Dyes and Pigments 99 (2013) 275–283

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Structurally modified indocyanine green dyes. Modification of the polyene linker

Innus Mohammad [a], Courtney Stanford [d], Martha D. Morton [c], Quing Zhu [b], Michael B. Smith [a,*]

[a] Department of Chemistry, University of Connecticut, 55 N. Eagleville Road, Storrs, CT 06269-3060, United States
[b] Department of Electrical and Computer Engineering, University of Connecticut, 371 Fairfield Way, Storrs, CT 06269-2157, United States
[c] Department of Chemistry, University of Nebraska, Hamilton Hall 838, Lincoln, NE 68588, United States
[d] The University of Iowa, E331 Chemistry Building, Iowa City, IA 52242-1294, United States

ARTICLE INFO

Article history:
Received 31 October 2012
Received in revised form
25 April 2013
Accepted 1 May 2013
Available online 10 May 2013

Keywords:
Indocyanine
Fluorescence
Near infrared
Dye
Synthesis
Absorbance

ABSTRACT

We have prepared a series of indocyanine green dicarboxylic acid derivatives (**9a,b, 4, 10, 13**) with modified polyene linkers in an attempt to increase the structural rigidity of the polyene linker and thereby the fluorescent yield. Incorporation of five- and six-membered rings into the polyene system led to lower florescent yield for **9a,b, 4** and **10**, but shortening the chain by two carbon atoms led to an increase in fluorescent yield for **13**.

1. Introduction

Fluorescence imaging is an optical imaging technique proven to be a useful tool for diagnostic imaging without the complications that often accompany the use of nuclear contrast agents [1]. In fluorescence imaging, a near infrared (NIR) fluorescent probe (>700 nm) is administered, and irradiation with NIR light allows collection of the remitted fluorescence for imaging. Fluorescence imaging has great potential for probing tumor molecular markers associated with tumor proliferation, growth, and metastasis. Indocyanine dyes are important abiotic molecules [2–5], and useful NIR probes. An important member of this family is the clinically approved indocyanine green (ICG, **1**; Scheme 1), which has many applications for fluorescence imaging [6,7]. ICG is the only FDA approved agent that can be used for human subjects, due to low toxicity and high absorbance in the NIR spectrum. However, applications of ICG are mitigated by several limitations, including optical instability in the body, low quantum yield, and promiscuous leakage in blood vessels. The use of ICG in molecular imaging probes may be limited due to loss of fluorescence after protein binding. Despite these problems, the NIR probe remains available after cell binding and internalization, due to the fact that ICG may be dissociated from the targeting antibody, thus activating fluorescence [8]. It is known, for example, that ICG binds to plasma proteins, and protein-bound ICG emits light with a peak wavelength of about 830 nm in the NIR. [9]. An attempt to improve the targeting capability of the dye, as it affects NIR imaging of cancerous tumors, led us to attach of a cancer targeting 2-nitroimidazole moiety to the fluorescent dye in order to improve detection of the tumor.

We prepared bis(carboxylic acid) derivative **2** using literature procedures, and used it to synthesize dye-conjugates **3** by linking two nitroimidazoacetate units to the dye via ethanolamine linkers [10,11]. The purpose of this research was and is to develop a non-invasive probe for the detection of cancerous tumors using NIR fluorescent dyes that contain a nitroimidazole moiety known to target hypoxic tumors [10,12]. The quantum yield for the dyes used in the study were about 0.06, which was sufficient for the NIR detection of hypoxic cancerous tumors *in vivo* [10]. To further develop this work, we targeted the synthesis of dyes with a larger

* Corresponding author.
E-mail address: michael.smith@uconn.edu (M.B. Smith).

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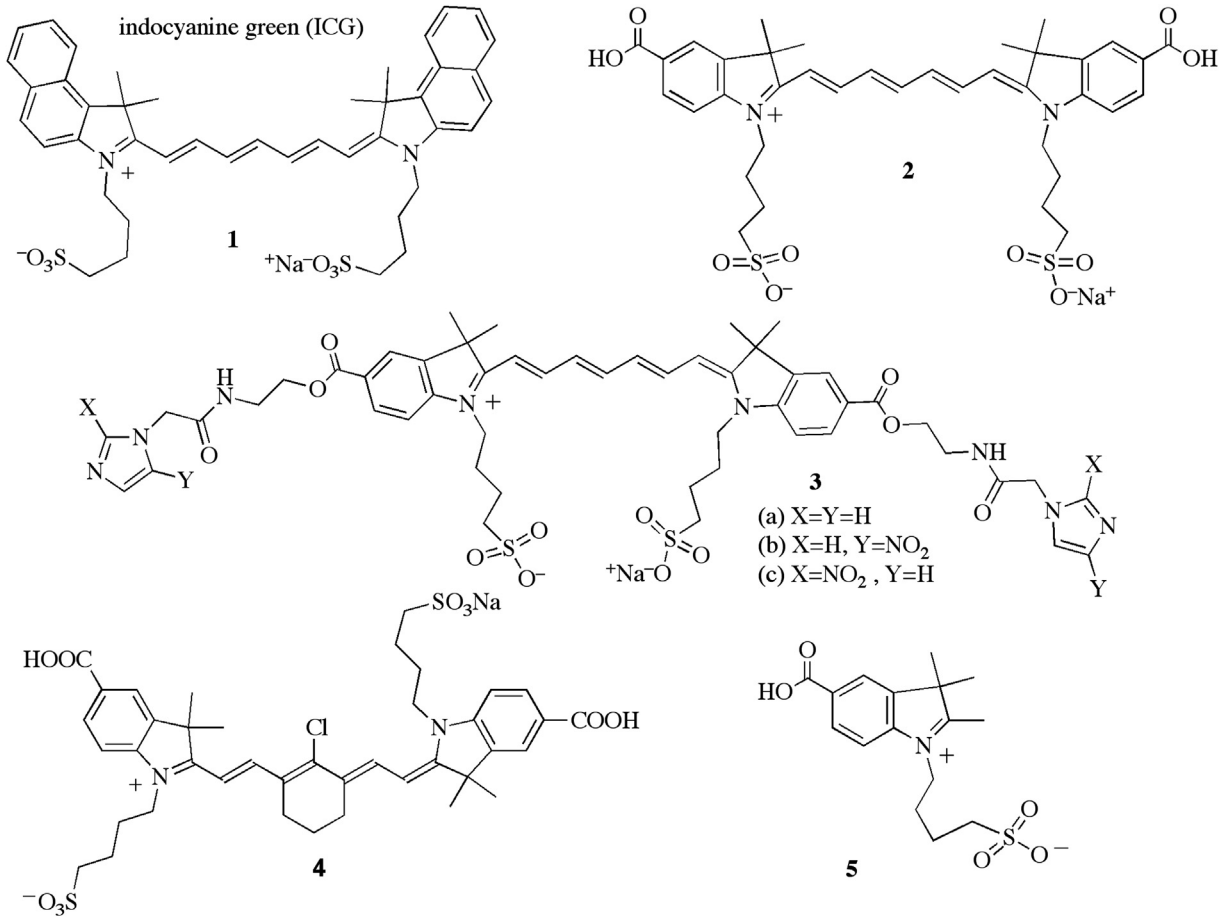
http://dx.doi.org/10.1016/j.dyepig.2013.05.001



Scheme 1. ICG, **1**, ICG-dicarboxylic acid derivative **2,** imidazole-ICG conjugate **3**, dye **4**, and indolium **5**.

quantum yield with the goal of increasing the sensitivity of our tumor detection system. However, any structural changes should retain the emission/absorption wavelengths in the NIR region. Once the synthetic work was completed, the next step was to examine parameters that influence the fluorescent yield of the dye, without significant perturbation of the emission and absorption wavelengths.

It is known that conversion of a nonplanar molecule to a similar but more planar and rigid molecule often leads to an increase in the quantum yield of fluorescence [13]. Decreases in fluorescence may be associated with a higher collisional probability observed with molecules that have a high degree of flexibility. In other words, molecules with more rigid structures have a lower probability of collision, which leads to a higher fluorescence potential, because the energy absorbed is not lost due to molecular vibration or collision. It has been shown, however, that rigidity (maintenance of a planar or near planar configuration) in the first excited S^1 excited state is more important than rigidity in the S_0 state [14]. One possible structural change that might lead to rigidity is incorporation of a ring into the polyene chain. Tung and co-workers reported just such a molecule as a near infrared fluorochrome, in their synthesis of compound **4** (reported as compound NIR820) [15]. In earlier work, Reynolds and Drexhage had prepared heptamethine pyrylium dyes that contained a ring in the polyene moiety [16]. These dyes were relatively unstable and exhibited a bathochromic shift. Slominskii et al. prepared a series of dyes containing a polyene moiety (methine dyes), including examples with a cyclohexane ring [17]. It is noted that the reaction of **4** with amines produced a new class of NIR amine tricarbocyanine dyes with excellent photostability properties [18]. Similarly, reaction with amines followed by conversion to the corresponding amide produced NIR amide tricarbocyanine dyes that were further modified to include lipoic acid residues used for cancer imaging due their ability to serve as highly sensitive NIR SERS reporter molecules [19].

For this study, we prepared **4** as well as several related indocyanine dyes, first with a six- or a five-membered ring incorporated into the polyene system (**9a,b** and **10**), and another derivative with a shorter polyene chain (**13**). Our primary interest was the synthesis of these compounds and determination of changes in florescent yield as a function of structure, if any. In Tung and coworker's work, **4** was used for protein labeling experiments and **4** was coupled to a peptide as an amino acid residue by initial conversion to the NHS activated ester [15,20]. Human transferrin (Tf) was used as a model protein and coupling of activated **4** with Tf gave a conjugate that facilitated florescence measurements in PBS solution as well as the protein binding studies.

Our long term goal for any new dye is to eventually prepare derivatives of **3** for targeting hypoxia in cancerous tumors rather than protein labeling experiments. The first step toward that goal requires synthesis of the dye and a study of the chemical and *in vitro* physical properties of the dye. Therefore, work from this laboratory reports the chemical synthesis, chemical properties, and *in vitro* stability studies in PBS and sucrose solution, as well as emission/absorbance data of the dyes. Dye-conjugates such as **3** are required for our *in vivo* studies and they were not prepared as part of this work, which focuses on identifying a new dye with improved fluorescent properties. It is important to point out that protein binding studies such as those published by Tung and co-workers

are beyond the scope of our current studies would take our research in a different direction.

Indeed, for this study we were primarily interested in the effects of a more rigid ring versus incorporation of more atoms into the structure, and also how the length of the conjugated chain would influence fluorescence. We found that incorporation of a ring diminished the fluorescence yield, but shortening the polyene chain increased the fluorescence yield.

2. Materials and methods

All glassware was oven-dried under vacuum, and all reactions in organic solvents were performed under a nitrogen atmosphere, unless otherwise noted. 3-Methyl-2-butanone, butane sultone, methylcyclohexene, phenylcyclohexene, cyclohexanone, and **11** were obtained from Aldrich Chemical Co. Indole derivative **5** and dye **2** were prepared using our previously reported procedure [10]. *N*-Formyl-*N*-methylaniline (**6**) was prepared in 68% yield by the reaction of commercially available N-methylaniline with formic acid and sodium formate [21]. All solvents were dried according to standard procedures. THF was distilled from sodium benzophenone ketyl, methylene chloride was distilled from calcium hydride, and dimethylformamide was vacuum distilled from calcium hydride. Thin-layer chromatography was done on Sorbent Technologies aluminum-backed TLC plates with fluorescent indicator and 0.2 mm silica gel layer thickness, and *p*-anisaldehyde or phosphomolybdic acid were used as developing agents. Column chromatography was done using 60 Å porosity, 32–63 μm silica gel. ^{1}H and ^{13}C NMR were collected on a Brüker Avance 300 (300.13 MHz for ^{1}H, 75.48 MHz for ^{13}C), Brüker DRX-400 (400.144 MHZ ^{1}H, 100.65 MHz ^{13}C) or a Brüker Avance 500 (500.13 MHz for ^{1}H, 125.65 MHz for ^{13}C). Chemical shifts are given in ppm downfield from TMS in the following format chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) coupling constants *J* in Hz. Mass spectrometry data was collected on a HP 5870B GC/MSD mass spectrometer with an HP-1 column, and high resolution mass spectrometry was done on a Micromass VB-QTOF tandem mass spectrometer. IR spectra were taken on FT/IR-410/C031560585 JASCO and Nexus 670 FT-IR E.S.P., neat, unless otherwise stated. UV–Vis spectra were recorded on Cary 50 spectrometer and fluorescence spectra on a Cary Eclipse. Melting points were taken on a Uni-melt capillary melting point apparatus and Digimelt MPA160 and recorded to a maximum of 270 °C. For products described as waxy solid, melting points could not be obtained.

2.1. N-((E)-(2-Methyl-3-((E)-(phenylimino)methyl)cyclohex-2-enylidene)methyl)aniline, 7a

A stirred solution of *N*-formyl-*N*-methylaniline (**6**, 2.11 g, 15.6 mmol) in chloroform (2 mL) at −5 °C was treated with phosphorous oxychloride (1.5 mL, 15.6 mmol), dropwise, and stirred for 1 h at 10 °C. Methylcyclohexene (0.6 mL, 5.2 mmol) was added dropwise and the solution stirred at 45 °C for 20 h. The reaction mixture was poured into a beaker containing vigorously stirred water (20 mL). Solid potassium carbonate (2 g, 14.5 mmol) was added carefully added. A solution of aniline hydrochloride salt (1.52 g, 11.7 mmol) in water (3 mL) was added and the mixture stirred at ambient temperature for 30 min. At this time, potassium carbonate (2 g, 14.5 mmol) was added portionwise and the resulting solution cooled to give a precipitate that was filtered, washed several times with cold water and stirred vigorously with acetone (2 × 3 mL) filtered and dried *in vacuo* to afford **7a** (0.68 g, 2.0 mmol, 38.6%) [22]. Mp = 207–210 °C; ^{1}H NMR (400 MHz, DMSO-*d6*) d 8.47 (s, 2 H), 7.48–7.43 (m, 8 H), 7.28–7.24 (m, 2 H), 2.60–2.57(m, 7 H), 1.98–1.91 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-*d6*) d 165.9, 147.7, 139.8, 129.6, 129.5, 129.3, 125.6, 118.8, 117.5, 115.1, 29.1, 23.5, 19.8, 14.3.

2.2. N-((E)-(2-phenyl-3-((E)-(phenylimino)methyl)cyclohex-2-enylidene)methyl)aniline, 7b

A stirred solution of *N*-formyl-*N*-methylaniline (**6**, 1.28 g, 9.5 mmol) in chloroform (2 mL) at −5 °C was treated with phosphorous oxychloride (0.9 mL, 9.5 mmol), dropwise, and stirred for 1 h at 10 °C. Phenylcyclohexene (0.5 g, 3.2 mmol) was added dropwise and then stirred at 45 °C for 20 h. The reaction mixture was poured into a beaker of vigorous stirred water (20 mL), and potassium carbonate (2 g, 14.5 mmol) was carefully added. A solution of aniline hydrochloride salt (0.92 g, 7.11 mmol) in water (3 mL) was added and the mixture stirred at ambient temperature for 30 min. After addition of potassium carbonate (2 g, 14.5 mmol) portionwise, the mixture was cooled and the resulting precipitate was filtered, washed several times with cold water, stirred vigorously with acetone (2 × 3 mL), filtered and dried *in vacuo* to afford **7b** as a dark red powder (0.85 g, 2.12 mmol, 67.5%) [22]. Mp = 246–250 °C; ^{1}H NMR (400 MHz, DMSO-d^6) d 11.01 (s, 2 H), 7.57–7.55 (m, 3 H), 7.42–7.40 (m, 2 H), 7.35 (t, *J* = 8 Hz, 4 H), 7.25 (bs, 2 H), 7.15 (t, *J* = 8 Hz, 2 H), 7.07 (d, *J* = 8 Hz, 4 H); ^{13}C (100 MHz, DMSO-*d6*) d 157.8, 150.5, 142.0, 139.8, 136.2, 135.3, 134.7, 130.6, 130.2, 129.8, 129.5, 128.3, 128.0, 125.8, 121.2, 118.7, 118.2, 114.6, 113.6, 23.9, 23.3, 21.9, 20.5, 19.8.

2.3. N-((E)-(2-chloro-3-((E)-(phenylimino)methyl)cyclohex-2-enylidene)methyl)aniline, 7c

Phosphorus oxychloride (22 mL, 0.12 mol) was added dropwise to dimethylformamide (**6**, 26 mL, 0.17 mol) at 0 °C, and the resulting solution was stirred for 30 min. Cyclohexanone (11 mL, 0.053 mol), was added and the reaction mixture was heated at reflux for 1 h. The solution was cooled to ambient temperature, 36 mL aniline/ethanol (1:1 (v/v)) was added dropwise and the solution stirred for 30 min. The reaction mixture was poured into 220 mL of ice cold water/concentrated HCl (10:1) and cooled for 2 h in an ice bath. Subsequent filtration gave a solid that was washed with cold water, diethyl ether and then acetone to give **7c**. (15.3 g, 42.7 mmol, 40.1%) [23]. Mp = 190–193 °C; ^{1}H NMR (400 MHz, DMSO-d^6) d 8.29 (s, 2 H), 7.44–7.42 (m, 4 H), 7.25–7.18 (m, 6 H), 7.05–7.02 (m, 2 H); ^{13}C NMR (100 MHz, MeOD-*d4*) d 173.7, 168.6, 151.0, 146.0, 145.1, 141.5, 131.2, 128.8, 123.5, 110.9, 102.6, 50.5, 49.3, 44.2, 27.3, 27.1, 26.2, 26.1, 22.4, 20.9.

2.4. N-((E)-(2-chloro-3-((E)-(phenylimino)methyl)cyclopent-2-enylidene)methyl)aniline, 8

A stirred solution of *N*-methylformanilide (**6**, 13.5 g, 99.9 mmol) in chloroform (13 mL) was treated with phosphorous oxychloride (14 mL, 153 mmol) at 10 °C and the stirred at ambient temperature for 1 h. Cyclopentanone (3.36 g, 39.9 mmol) was added and the solution stirred at 50 °C for 4 h, cooled to ambient temperature and potassium carbonate (10 g, 72.5 mmol) was added, followed by aniline (8.4 g, 90.2 mmol), concentrated HCl (7.5 mL) and water (50 mL) in that order. Subsequent stirring for 1 h at ambient temperature was followed by addition of CH_2Cl_2, A solid precipitated formed, which was filtered and washed with acetone (5 × 10 mL) and water (5 × 20 mL) to afford **8** (10 g, 29.0 mmol, 72.7%) [23]. Mp = 195–198 °C; ^{1}H NMR (400 MHz, DMSO-d^6) d 11.40 (bs, 2 H), 8.30 (s, 2 H), 7.62–7.60 (m, 4 H), 7.46 (t, *J* = 8 Hz, 4 H), 7.27–7.24 (m, 2 H), 3.02 (s, 4 H); ^{13}C NMR (100 MHz, DMSO-*d6*) d 142.8, 140.5, 139.6, 139.3, 138.1, 130.2, 129.7, 129.5, 126.0, 122.9, 118.5, 116.3, 114.9, 110.6, 26.1.

2.5. Sodium 2-((E)-2-((E)-3-((Z)-2-(5-carboxy-3,3-dimethyl-3-(4-sulfonatobutyl)Indolin-2-ylidene)ethylidene)-2-methylcyclohex-1-enyl)vinyl)-3,3-dimethylindole-1-sulfonate-5-carboxylic acid, 9a

A vigorously stirred solution of 3-(5-carboxy-2,3,3-trimethyl-3*H*-indolium-1-yl)propane-1-sulfonate (**5**, 0.107 g, 0.32 mmol) and **7a** (0.05 g, 0.15 mmol) in acetic anhydride (1 mL) and acetic acid (0.5 mL) was treated with sodium acetate (0.041 g, 0.5 mmol) and heated at reflux (120 °C) for 45 min. The reaction mixture was cooled to ambient temperature and anhydrous diethyl ether (5 mL) was added. The resulting precipitate was isolated by vacuum filtration to give a crude solid that was recrystallized (methanol: water) to give **9a** as a green solid (0.030 g, 0.04 mmol, 25%). Mp = charring at 255–260 °C; ^{1}H NMR (400 MHz, MeOD) d 8.20 (d, J = 16 Hz, 2 H), 8.09–8.06 (m, 2 H), 7.34 (d, J = 8 Hz, 2 H), 6.34 (d, J = 12 Hz, 2 H), 4.21–4.18 (m, 4 H), 2.91–2.88 (m, 4 H), 2.66–2.63 (m, 4 H), 2.49 (s, 3 H), 2.05–1.90 (m, 10 H), 1.76 (s, 12 H); ^{13}C NMR (100 MHz, MeOD) d 173.4, 158.4, 146.8, 142.3, 134.1, 124.6, 111.4, 102.6, 52.0, 45.2, 28.7, 27.4, 26.6, 23.8, 15.4; HRMS (TOF). Calcd for $C_{41}H_{51}N_2O_{10}S_2$ (M^+ + H) 795.2985 Obs. 795.2964.

2.6. Sodium 2-((E)-2-((E)-3-((Z)-2-(5-carboxy-3,3-dimethyl-3-(4-sulfonatobutyl)Indolin-2-ylidene)ethylidene)-2-phenylcyclohex-1-enyl)vinyl)-3,3-dimethylindole-1-sulfonate-5-carboxylic acid, 9b

A vigorously stirred solution of 3-(5-carboxy-2,3,3-trimethyl-3*H*-indolium-1-yl)propane-1-sulfonate (**5**, 0.36 g, 1.06 mmol) and **7b** (0.2 g, 0.5 mmol) in acetic anhydride (1.5 mL) and acetic acid (1 mL) was treated with sodium acetate (0.139 g, 1.7 mmol) and heated at reflux (120 °C) for 45 min. The reaction mixture was cooled to ambient temperature and anhydrous diethyl ether (15 mL) was added. The resulting precipitate was isolated by vacuum filtration to give a crude solid that was recrystallized (methanol: water) to give **9b** as a green solid (0.025 g, 0.03 mmol, 57%). Mp = charring at 255–260 °C; ^{1}H NMR (400 MHz, MeOD) d 8.04 (d, J = 8 Hz, 2 H), 7.91 (s, 2 H), 7.63 (t, J = 8 Hz, 3 H), 7.34 (t, J = 8 Hz, 4 H), 7.26 (d, J = 4 Hz, 2 H), 6.32 (d, J = 12 Hz, 2 H), 4.16 (m, 4 H), 2.88 (t, J = 8 Hz, 4 H), 2.77 (m, 4 H), 2.07 (m, 2 H), 1.93 (m, 8 H), 1.21 (s, 12 H); ^{13}C NMR (100 MHz, MeOD) d 175.6, 169.2, 156.8, 147.4, 142.8, 132.3, 128.7, 128.6, 124.6, 112.0, 106.0, 51.7, 50.4, 45.1, 27.9,27.2, 23.5; HRMS (TOF). Calcd for $C_{46}H_{53}N_2O_{10}S_2$ (M^+ + H) 857.3142 Obs. 857.3097.

2.7. Sodium 2-((E)-2-((E)-3-((Z)-2-(5-carboxy-3,3-dimethyl-3-(4-sulfonatobutyl)Indolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-enyl)vinyl)-3,3-dimethylindole-1-sulfonate-5-carboxylic acid, 4 [15]

A vigorously stirred solution of 3-(5-carboxy-2,3,3-trimethyl-3*H*-indolium-1-yl)propane-1-sulfonate (**5**, 0.1 g, 0.29 mmol) and **7c** (0.05 g, 0.14 mmol) in acetic anhydride (1 mL) and acetic acid (0.5 mL) was treated with sodium acetate (0.039 g, 0.47 mmol) and heated at reflux (120 °C) for 45 min. The reaction mixture was cooled to ambient temperature and anhydrous diethyl ether (5 mL) was added. The resulting precipitate was isolated by vacuum filtration to give a crude solid that was recrystallized (methanol: water) to give **4** as a green solid (0.055 g, 0.07 mmol, 47%) [15]. Mp = charring at 130–140 °C; ^{1}H NMR (400 MHz, MeOD) d 8.49 (d, J = 16 Hz, 2 H), 8.13-8.10 (m, 4 H), 7.45 (d, J = 8 Hz, 2 H), 6.43 (d, J = 12 Hz, 2 H), 4.26 (t, J = 8 Hz, 4 H), 2.90–2.88 (m, 4 H), 2.80–2.77 (m, 4 H), 2.05–1.99 (m, 10 H), 1.77 (s, 12 H); ^{13}C NMR (100 MHz, MeOD) d 174.9, 169.8, 152.1, 147.2, 146.3, 142.7, 132.4, 130.0, 124.7, 112.1, 103.7, 45.3, 28.5, 28.3, 27.4, 27.2, 23.6, 22.1; HRMS (TOF). Calcd for $C_{40}H_{48}N_2O_{10}S$ (M^+ + H) 815.2439 Obs. 815.2451.

2.8. Sodium 2-((E)-2-((E)-3-((Z)-2-(5-carboxy-3,3-dimethyl-3-(4-sulfonatobutyl)Indolin-2-ylidene)ethylidene)-2-chlorocyclopent-1-enyl)vinyl)-3,3-dimethylindole-1-sulfonate-5-carboxylic acid, 10

A vigorously stirred solution of 3-(5-carboxy-2,3,3-trimethyl-3*H*-indolium-1-yl)propane-1-sulfonate (**5**, 0.197 g, 0.58 mmol) and **8** (0.1 g, 2.9 mmol) in acetic anhydride (1.5 mL) and acetic acid (1 mL) was treated with sodium acetate (0.084 g, 1.02 mmol) and heated at reflux (120 °C) for 45 min. The reaction mixture was cooled to ambient temperature and anhydrous diethyl ether (10 mL) was added. The resulting filtrate was isolated by vacuum filtration to give a crude solid that was recrystallized (methanol: water) to give **10** as a green solid (0.06 g, 0.07 mmol, 25%). Mp = charring at 145–150 °C; ^{1}H NMR (400 MHz, MeOD) d 8.10–8.08 (m, 4 H), 7.97 (d, J = 16 Hz, 2 H), 7.40 (d, J = 8 Hz, 2 H), 6.25 (d, J = 16 Hz, 2 H), 4.23 (t, J = 8 Hz, 4 H), 2.90–2.87 (m, 4 H), 2.02–1.94 (m, 8 H), 1.75 (s, 12 H); ^{13}C NMR (100 MHz, DMSO-*d6*) d 167.8, 167.0, 153.9, 148.6, 145.9, 141.2, 138.4, 137.3, 130.8, 123.4, 122.6, 103.9, 99.4, 53.8, 51.3, 51.1, 50.7, 48.8, 44.5, 28.2, 27.7, 27.4, 26.1, 23.0, 22.7, 22.5, 20.1; HRMS (TOF). Calcd for $C_{39}H_{45}ClN_2O_{10}S_2$ (M^+ + H) 801.2282 Obs. 801.2367.

2.9. N-((1E,3E)-3-(phenylimino)prop-1-enyl)benzenamine hydrochloride (12)

A solution of propanedial bis(dimethyl acetal) (**11**, 5.25 mL, 0.03 mol) and HCl (4.25 mL) in distilled water (90 mL) was treated with a solution of distilled water (70 mL), HCl (5 mL) and aniline (3.7 mL, 0.04 mol) at 50 °C. The resulting solution was stirred at 50 °C for 30 min, cooled, and the resulting precipitate was isolated by vacuum filtration and dried *in vacuo* to yield **12** as an orange solid (6.35 g, 24.5 mmol, 81.8%) [24]. Mp = charring at 150 °C; ^{1}H NMR (400 MHz, $CDCl_3$) d 8.72 (d, J = 12 Hz, 2 H), 7.45–7.38 (m, 9 H), 7.24 (t, J = 8 Hz, 2 H), 6.28 (t, J = 12 Hz, 1 H); ^{13}C NMR (100 MHz, DMSO-d^6) d 159.6, 139.8, 131.1, 131.0, 127.6, 118.8, 118.7, 99.5, 99.4.

2.10. Sodium 4-[2-[(1E,3E,5Z)-7-[1,1-dimethyl-3-(4-sulfonatobutyl) indol-2-ylidene]penta-1,3-dienyl]-1,1-dimethylindol-3-ium-3-yl] butane-1-sulfonate, 13

A vigorously stirred solution of 3-(5-carboxy-2,3,3-trimethyl-3*H*-indolium-1-yl)propane-1-sulfonate (**5**, 0.14 g, 0.41 mmol) and **12** (0.05 g, 0.19 mmol) in acetic anhydride (1 mL) and acetic acid (0.5 mL) was treated with sodium acetate (0.054 g, 0.66 mmol) and heated at reflux (120 °C) for 45 min. The reaction mixture was cooled to ambient temperature and anhydrous diethyl ether (5 mL) was added. The resulting precipitate was isolated by vacuum filtration to give a crude solid that was recrystallized (methanol: water) to give **13** as a blue solid (0.09 g, 0.12 mmol, 63.4%). Mp = decomposition at 287 °C; ^{1}H NMR (400 MHz, MeOD) d 12.98–12.92 (bs, 2 H), 8.43 (t, J = 12 Hz, 2 H), 8.17 (s, 2 H), 7.99 (d, J = 8 Hz, 2 H), 7.53 (d, J = 8 Hz, 2 H), 6.74–6.67 (m, 1 H), 6.49 (d, J = 16 Hz, 2 H), 4.15 (m, 4 H), 1.18–1.76 (m, 8 H), 1.72 (s, 12 H); ^{13}C NMR (100 MHz, MeOD) d 175.7, 169.2, 156.8, 147.4, 142.8, 132.3, 128.7128.6, 124.6, 112.0, 106.0, 51.7, 50.4, 45.1, 27.9, 27.2, 23.5; HRMS (TOF). Calcd for $C_{35}H_{43}N_2O_{10}S_2$ (M^+ + H) 715.2359 Obs. 715.2321.

All UV–Vis spectra were recorded on Cary 50 spectrometer and fluorescence spectra on a Cary Eclipse. Fluorescence was measured in a 10 mm fluorescence cuvette and sample concentrations were maintained below 0.1 absorbance by diluting the stock solutions to the corresponding concentrations. All UV absorbance measurements were done with solvent background correction, at the concentrations listed in Table 1.

Table 1
Dye solutions used for UV–Vis analysis.

Dye in PBS	Stock solution concentration in μM
9a	542.7
9b	428.5
4	354.3
10	734.1
13	420.7
Dye in Ethanol	Stock solution concentration in μM
9a	636.5
9b	417.1
4	728.5
10	307.7
13	144.8
Dye in 9.25% sucrose	Stock solution concentration in μM
9a	681.4
9b	731.9
4	700.2
10	1202.4
13	986.2

3. Results and discussion

3.1. Synthesis

The ultimate goal of our work is to develop nitroimidazole derivatives covalently linked to indocyanine green derivatives for use as NIR florescent dyes that target hypoxia in cancerous tumors. For this purpose, we chose a *bis*(carboxylic acid) derivative of ICG as the prototype dye, specifically **2**. The choice was dictated by the need to attach nitroimidazole units, and suggested by our previous work that used functionalized indocyanine carboxylic acid derivatives [10]. The fluorescence quantum yield of **2** (0.066) is slightly greater than the commercial ICG (0.012) [10], probably due to the observation that formation of fluorescence-quenched aggregates is suppressed because dye–dye interaction and aggregation is reduced by hydrophilic, non-charged but sterically demanding substituents [25,26]. It is noted that the synthesis of **2** may lead to a mixture of stereoisomers due to rotation about each of the four C–C single bonds of the polyene unit. As seen in Fig. 1 (a), which is the HPLC trace for **1**, there is a clear indication that this compound is a mixture of stereoisomers. We can find no instance in the literature where a similar stereochemical analysis was reported in connection for a study. Our analysis of synthetic **2** in Fig. 1(b), on the other hand, shows two peaks, indicating there are fewer stereoisomers when compared with **1**. Many polyenes exist as the lower energy all-*E* isomer, but solvent effects as well as structural features of the molecule can stabilize other isomers, leading to mixtures [27]. The major stereoisomer of **2** may be the all-*E* isomer, but we have no evidence to absolutely rule out other isomers. It is clear, however, that **2** is relatively pure when compared to **1**. The dyes used in our study are dicarboxylic acid derivatives, were prepared in a manner identical to **2**, and we believe the stereochemistry of all dyes are similar to **2**. However, the compounds we have prepared, as well as those similar compounds reported in the literature, are probably not a single isomer and isomers would arise from bond rotation associated with the polyene linker. The interactions of the polar substituents incorporated into these dyes may lead to stabilization and the relatively small mixture of observed isomers.

A straightforward synthesis of **2** was reported by Lindsey and co-workers [28], in which the reaction of *p*-hydrazinobenzoic acid with 3-methyl-2-butanone gave a 71% yield of 2,3,3-trimethyl-3*H*-indole-5-carboxylic acid via a Fischer indole synthesis [10]. Subsequent treatment with butanesultone gave a 32% yield of 3-(5-Carboxy-2,3,3-trimethyl-3*H*-indolium-1-yl)propane-1-sulfonate, **5** [10,29]. Ozinskas and co-workers prepared **5** by a similar route, in 98% yield [28]. In this later case, treatment of 2,3,3-trimethyl-3*H*-indole-5-carboxylic acid with ethyl iodide at 120 °C in a sealed tube gave a *N*-ethyl derivative of **5** in 26% yield, whereas heating with butanesultone at 180 °C gave **5** in 82% yield [15]. Licha et al. prepared **6** by a similar route [25]. In all cases the dyes were assembled by condensation of **5** with the commercially available glutaconic aldehyde dianilide·HCl [25,28,30]. In our hands, 2,3,3-trimethyl-3*H*-indole-5-carboxylic acid was prepared in 68% yield, **5** in 88% yield and **2** in 85% yield [10].

Both the dye **2** and dye-conjugates **3** showed absorption at about 754 nm, and emission at about 778 nm in PBS (see Scheme 3 and Table 1) [10]. Spectral data for **2** had been reported previously in DMSO, with emission at 817 nm and absorption at 794 nm, molar absorbtivity of 286,000 and a fluorescence quantum yield of 0.12 [31]. Our stated goal was to modify the structure of **2** to increase rigidity and thereby increase the fluorescent quantum yield. Any structural modification of the polyene unit must produce a dye with absorption/emission in the NIR region. In order to introduce rigidity into the molecule, we targeted two new structural types: one introduced a ring into the polyene unit as observed previously with **4**, but with modification of the group attached to the cyclohexene ring and we included a five-membered ring derivative, and the second type of structurally modification shortened the polyene chain.

N-Formyl-*N*-methylaniline (**6**) was prepared by the reaction of *N*-methylaniline with formic acid and sodium formate [21], and then treated first with $POCl_3$ and then with 1-methylcyclohexene. As shown in Scheme 2, subsequent reaction with aniline hydrochloride gave anilide **7a** in 39% yield [22]. 1-Phenylcyclohexene was converted to **7b** in 68% yield using an identical procedure [22]. The direct treatment of *N*-formyl-*N*-methylaniline with $POCl_3$ in the presence of cyclohexanone and aniline hydrochloride led to a 79% yield of **7c** [22]. The identical reaction with cyclopentanone led to **8** in 73% yield [23]. With these anilides in hand, coupling with indolium sulfonate **5** was accomplished using the standard procedure from our previous work, as shown in Scheme 3: anilide and indole heated to 120 °C in acetic acid-acetic anhydride, buffered with sodium acetate [10]. Using this procedure, **7a** was converted to **9a** in 41% yield, **7b** to **9b** in 37% yield, **7c** to **4** in 22% yield, and **8** to **10** in 25% yield. As noted earlier, Tung and co-workers prepared **4** in 67% yield by heating commercially available **7c**, obtained from Aldrich, with **5** in ethanol at reflux for two hours in the presence of sodium acetate [15].

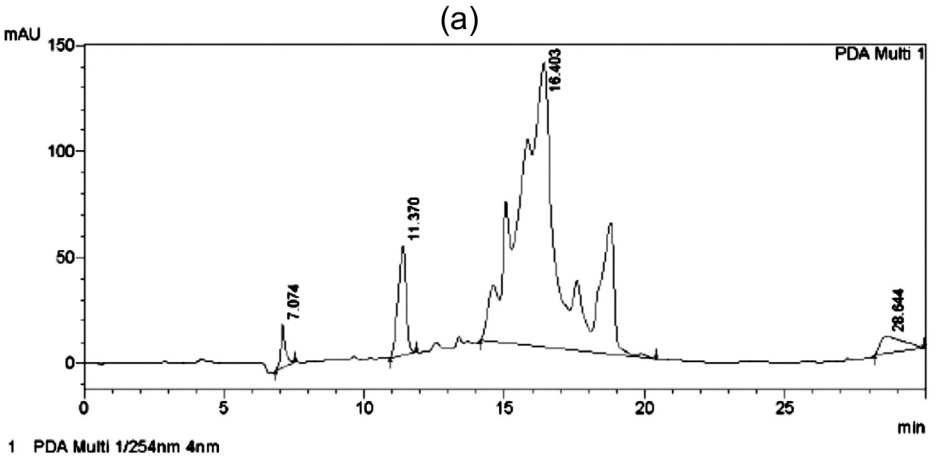
We prepared a single example of the second target type (**13**, see Scheme 4), with two carbons less in the polyene linker. Reaction of the bis(dimethyl acetal) of propanedial (**11**) with aniline, in aqueous HCl led to anilide **12** in 90% yield [24]. Subsequent reaction with indole **5** under the standard conditions gave a 30% yield of dye **13**.

3.2. Absorption and fluorescence properties

All derivatives were characterized using an UV–Vis spectrophotometer and a fluorescence spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA). The wavelength range of both spectrophotometers is 250 nm–1100 nm. The dyes were suspended in either Phosphate Buffer Saline (PBS) or a 9.25% aqueous sucrose solution, and the absorption and fluorescence spectra were recorded. These two solvent systems were chosen to be compatible with our previously reported *in vivo* studies with **3** and related compounds.

Quantum yields were calculated relative to the standard samples that have a fixed fluorescence quantum yield value, according to the following equation [32]:

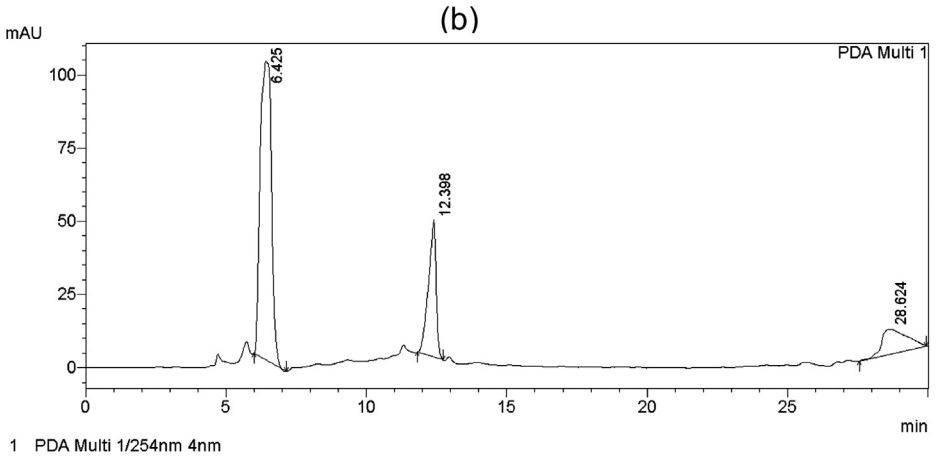
$$\Phi_X = \Phi_{ST}(\mathrm{Grad}_X/\mathrm{Grad}_{ST})\left(h_X^2/h_{ST}^2\right)$$



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	7.074	231018	20497	1.497	9.574
2	11.370	1022011	51841	6.621	24.214
3	16.403	13736929	134118	88.998	62.645
4	28.644	445118	7636	2.884	3.567
Total		15435077	214092	100.000	100.000



PeakTable

PDA Ch1 254nm 4nm

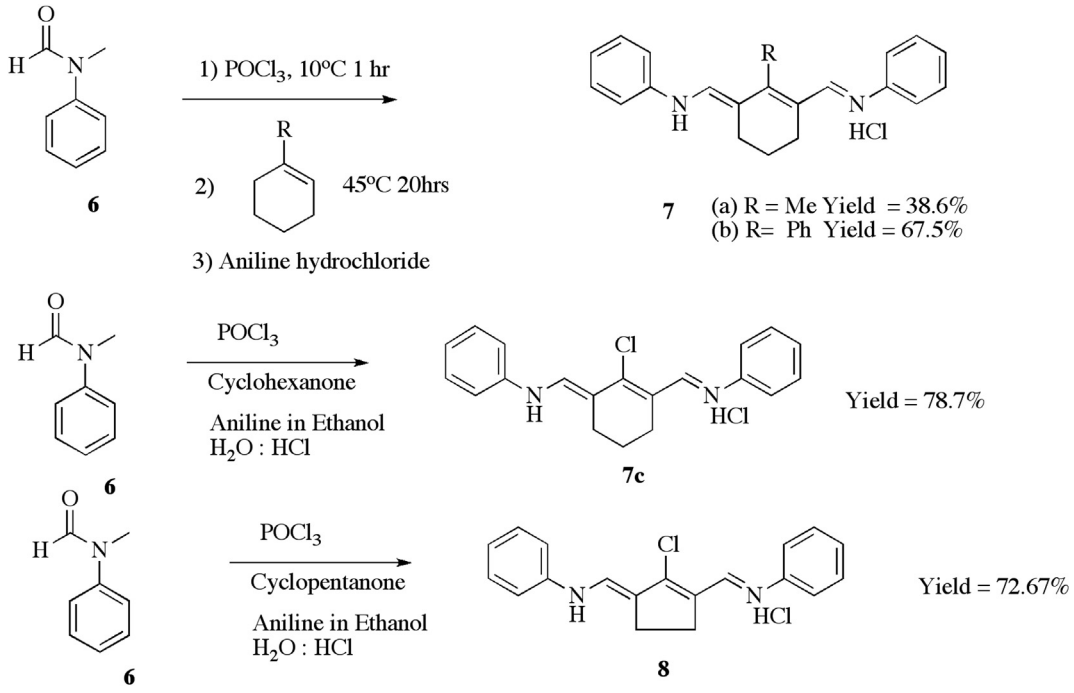
Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.425	2843675	101983	67.087	64.888
2	12.398	880680	46713	20.777	29.722
3	28.624	514457	8471	12.137	5.390
Total		4238812	157167	100.000	100.000

Fig. 1. HPLC of commercial ICG dye **1** (a) and synthetic dye conjugate **2** (b).

Where ST = standard and X = unknown, respectively, Φ is the fluorescence quantum yield, Grad is the gradient from the plot of integrated fluorescence intensity versus absorbance, and h the refractive index of the solvent. Our results are shown in Table 2.

The florescence yield of **9a,b, 4, 10** and **13** were measured in ethanol using Rh101 as the standard, in PBS and in a 9.25% sucrose solution using **2** as the standard. All results are shown in Table 2. It is clear that incorporating a ring into the polyene moiety as in **9a,b, 4** and **10** had little or no effect, or led to a slight decrease in the quantum yield. Although each of these derivatives should be more rigid in terms of rotation about the C–C bonds of the polyene, the increase in the number of atoms may mitigate any benefits. On the other hand, the chain-shortened derivative **13** showed a significant enhancement in the quantum yield, with quantum yields ranging from a low of 0.028 to a high of 0.34.

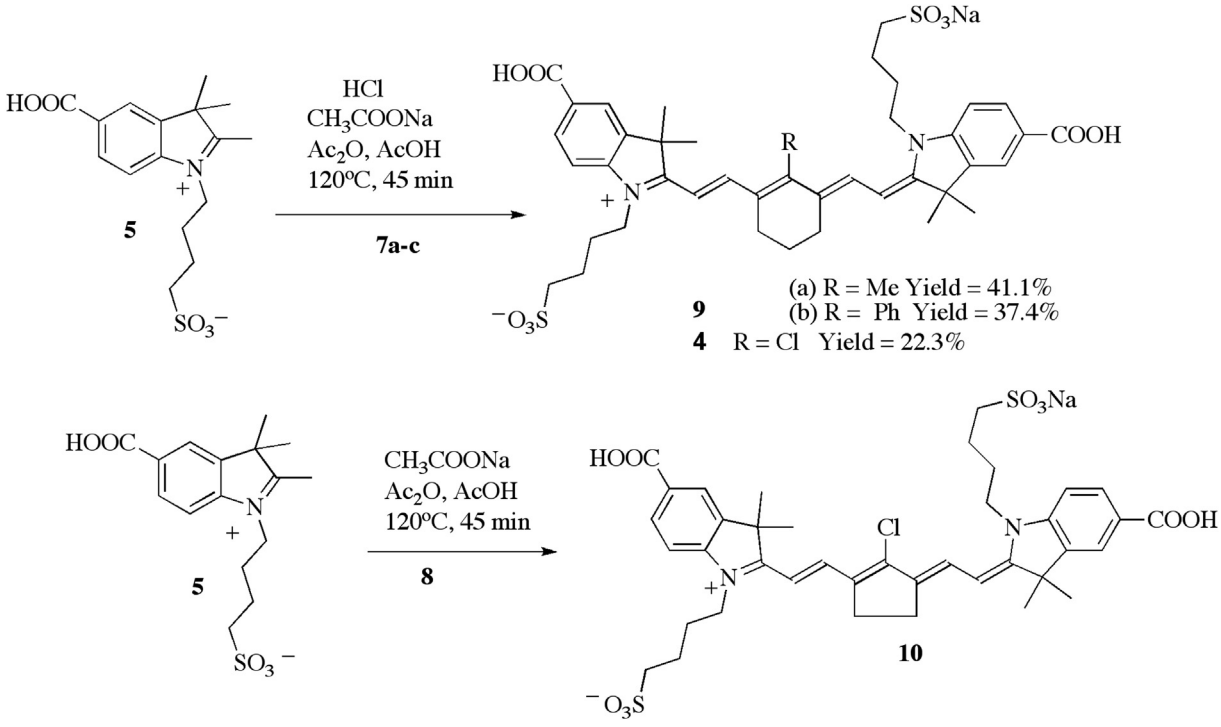
Using Rh101 as the standard, the dye **13** absorbed at 657 nm and emission was measured at 676 nm. These values are in the useful range for NIR analysis, although a different filter would be required for a NIR analysis using any dye conjugate prepared from **13** that is structurally related to **3**. Using **2**, absorbance was at 655 nm and emission at 669 nm in PBS, and absorbance at 655 nm and emission at 672 nm in sucrose solution. The chloro and methyl derivatives (**4** and **9a**) showed slight shifts in the absorption and emission peaks, relative to **2**, although **10** showed a peak past 800 nm. However, the emission peaks shifted to past 800 nm for all three using Rh101 and for **9a,c** using **2** (see Table 2). Tung and coworkers reported data for



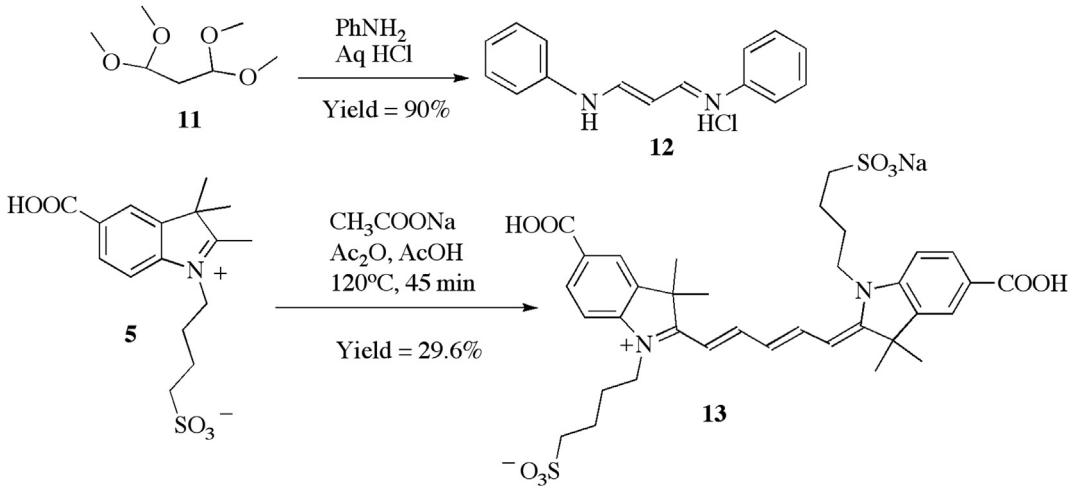
Scheme 2. Synthesis of dianilides **7** and **8.**

4 in methanol, and reported that **4** absorbs maximally at 790 nm and emits maximally at 820 nm in methanol with a molar extinction coefficient of 184,000, and a quantum yield of 0.08 [15]. We found that **4** absorbs at 812 nm and emits at 826 nm, in ethanol, with a quantum yield of 0.025 and a molar extinction coefficient of 188,928. Our data was reported using Rh101 as a standard. Tung used the same method to determine relative fluorescence quantum yield, indicating the use of a standard, but that standard was not identified. As seen in Table 2, phenyl derivative **9b** did not show the shifts observed for **4**. In all cases, the quantum yield was less than **2**, although that of phenyl derivative **9b** was close to **2**.

It is clear from our results that incorporation of a ring in the polyene chain does not increase the florescence yield. While the ring may diminish the number of stereoisomers that arise due to



Scheme 3. Synthesis of dicarboxylic acid indocyanine dyes **9** and **10.**



Scheme 4. Synthesis of polyene shortened dicarboxylic acid indocyanine dye **13.**

rotation about the C–C bonds, any benefit may be offset by the increased number of atoms. However, our analysis of isomers in Fig. 1(b) suggests there are a limited number of isomers in the dye. Loss of two carbon atoms from the polyene chain led to a significant increase in fluorescent yield for 13. It is not clear why this relatively minor structural change leads to such an increase, but one explanation is that the diminished number of C–C bonds leads to fewer isomers due to rotation. Since our analysis of isomers in Fig. 1(b) suggests there are a limited number of isomers in the dye it is not at all clear this is the explanation. It is certainly possible that fewer structural isomers can account for the increase in florescence yield. It is also possible that brining the two indole moieties closer together may restrict rotation and lead to an increase in rigidity. At this time our explanation remains speculative.

Table 2
Photophysical properties of the dyes in different solvents against against Rh101 as standard.

Dye	λ_{abs} (in nm)	Rh101 standard	In ethanol	Φ
		$\lambda_{emission}$ (in nm)	Extinction coefficient (ε) ($M^{-1}cm^{-1}$)	
2	759	778	191.362	0.146
9a	783	803	182.534	0.024
9b	775	791	250.265	0.127
4	796	811	188.928	0.059
10	820	840	144.783	0.041
13	657	676	210.347	0.344
Dye	λ_{abs} (in nm)	Diacid 2 standard	In PBS	Φ
		$\lambda_{emission}$ (in nm)	Extinction coefficient (ε) ($M^{-1}cm^{-1}$)	
2	756	778	236.868	0.066
9a	774	791	183.510	0.024
9b	766	779	209.539	0.055
4	789	802	210.351	0.028
10	812	826	254.619	0.025
13	655	669	220.417	0.289
Dye	λ_{abs} (in nm)	Diacid 2 standard	In 9.25% sucrose soln.	Φ
		$\lambda_{emission}$ (in nm)	Extinction coefficient (ε) ($M^{-1}cm^{-1}$)	
2	755	779	211.000	0.072
9a	776	791	207.096	0.030
9b	767	787	240.440	0.068
4	790	815	230.286	0.031
10	813	833	113.211	0.024
13	655	672	261.971	0.338

3.3. Optical stability in solution

The photostability of all dyes prepared for this study were examined in both PBS solution and in 9.25% sucrose solution. The dyes were dissolved in PBS solution and 9.25% sucrose solution, and the absorbance of each solution was maintained at approximately 0.82. Each sample was irradiated under a 500 W halogen lamp, maintained at a distance of 300 mm, for 14 h. An aqueous solution of sodium nitrite (50.0 g/L) was placed between the samples and the lamp to filter the light to less than 400 nm, and also to mitigate heat from the lamp.

The initial absorbance data was measured and then measurements were carried out each hour. As shown in Fig. 2 (in PBS solution) and Fig. 3 (in the sucrose solution), all dyes that contained a ring, **9a–c** and **10**, showed loss of absorbance over the course of three hours. This data stands in sharp contrast to dye **13**, which showed significantly improved stability in both PBS and in sucrose. It is noted that we also examined the stability in ethanol, but unavoidable evaporation of ethanol over the course of the experiment led slight changes in concentration and we deemed the data unreliable. This experiment was repeated with the same result, so it is not included here.

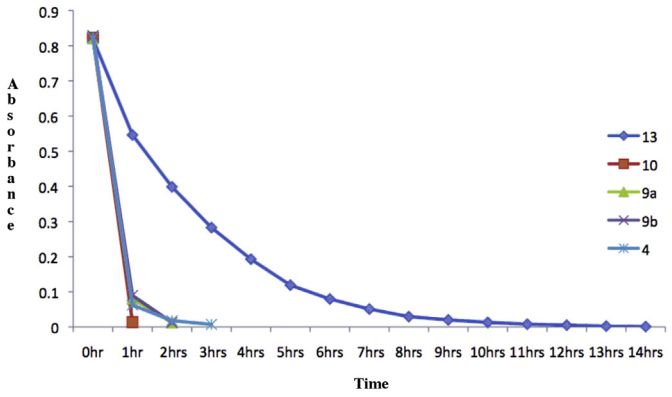


Fig. 2. Photostabilty of indocyanine dyes **4**, **9a,b**, **10**, **13** in PBS solution.

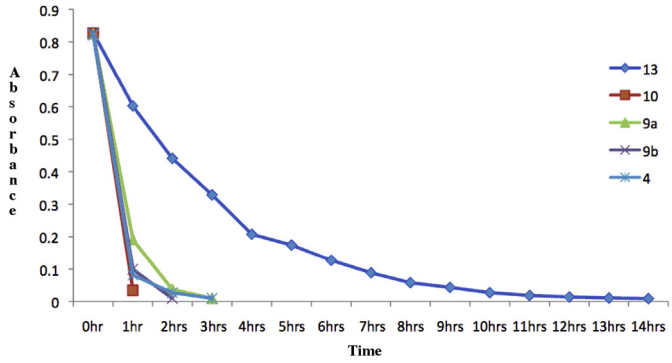


Fig. 3. Photostability of indocyanine dyes **4**, **9a,b**, **10**, **13** in 9.25% sucrose solution.

4. Summary

We have successfully synthesized new derivatives (**9a,b, 4, 10** and **13**) of the known ICG carboxylic acid derivative **2**. The spectral characteristics of all dyes are reported, including their absorption and fluorescence properties. Incorporation of rings into the polyene structure of the dye led to a shift of the emission band, and a decrease in quantum yield, relative to dye **2**. Shortening the chain by two carbons, as in **13**, led to a small shift in absorbance and emission, but also gave a significant increase in the quantum yield. We are currently preparing nitroimidazole derivatives of **13** as analogs of our first generation dye-conjugate **3**, and will proceed with *in vivo* studies in mice for NIR imaging of hypoxic cancerous tumors. That study will be the subject of a future publication as this work is focused only on the preparation and characterization of the structurally modified dyes.

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