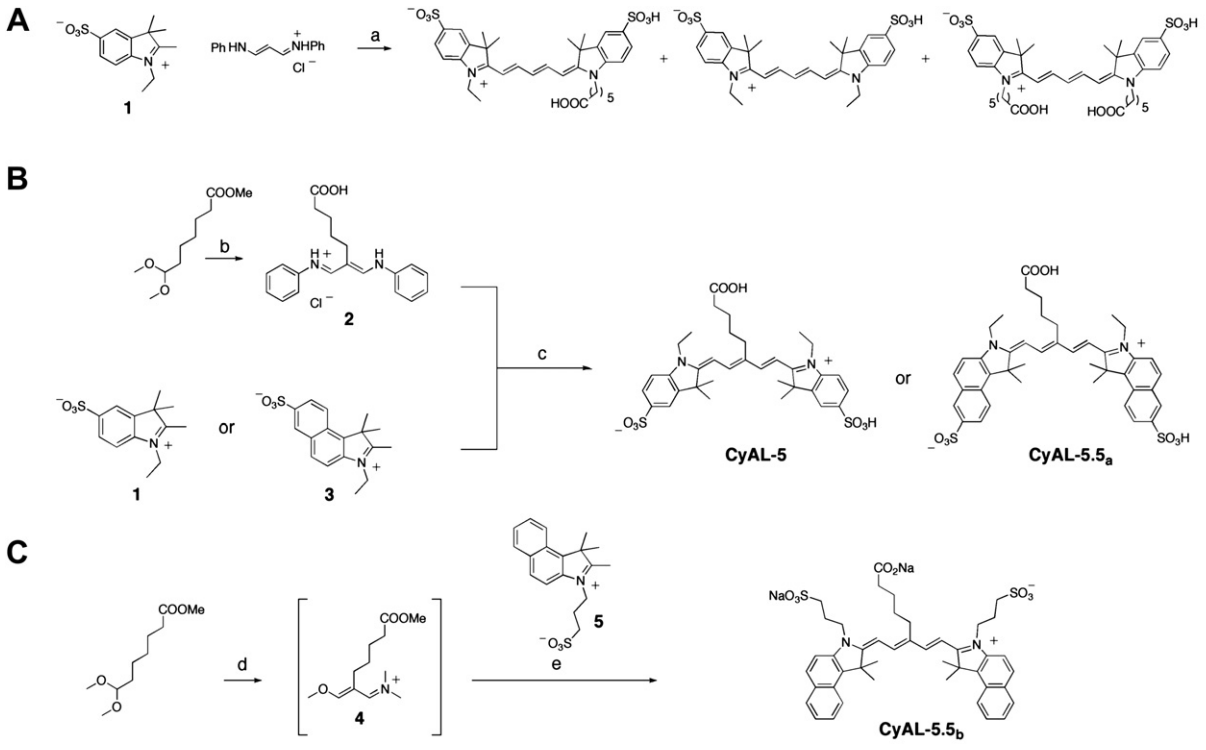

Scheme 1. (A) Traditional asymmetric synthetic procedure giving multiple undesired side products. (B and C) Multistep and one-pot synthetic procedures for the symmetric CyAL fluorophores.

alkylated with ethyl iodide to afford 1-ethyl-2,3,3-trimethyl-3*H*-indolium-5-sulfonate (**1**) and 3-ethyl-1,1,2-trimethyl-1*H*-benz[*e*]indolium-7-sulfonate (**3**), respectively, according to documented procedures [11,17]. 3-(1,1,2-Trimethyl-1*H*-benz[*e*]indolium-3-yl) propane-1-sulfonate (**5**) was synthesized in one step from commercially available 1,1,2-trimethyl-1H-benz[*e*]indole by alkylation with 1,3-propanesultone [14]. Methyl 7,7-dimethoxyheptanoate was purchased from AA Pharmaceuticals Inc. (Brighton, MA). All solvents were at least of reagent grade and were used without further purification. ^{1}H NMR spectra (400 MHz) were collected on a Bruker Advance-400 NMR spectrometer at ambient temperature. Chemical shifts were measured using tetramethylsilane (TMS) as an internal standard. High-resolution electrospray ionization (ESI) mass spectra were obtained on a Bruker Daltonics APEX IV 4.7 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS) in the Department of Chemistry Instrumentation Facility at the Massachusetts Institute of Technology. Low-resolution mass spectra were acquired on a Micromass ZQ 4000 mass spectrometer. Absorption spectra and extinction coefficients were obtained on a Varian Cary 50-Bio UV–visible spectrophotometer. Emission spectra were collected on a Varian Cary Eclipse fluorescence spectrophotometer. Fluorescence quantum yield measurements were performed on at least three samples for each dye in PBS, pH 7.0 with a maximum absorption for each sample of less than 0.1, using Cy-5 ($\Phi = 0.27$) and Cy-5.5 ($\Phi = 0.23$) as standards [18]. The standard deviation for both the extinction coefficient and quantum yield measurements is less than 10%.

2.2. Synthesis of malonaldehyde dianil intermediate (*2*)

Phosgene (50 mmol of a 20% w/w solution in toluene) was added to *N,N*-dimethylformamide (3.9 mL, 50 mmol) with stirring in an ice bath over 5 min to give a white paste. This mixture was allowed to stand until no further gas evolution was observed (~30 min). To the mixture was added methyl 7,7-dimethoxyheptanoate (5.1 g, 25 mmol) and the reaction was heated to 70 °C for 1 h. After cooling, the solvent was removed by rotary evaporation giving a yellow–brown oil. The oil was suspended in water (20 mL) then 5 mL of 10% aqueous HCl and aniline hydrochloride (6.5 g, 50 mmol) were added. The resulting mixture was sealed in a thick-walled glass pressure tube and heated at 120 °C in an oil bath for 1.5 h. After heating, the reaction solution was cooled slowly to room temperature over 2 h, during which the product crystallized (if the reaction is cooled too quickly, a sticky precipitate is formed). After filtration and washing with water, **2** is obtained as a yellow solid (2.05 g, 23%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.72 (d, 2H, $J = 12.0$ Hz), 7.58 (d, 4H, $J = 8.4$ Hz), 7.51 (t, 4H, $J = 8.0$ Hz), 7.30 (t, 4H, $J = 7.2$ Hz), 2.77 (t, 2H, $J = 6.4$ Hz), 2.27 (t, 2H, $J = 7.6$ Hz), 1.71–1.64 (m, 2H), 1.50–1.42 (m, 2H). HRMS–ESI $[M]^+$ *m/z* calcd. for $[C_{20}H_{23}N_2O_2]^+$ 323.1754, found 323.1743.

2.3. Synthetic protocol for CyAL-5 (an analogous protocol is used to prepare CyAL-5.5$_a$)

Four equivalents of indolium **1** (107 mg, 0.4 mmol) were dissolved with one equivalent of **2** (32 mg, 0.1 mmol) in 1 mL acetic acid/acetic anhydride/triethylamine (5:5:1). The desired dyes were then formed by heating the reaction solution at 115 °C for 45 min in a sealed thick-walled glass pressure tube. After solvent removal in vacuo, the crude product was purified by C18 cartridge chromatography eluting with 30% acetonitrile and 0.1% trifluoroacetic acid in water. CyAL-5 Yield, 26 mg, 39%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.18 (d, 2H, $J = 14$ Hz), 7.82 (s, 2H), 7.65 (d, 2H, $J = 8.2$ Hz), 7.35 (d, 2H, $J = 8.4$ Hz), 6.18 (d, 2H, $J = 14$ Hz), 4.24–4.19 (m, 4H), 2.63 (t, 2H,

J = 7.4 Hz), 2.31 (t, 2H, J = 8.0 Hz), 1.71 (s, 12H), 1.68 (t, 2H, J = 7.8 Hz), 1.50–1.47 (m, 2H), 1.28 (t, 6H, J = 7.2 Hz). HRMS–ESI $[M - 2H]^-$ m/z calcd. for $[C_{34}H_{41}N_2O_9S_2]^-$ 669.2310, found 669.2252. CyAL-5.5$_a$ Yield, 31 mg, 40%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.27–8.24 (m, 4H), 8.20 (d, 2H, J = 8.8 Hz), 8.16 (d, 2H, J = 8.8 Hz), 7.87 (d, 2H, J = 8.6 Hz), 7.76 (d, 2H, J = 8.8 Hz), 6.21 (d, 2H, J = 14.3 Hz), 4.31 (m, 4H), 2.68–2.64 (m, 2H), 2.09 (t, 2H, J = 7.0 Hz), 1.99 (s, 12H), 1.71 (t, 2H, J = 6.6 Hz), 1.53 (t, 2H, J = 7.2 Hz), 1.36 (t, 6H, J = 7.0 Hz). HRMS–ESI $[M - 2H]^-$ m/z calcd. for $[C_{42}H_{45}N_2O_9S_2]^-$ 769.2623, found 769.2493.

2.4. Synthetic protocol for CyAL-5.5$_b$

Oxalyl chloride (0.436 mL, 5 mmol) was added to *N,N*-dimethylformamide (0.386 mL, 5 mmol) with stirring in an ice bath over 5 min to give a white solid. After stirring for an additional 5 min, methyl 7,7-dimethoxyheptanoate (0.51 g, 2.5 mmol) was added and the mixture was heated at 70–75 °C for 1 h to generate reactive aminoformylation intermediate **4**. To the crude **4** was added acetic acid (8 mL), triethylamine (2 mL) and **5** (1.66 g, 5 mmol). The resulting mixture was heated in a sealed, thick-walled glass pressure tube on an oil bath at 120 °C for 2 h. Following solvent removal under reduced pressure, the residue was dissolved in water (50 mL), the pH was adjusted to 12 by careful addition of solid NaOH, and the resulting solution was heated at 70 °C for 3.5 h. Following this saponification of the methyl ester, the pH was adjusted to 7 with trifluoroacetic acid and the product was purified by reverse phase flash chromatography on a 70 g Varian Mega BE-C18 cartridge (cat# 12256081) eluting with 30% acetonitrile in water to afford the sodium salt of CyAL-5.5$_b$ as a dark blue solid. Yield, 0.60 g, 29%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.32 (d, 2H, J = 14.0 Hz), 8.22 (d, 2H, J = 8.5 Hz), 8.08 (d, 2H, J = 8.7 Hz), 8.06 (d, 2H, J = 5.7 Hz), 7.84 (d, 2H, J = 9.16 Hz), 7.67 (t, 2H, J = 7.3 Hz), 7.50 (t, 2H, J = 7.6 Hz), 6.33 (d, 2H, J = 13.7 Hz), 4.5 (m, 4H), 2.71 (t, 2H, J = 7.6 Hz), 2.65 (t, 4H, J = 6.4 Hz), 2.34 (t, 2H, J = 7.2 Hz), 2.09 (m, 4H), 1.98 (s, 12H), 1.78 (m, 2H), 1.19 (m, 2H). LRMS–ESI $[M]^+$ m/z calcd. for $[C_{44}H_{51}N_2O_8S_2]^+$ 799.3, found 799.3.

3. Results and discussion

In this new synthetic approach, we have shifted the location of the carboxylic acid moiety from the indolium or benz[*e*]indolium groups to the polymethine backbone of the dye molecule. This results in generation of symmetric, monofunctional carbocyanine fluorophores that are more easily prepared and purified than most traditional asymmetric carbocyanine dyes. Similar symmetric monofunctional Cy-5 analogs have been prepared through an intramolecular exchange reaction to generate dyes with a variety of functional groups attached to the polymethine backbone [16]. However, this procedure introduces an extra aromatic group on the fluorophore periphery, resulting in increased hydrophobicity and potential for aggregation in aqueous solution. Therefore, we developed in this work, a modified malonaldehyde dianil derivative bearing an alkyl carboxylic acid group. The malonaldehyde derivative (**2**) was synthesized in 23% yield via the Vilsmeier–Haack–Arnold aminoformylation of methyl 7,7-dimethoxyheptanoate (Scheme 1B). Malonaldehyde dianil **2** is suitable for condensation with the appropriate indolium or benz[*e*]indolium to yield the corresponding symmetric monofunctional dyes.

The synthesis of symmetric carbocyanine dyes often proceeds more smoothly and in higher yield than the corresponding asymmetric carbocyanines. This is in part due to the mixture of dye products that are generated in the preparation of the asymmetric dyes (Scheme 1A). The symmetric, water-soluble alkyl carboxylic acid derivatized dyes (CyAL-5 and CyAL-5.5$_a$) are prepared by condensation of malonaldehyde dianil **2** with indolium **1** or benz[*e*] indolium **3** using a mixture of acetic anhydride, acetic acid and triethylamine as solvent in 39, and 40% yield for CyAL-5 and CyAL-5.5$_a$, respectively (Scheme 1B).

Table 1
Optical properties of the fluorophores in PBS, pH 7.0.

	$\lambda_{max,abs}$ (nm)[a]	$\lambda_{max,em}$ (nm)	ε (M^{-1} cm^{-1})[b]	Φ[c]
CyAL-5	643	661	230,000	0.13
CyAL-5.5$_a$	674	692	160,000	0.12
CyAL-5.5$_b$	674	693	130,000	0.08

[a] Spectra were obtained in PBS, pH 7.0.
[b] Extinction coefficients, at the dye absorption maxima, were performed in triplicate.
[c] Emission spectra were excited at 620 nm for CyAL-5 and 640 nm for CyAL-5.5$_a$ and CyAL-5.5$_b$. Cy-5 and Cy-5.5 were used as fluorescence standards, respectively [10,11]. The data are the average of at least 3 replicates and errors were <10%.

In an effort to optimize the synthesis and improve the overall reaction yield a modified multi-stage procedure was developed that does not require isolation of the malonaldehyde dianil precursor. The initial products generated from aminoformylation of alkyl acetals are 3-methoxy *N,N*-dimethylpropeniminium derivatives (compound **4**, Scheme 1C) [19]. We have found that the propeniminium intermediates react readily with indoliums or benz[*e*]indoliums to generate carbocyanine fluorophores and therefore isolation of malonaldehyde dianil **2** is unnecessary. In a one-pot procedure the initial aminoformylation product of methyl 7,7-dimethoxyheptanoate was allowed to react with **5** generating the methyl ester of CyAL-5.5$_b$. 3-(1,1,2-Trimethyl-1**H**-benz[**e**]indolium-3-yl)propane-1-sulfonate (**5**) was employed in place of benz[*e*]indolium **3** because it can be prepared easily in one step with 90% or greater yield from commercially available 1,1,2-trimethyl-1H-benz[e]indole [14]. Once generated, the methyl ester of the fluorophore is hydrolyzed by heating in pH 12 aqueous NaOH to yield CyAL-5.5$_b$ in 29% overall

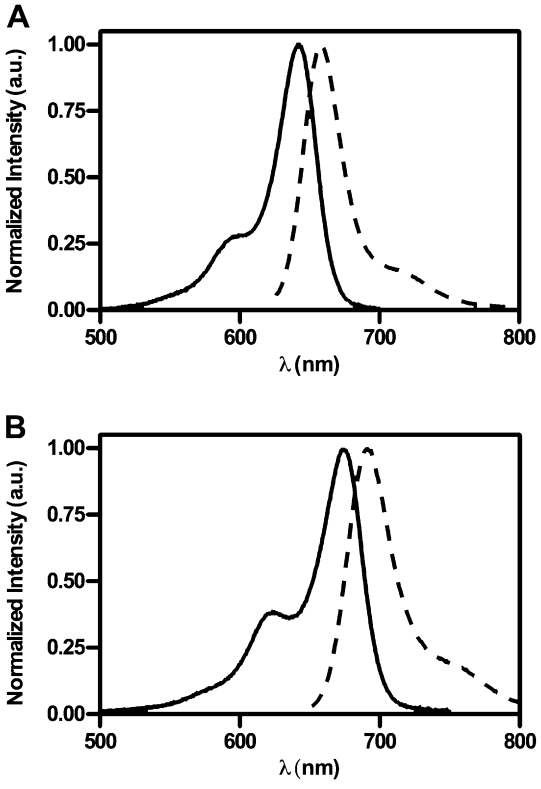

Fig. 1. Absorption (solid lines) and emission (dashed lines) spectra of CyAL-5 (A) and CyAL-5.5$_a$ (B) in PBS, pH 7.0.

yield. This is a significant improvement over the 9% overall yields (based on methyl 7,7-dimethoxyheptanoate) obtained for the synthesis of CyAL-5 and CyAL-5.5$_a$.

All three fluorophores are easily purified by reverse phase column chromatography using inexpensive pre-packed C18 cartridges. The obtained dyes are of high purity (Fig. S1), are suitable for both chemical and optical characterization, and can be converted into their corresponding reactive succinimidyl esters by treatment with 4 equivalents of *N,N′*-disuccinimidyl carbonate and 8 equivalents of triethylamine in anhydrous DMF. The modified fluorophores are water-soluble, have extinction coefficients greater than 100,000 M^{-1} cm^{-1}, and fluorescence quantum yields between 8 and 13%. These fluorescence quantum yield values agree with the reported quantum yields of analogous carbocyanine dyes with similar sulfonation patterns [10,11]. The optical properties of the fluorophores are summarized in Table 1 and photostability studies are shown in Fig. S2. Fig. 1 shows both CyAL-5 and CyAL-5.5$_a$ have absorption and emission spectra in the far-red to near infrared region that match well with common filter sets used for imaging commercially available Cy-5 and Cy-5.5 [18].

4. Conclusions

Two new synthetic procedures for the synthesis of monofunctional pentamethine carbocyanine dyes have been developed. By incorporating an alkyl carboxylic acid group into the malonaldehyde intermediate, instead of attaching the acid functionality to the indolium or benz[*e*]indolium moieties as in conventional reactions, the bioconjugatable cyanine dyes are formed cleanly in simple condensation reactions with good yields. The new fluorescent labels have large extinction coefficients, bright far-red/NIR emission, and are water-soluble. These new carbocyanine fluorophores, because of their straightforward synthesis, easy purification, and excellent optical characteristics are well suited for use in a variety of biochemical and in vivo imaging applications.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.dyepig.2010.12.008.

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