

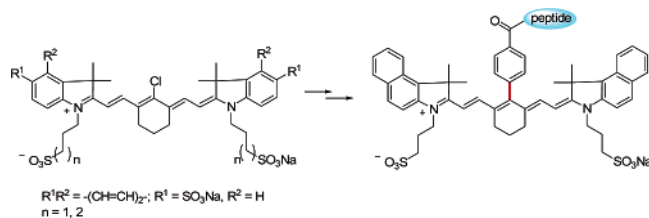
Heptamethine Cyanine Dyes with a Robust C–C Bond at the Central Position of the Chromophore

Hyeran Lee, J. Christian Mason, and Samuel Achilefu*

Department of Radiology, Washington University,
St. Louis, Missouri 63110

achilefu@mir.wustl.edu

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Novel, highly fluorescent, monofunctional, water-soluble heptamethine cyanine dyes containing a robust C–C bond at the central position of the near-infrared fluorophore system were prepared by the Suzuki–Miyaura method. The reaction proceeded efficiently to replace the *meso*-chlorine atom with a carboxy-functionalized aryl moiety and afforded the desired compounds in high yields. This methodology is particularly attractive due to its versatility and the utilization of environmentally friendly water as solvent. The new compounds possess excellent spectral properties and readily label bioactive molecules on solid support. The results demonstrate the potential of using the new compounds as fluorescent antennae for molecular imaging, spectroscopy, microscopy, and chemical or biological molecular recognition studies.

Molecules that absorb and emit light in the near-infrared (NIR) wavelengths have become the central focus of numerous analytical, biological, and spectroscopic studies because their spectral, chemical, and biological properties facilitate the detection of minute molecular processes in solutions and living tissues. The excellent safety profile of the NIR heptamethine cyanine fluorochrome indocyanine green (ICG, Figure 1) in humans has spurred interest in the development of ICG derivatives, including Cy dyes and cypate (Figure 1) for in vivo molecular imaging by NIR optical methods.^{1,2}

However, the low fluorescence quantum yield, short fluorescence lifetime, propensity to photobleach, and poor chemical stability of these dyes have limited their use in chemical and life sciences. To overcome these limitations, numerous studies have shown that incorporating a rigid cyclohexenyl ring in the polymethine chain increases photostability and enhances the quantum yield in comparison to cyanine dyes with an open polymethine chain.^{3–5} The central cyclohexenyl group offers

an additional reactive site for labeling bioactive molecules via functionalization of the *meso*-chloro cyclohexenyl group. Furthermore, incorporating the alkylsulfonato functionality on the nitrogen atom of the heterocyclic moiety improves photostability and water solubility.

A common approach to prepare monofunctionalized NIR fluorescent probes using the above strategy is stepwise condensation of two different heterocyclic moieties in which one of the heterocyclic bases contains a monocarboxy functionality to give an asymmetric dye.⁶ However, the purification process using this route is problematic due to the formation of undesirable symmetric dyes as byproducts. Interestingly, the chloro-substituted heptamethine cyanine dyes can readily undergo nucleophilic substitution at the *meso* position via a S_N1 mechanistic pathway to replace the chlorine atom with more versatile functionalities.³ This has led to the development of an alternative pathway to the monofunctionalized dyes by direct derivatization of the chloro-substituted heptamethine cyanine probes at the central position of the polymethine chain.^{3,7,8} Using this strategy, functionalized, water-soluble heptamethine cyanine dyes containing aryl–ether, aryl–thioether, aryl–amine, alkyl–ether, and alkyl–thioether linkages suitable for labeling biomolecules have been prepared and utilized in analytical and molecular imaging applications.^{9–14} Unfortunately, the enol and thioenol ether linkages are chemically labile, thereby rendering the fluorochromes susceptible to cleavage and subsequent loss of the fluorescence signal.^{11,13,15} Additionally, the photostability of the alkyl–thioether fluorophore was found to be very poor.¹⁶ To circumvent these problems and further optimize the chemical and photochemical properties of NIR fluorophores, a direct carbon substitution of the *meso*-chlorine atom in the polymethine chain is highly desirable.

Previously, Johannes et al.¹⁷ reported palladium-catalyzed C,C-coupling reactions of bromo- or iodo-substituted indocyanines and indodicarbocyanines with aryethynes, styrenes, and heteroarylstannans. However, reports on the cross-coupling

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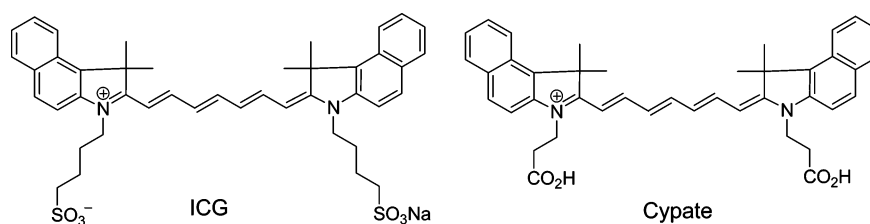


FIGURE 1. Structures of representative cyanine dyes.

reactions of the chloro-substituted carbocyanines are lacking probably because of the relatively inert nature of chloro-substituted derivatives in comparison to bromo- or iodo-substituted analogues to oxidative addition to a palladium(0) complex, which is believed to be the rate-determining step in the cross-coupling reaction of organometallics.¹⁸ The relative reactivity of these halogens decreases in the order of $I > Br \gg Cl$. Unfortunately, the majority of carbocyanines that is easily accessible from commercial sources is the chloro analogues due to their easy and clean preparation. Moreover, commercial dyes that are suitable for biomedical applications are highly charged due to polysulfonation for enhanced water solubility, which can further complicate the cross-coupling reactions. Accordingly, it is highly desirable to develop a more universal and facile synthetic methodology to perform regioselective C–C cross-coupling reactions of water-soluble chloro-substituted carbocyanines.

Among several organometallic reagents, organoboronic acids are convenient reagents for the cross-coupling reactions because they are generally thermally stable and inert to water and oxygen, thus allowing their handling without special precautions.¹⁸ Numerous attempts to cross-couple hydrophilic chloro-substituted heptamethine cyanine dyes with arylboronic acids utilizing published methods were unsuccessful and only resulted in hydrolyzing the chlorine atom to give bis(aminodien)one or decomposing the dye species after prolonged heating, depending on the choice of the palladium catalyst. For these reasons, we explored suitable reaction conditions to perform C–C cross-coupling reactions of hydrophilic chloro-substituted heptamethine cyanines with arylboronic acids.

The synthesis and peptide labeling reactions of the new highly fluorescent, monofunctional, water-soluble heptamethine cyanine dyes containing a robust C–C bond at the central position of the chromophore by the Suzuki–Miyaura method are shown in Scheme 1.

The precursor chloro cyanine dyes **1a–1c** utilized in the C,C-coupling reactions can be efficiently synthesized via condensation reaction of a heterocyclic base with Vilsmeier–Haack reagents by literature methods.¹⁹ Thus, separate reactions of chloro-substituted benz[e]indolium dyes **1a,b** with 4-(OH)₂B–C₆H₄–CO₂H in the presence of Pd(PPh₃)₄ using aqueous reaction conditions proceeded efficiently to afford the desired dyes **2a,b** in 68–83% yields, respectively.

The versatility of this coupling methodology was further evaluated with the highly hydrophilic tetrasulfonate **1c** under similar reaction conditions. The reaction proceeded efficiently to replace the *meso*-chlorine atom with a carboxy-functionalized aryl moiety and afforded the desired compound **2c** in 73% yield. This methodology is particularly attractive due to its versatility

and the utilization of environmentally friendly water as solvent. Additionally, water-soluble NIR bioconjugatable fluorochromes can be prepared directly in a one-pot procedure from commercially available chloro-substituted dyes such as IR-820. Furthermore, this route offers fluorochromes in an analytically pure form at the gram scale because of the easy purification by simple crystallization. Spectral properties of the newly synthesized dyes **2a–c** are shown in Table 1 in comparison to those of indocyanine green (ICG). The absorption spectra of these dyes show a characteristic band broadening, which is typical of heptamethine dyes with the absorption maxima in the range of 770–800 nm. They exhibit a hypsochromic shift (~20 nm) from their parent chloro dyes **1a–c** (795–820 nm), indicating a direct interaction of the aryl group with the chromophore system. Their exceptionally high molar absorptivities of 240 000 M⁻¹ cm⁻¹ for dyes **2a,b** and 220 000 M⁻¹ cm⁻¹ for dye **2c** exceed those of many organic dyes. Although the maximum emission wavelengths vary from 770 to 811 nm with small Stoke's shifts of 10–15 nm, the broad emission bands allow flexibility in the choice of excitation and fluorescence wavelengths in analytical and biological assays. The relative fluorescence quantum yields of dyes **2a–c** measured in MeOH using ICG as a standard show moderate improvement with the values in the range of 0.088–0.10 compared to 0.078 for ICG.²⁰ Also noteworthy is the fact that the relatively low fluorescence quantum yields of these compounds are compensated by their very high molar absorptivities, as reflected in their impressive brightness (Table 1). The brightness or fluorescence intensity per dye molecule, which is the product of the fluorescence quantum yield and molar absorptivity,²¹ is a useful index to predict the sensitivity of detecting small amounts of the fluorescent probes, especially in heterogeneous media such as cells and tissues. However, these dyes are expected to have relative fluorescence quantum yields of about 0.3 to approach the brightness of fluorescein, which has a high fluorescence quantum yield of 0.85 in aqueous solution above pH 7²² and a brightness of about 69 000 M⁻¹ cm⁻¹.²¹

The representative absorption and fluorescence spectra of these dyes are shown in Figure 2 for the dye **2c**.

The conjugation efficacy of dyes **2a–c** was evaluated via solid-phase chemistry by reacting **2b** with ϵ -aminohexanoic acid (ϵ -Ahx). The *N*-Fmoc-protected ϵ -Ahx was coupled to a Rink amide resin by standard Fmoc peptide chemistry.^{23,24} After

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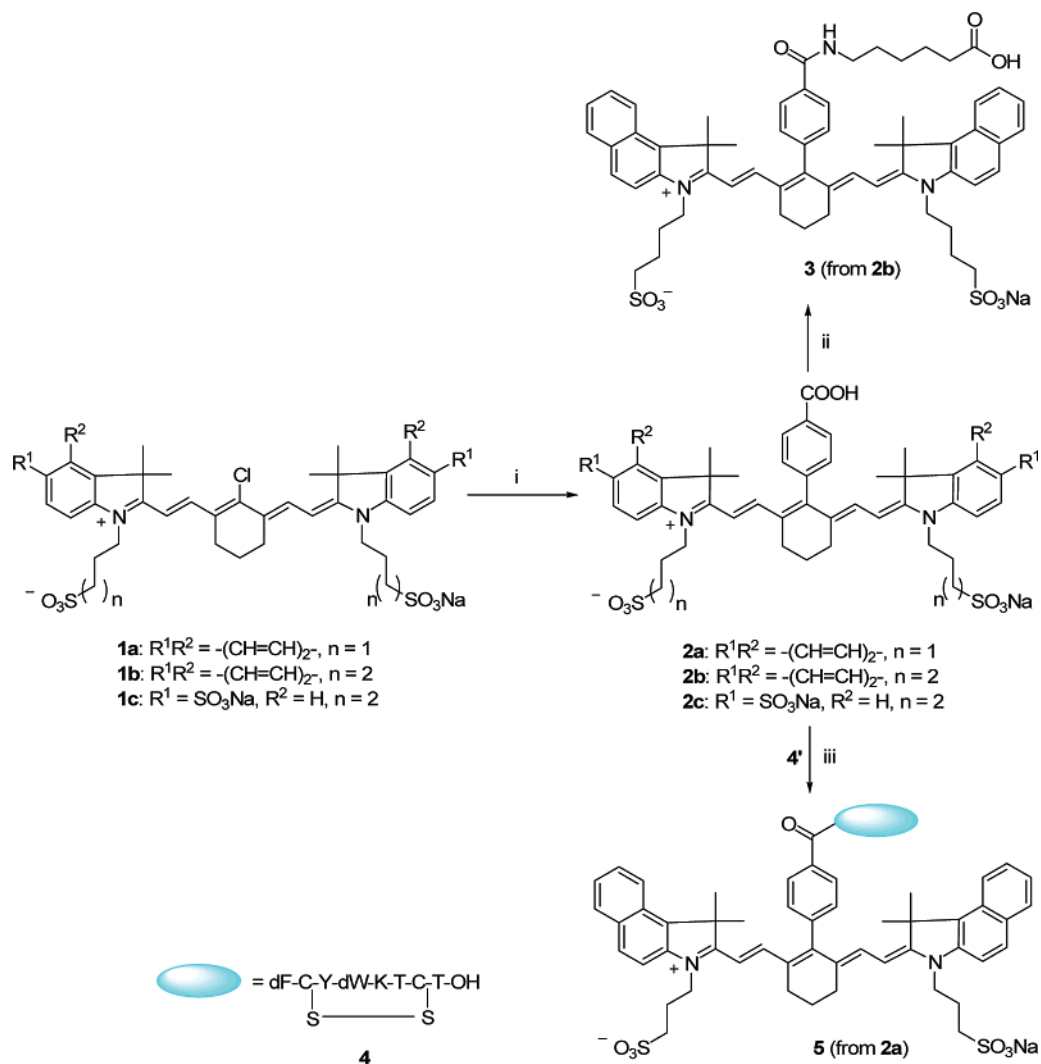
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SCHEME 1. C,C-Coupling of *Meso*-Chloro-Substituted Heptamethine Cyanine Dyes^a

^a Reaction conditions: (i) 4-B(OH)₂-C₆H₄-COOH/Pd(PPh₃)₄/H₂O, reflux; (ii) (a) ϵ -Ahx/HBTU/HOBt/DMF-MeOH, (b) TFA; (iii) (a) HBTU/HOBt/DMF-MeOH, (b) TFA.

TABLE 1. Comparative Spectral Properties of **2a–c** and ICG in Methanol

compd	$\lambda_{\text{max,abs}}$ (nm)	$\lambda_{\text{max,em}}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)	Φ_{F}^a	Φ_{B}^b
2a	800	811	240 000	0.088	1.3
2b	800	811	240 000	0.092	1.4
2c	770	785	220 000	0.10	1.4
ICG	785	807	204 000	0.078 ^c	1

^a Relative fluorescence quantum yield. ^b Relative brightness referenced to ICG. ^c Quantum yield standard.²⁰

removing the Fmoc with 20% piperidine in DMF, treatment of **2b** with the resin-bound ϵ -Ahx in the presence of *N*-hydroxybenzotriazole (HOBt), 2-(1-*H*-benzotriazole-1-yl)-1,1,1,3-tetramethyluronium hexafluorophosphate (HBTU), and DIEA gave the desired product **3**. The absorption maximum of the compound was retained at 803 nm after TFA-mediated cleavage from the resin and HPLC purification. This result suggests that compounds **2a–c** possess excellent chemical stabilities that are needed for solid-phase peptide synthesis and harsh cleavage conditions.

To further demonstrate the feasibility of labeling biologically relevant molecules with dye **2a**, a somatostatin receptor-avid

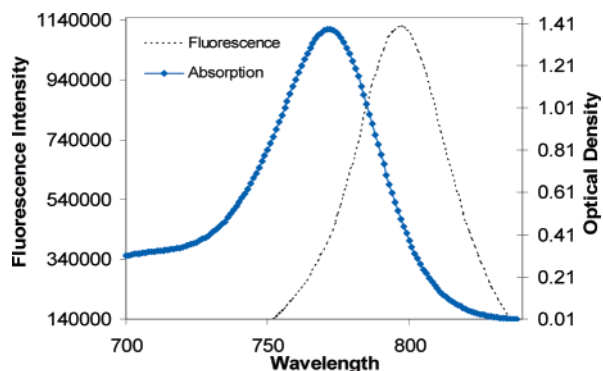


FIGURE 2. Absorption and emission spectra of dye **2c** in 20% aqueous DMSO solution.

octapeptide (octreotate **4'**) was utilized. Previous studies have shown that targeting this receptor with fluorescent- or radio-labeled peptides facilitates the visualization of tumors in humans and small animals.^{25,26} Octreotate was assembled on solid support using Wang resin,²³ and the N-terminal Fmoc was removed as described above. The hydrophilic dye was suspended

in DMF, and drops of MeOH were added until a clear solution was obtained. Subsequent activation with a mixture of HOBt/HBTU coupling reagents and reaction with the amino group of the peptide on solid support afforded the desired dye–peptide conjugate **5** after removal of all side-chain amino acid protecting groups and cleavage from the resin using TFA. The new dye–peptide conjugate was purified by HPLC and characterized as described previously. The absorption and fluorescence spectra of the dye in compound **5** were practically unchanged, demonstrating the feasibility of using these dyes to label biomolecules.

In conclusion, we have developed a new method to prepare a monofunctional NIR dye by substituting the *meso*-chlorine atom of the cyclohexenyl ring with a robust C–C linkage via the Suzuki–Miyaura method. The reaction was conducted under environment friendly aqueous conditions, and high yields of the desired compounds with overall improved chemical and photophysical properties were obtained. We successfully labeled bioactive molecules with the dyes, demonstrating the feasibility of their use for optical molecular imaging of diseases and other NIR fluorescence studies. Because of their superior spectral properties, we are currently labeling a variety of bioactive molecules with these dyes for biological imaging and spectroscopy studies. Future studies will also include comparative photostability studies with other cyanine dyes.

Experimental Section

Synthesis of Chloro Dyes 1a–1c. Condensation of heterocyclic salts and dianil was conducted in a 1:1 mixture of ethanol and acetic anhydride in the presence of sodium acetate by using a general procedure.^{3,14} The compounds were recrystallized from methanol/ether.

Synthesis of Cyanine Dyes 2a–2c. Precursor chloro dyes **1a–1c** (1.0 mmol) and 4-carboxyphenylboric acid (1.8 mmol) in H₂O were heated under reflux in the presence of Pd(PPh₃)₄ (0.065 mmol) for 6–9 h. The reaction progress was monitored by visible/near-infrared spectroscopy for aliquots diluted with methanol until absorption of the starting chloro cyanine disappears. The reaction mixture was then cooled to room temperature, and H₂O was removed in vacuo. The solid was isolated by precipitation with MeOH/acetone, and the precipitate was further washed with acetone. Dye **2c** obtained was additionally crystallized from EtOH.

Dye 2a. Yield 68%. ¹H NMR: δ 1.41 (s, 12H), 2.04 (m, 6H), 2.59 (t, *J* = 6 Hz, 4H), 2.78 (t, *J* = 6 Hz, 4H), 4.42 (t, *J* = 7 Hz, 4H), 6.46 (br d, *J* = 14 Hz, 2H), 7.14 (br d, *J* = 14 Hz, 2H), 7.45 (d, *J* = 9 Hz, 2H), 7.47 (t, *J* = 8 Hz, 2H), 7.57 (t, *J* = 8 Hz, 2H), 7.78 (d, *J* = 9 Hz, 2H), 8.01 (m, 6H), 8.24 (d, *J* = 8 Hz, 2H). λ_{max} (in 25% aqueous DMSO): 800 nm (ε 240 000 M⁻¹ cm⁻¹). Φ_F (MeOH): 0.088. ESI-MS (negative matrix), *m/z*: 883 (M⁺ – Na, 100%).

Dye 2b. Yield 83%. ¹H NMR: δ 1.40 (s, 12H), 1.76 (m, 8H), 2.01 (m, 2H), 2.55 (m, 4H), 2.76 (m, 4H), 4.26 (m, 4H), 6.32 (br d, *J* = 14 Hz, 2H), 7.13 (br d, *J* = 14 Hz, 2H), 7.46 (m, 4H), 7.58 (t, *J* = 8 Hz, 2H), 7.73 (d, *J* = 9 Hz, 2H), 8.01 (m, 6H), 8.25 (d,

J = 8 Hz, 2H). λ_{max} (in 25% aqueous DMSO): 800 nm (ε 240 000 M⁻¹ cm⁻¹). Φ_F (MeOH): 0.092. MS-ESI, *m/z*: 913 (M⁺ – Na, 100%).

Dye 2c. Yield 73%. ¹H NMR: δ 1.10 (s, 12H), 1.58 (m, 2H), 1.72 (m, 4H), 2.45 (t, *J* = 6 Hz, 4H), 2.72 (m, 4H), 3.32 (t, *J* = 6 Hz, 4H), 4.11 (m, 4H), 6.28 (br d, *J* = 14 Hz, 2H), 7.02 (br d, *J* = 14 Hz, 2H), 7.30 (d, *J* = 8 Hz, 4H), 7.41 (d, *J* = 8 Hz, 2H), 7.60 (d, *J* = 8 Hz, 2H), 7.61 (s, 2H), 8.19 (d, *J* = 8 Hz, 2H). λ_{max} (in 25% aqueous DMSO): 770 nm (ε 220 000 M⁻¹ cm⁻¹). Φ_F (MeOH): 0.100. ESI-MS (negative matrix), *m/z*: 971 (M⁺ – 3Na, 100%).

Synthesis of Cyanine Dye 3 from 2b. The resin-bound ε-Ahx was added to a solution of the cyanine dye preactivated with HBTU/HOBt/DIEA in DMF/MeOH. The resulting mixture was agitated for 5 h at room temperature. The resin was then washed with DMF and DCM, cleaved with a TFA solution, and concentrated in vacuo. The product was precipitated in cooled *tert*-butyl methyl ether and purified by semipreparative HPLC to afford dye–ε-Ahx conjugate **3**. λ_{max} (in 25% aqueous DMSO): 803 nm. ESI-MS, *m/z*: 1025 (M⁺ – Na, 100%).

Synthesis of Cyanine–Octreotate 5 from 2a. The resin-bound peptide was assembled starting from Wang resin based by conventional Fmoc chemistry, as described previously.²³ To the resulting resin-bound peptide was added a solution of the cyanine dye (3 equiv) preactivated with HBTU/HOBt (3 equiv) in DMF/MeOH. The resulting mixture was mixed for 5 h at room temperature. The resin was then washed with DMF, MeOH, and DCM and cleaved with a cleavage mixture consisting of 95% TFA, 2.5% phenol, 2.5% thioanisole, and 5% H₂O for 90 min. The resin was filtered, and the filtrate was precipitated in cold *tert*-butyl methyl ether and purified by semipreparative HPLC to afford dye–octreotate conjugate **5**. ESI-MS, calcd *m/z*: 1916. Observed for ESI-MS, *m/z*: 1916 (M⁺ – Na, 100%).

UV–Vis and Fluorescent Spectroscopic Analysis. Stock solutions (3.0 mM) of the dye and its conjugates were prepared by dissolving them in DMSO. UV–Vis and fluorescence measurements were carried out by sequentially adding 0.5–2.0 μL aliquots of the stock solutions via a micropipet into 3 mL of 20% aqueous DMSO solution in a quartz cuvette and stirring for equilibration prior to acquiring the spectra. The molar extinction coefficient was obtained using Beer's law at 0.1–0.6 μM concentration of the dye. The relative fluorescence quantum yield was determined using the equation²⁷

$$\Phi_{F(x)} = (A_s/A_x)(F_x/F_s)(n_x/n_s)^2\Phi_{F(s)}$$

where Φ_{F(x)} is the fluorescence quantum yield, *A* is the absorbance, *F* is the area under the emission curve, *n* is the refractive index of the solvents used in measurement, and the subscripts *s* and *x* represent the standard and unknown, respectively. Indocyanine green (ICG) was used as a reference standard.

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Supporting Information Available: Characterization for all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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