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Ultrabright and Serum-Stable Squaraine Dyes

Yogesh Yadav[⊥],

Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, United States

Eric Owens[⊥],

Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, United States;
Center for Diagnostics and Therapeutics, Atlanta, Georgia 30303, United States

Shinsuke Nomura

Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States; Department of Surgery, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

Takeshi Fukuda, Yoonji Baek, Satoshi Kashiwagi, Hak Soo Choi

Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

Maged Henary

Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, United States;
Center for Diagnostics and Therapeutics, Atlanta, Georgia 30303, United States;

Abstract

Highly stable symmetric and asymmetric squaraine fluorophores have been synthesized featuring an internal salt bridge between a quaternary ammonium cation and the central oxycyclobutenolate ring of the chromophore. Some of our newly synthesized symmetric and asymmetric compounds display increased molar absorptivity, quantum yield in serum, and thermal/photochemical stability over previously reported squaraine-based dyes. Consequently, both classes show great promise in resurfacing the normal environment-labile squaraine dyes as novel imaging agents and scaffolds for fluorescence sensing. Furthermore, incorporating a covalent attachment point away from the conjugated system allows for biological tagging applications without disturbing the optimum optical characteristics of the newly designed fluorophore.

Graphical Abstract

Corresponding Authors: **Hak Soo Choi** – Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States; Phone: +1 617-726-5784; hchoi12@mgh.harvard.edu, **Maged Henary** – Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, United States; Center for Diagnostics and Therapeutics, Atlanta, Georgia 30303, United States; Phone: +1 404-413-5566; mhenary1@gsu.edu.

[⊥]Y.Y. and E.O. equally contributed.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00617>.

¹H NMR and ¹³C NMR, mass spectroscopy analyses, and optical properties of the final squaraine dyes **9–12** and **16–19** (PDF)
Molecular formula strings of the reported compounds (CSV)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00617>

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INTRODUCTION

Biological sensors and imaging agents that absorb and fluoresce within the near-infrared (NIR) region of the electromagnetic spectrum continue to garner sustained research interest because of their inherent optical clarity for *in vivo* and *in vitro* use. The NIR region features a reduction in light scattering, low light absorption by biomolecules, and the removal of fluorescence signal competition arising from biological sources. Designing a NIR-absorbing and fluorescing compound must satisfy an array of diverse and crucial characteristics, including (1) the compound must display absorbance and fluorescence above 630 nm, which is red-shifted enough to be easily filtered from lower wavelength biomolecular autofluorescence, (2) a sufficient extinction coefficient and quantum yield (QY) in serum/water to be used in living/aqueous systems, (3) sustained water solubility to avoid micro-crystallization and reduction in optical performance, and (4) high photo- and chemical stability to retain absorbance and fluorescence abilities in harsh conditions such as covalently labeling a biomolecule with a NIR dye (Figure 1—top). Many fluorophore subclasses, including fluorescein,^{1,2} BODIPY,^{3–5} and aminocoumarin-^{6,7} based fluorophores (Figure 1—bottom), satisfy some of these characteristics, and they have been explored with great interest.

One particular interesting class of NIR fluorescent compounds centers on an oxocyclobutenolate ring and are called squaraine dyes.^{8–10} These compounds are characterized by extremely high fluorescence QY (20–30%), which is highly desirable for increasing meaningful signal; however, because of their initial discovery, they have been reported by various researchers to be unstable because of their highly chemically and photolytically labile oxocyclobutenolate ring, which quickly diminishes their widespread utility.¹¹ The delocalized cation between the terminal heterocyclic rings helps to resolve this but adds only minor stability to the overall structure; various other strategies such as dye polymerization,¹² rotaxane protection of the squaraine core,¹³ polymer incorporation,¹⁴ or dextran enveloping¹⁵ have been employed to harness the characteristically remarkable QY of these compounds. All of these methods have been shown to impart a subtle increase on the stability by protecting the electrophilic core of the chromophore, but these low-yielding (10%) and tedious synthesis and purification processes have not offered a substantial breakthrough in increasing squaraine dye stability or overall utility. In addition, these fluorophores cannot be utilized for many specific labeling techniques that require a

relatively small molecular size—such as imaging *in vivo* or in biomolecular labeling, which renders squaraine compounds useless in these increasingly demanding applications.^{16–18} Many efforts have been exhausted in the attempt to increase the stability of squaraine fluorophores, and herein, we report a facile scaffold for producing the most stable squaraine compounds synthesized with unparalleled brightness in serum as determined by their measured extinction coefficients (ϵ), QY (Φ), and optical brightness ($\epsilon \times \Phi$). Using our generic fluorophore platform, we are able to harness the optical prowess of squaraine-based NIR fluorophores without sacrificing stability.

RESULTS AND DISCUSSION

We initiated this finding by synthesizing water-soluble squaraine fluorophores (Scheme 1) as potential probes for image-guided surgery. To begin, we utilized Fischer indole synthesis, which proceeds well in acetic acid, to install various halogens on the ring for influencing physicochemical descriptors for *in vivo* studies. Alkylating the heterocyclic nitrogen with a propyl-based quaternary ammonium side chain is followed by the corresponding dicationic salt, which is allowed to react with squaric acid under dean-stark conditions. Having the final fluorophores in hand, we initially confirmed that we successfully installed improved water solubility groups, and we are investigating the optical profile and the ability of our squaraine dyes to survive the intravenous injection (Figure 2).

Similar to the above synthesis, we prepared additional analogues containing only a single quaternary ammonium arm, as shown in Scheme 2. The synthetic route begins with methylation and subjecting the precursor **13** to a reaction with diisopropyl squarate. Careful reaction monitoring and using triethyl amine afford the single heterocyclic adduct, which is then reacted with an ethanolic sodium hydroxide solution to afford the intermediate **15** for reaction with a second heterocyclic equivalent (**5–8**) under identical reaction conditions, to result in the final asymmetric dyes **16–19** in high yields.

After the synthesis of the asymmetric compounds **16–19** successfully, we began to realize that we could, in fact, use these new fluorophores for *in vivo* experiments. Biological experiments often require a reactive handle for covalent attachment of a fluorophore, and at this point, our compounds lacked a reactive functionality; therefore, using the chemistry optimized, as shown in Scheme 2, we prepared the derivative **22** (Scheme 3) bearing the reactive carboxylic acid functionality for further covalent modification. Having confirmed that we could synthesize a biologically relevant squaraine dye, we fully examined the optical profile in various solvents [ethanol (EtOH), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), and fetal bovine serum (FBS)] to confirm the NIR properties and ensure that our scaffold still featured the positive properties of squaraine dyes.

Importantly for future *in vivo* experimentation/applications, all dyes dissolve immediately in aqueous systems without the need for a cosolvent and show NIR absorption and fluorescence characteristics with common Stokes shift values (8–15 nm). The sharpness of the absorption band in serum is highly remarkable as other fluorophores display an aggregation, which reduces their fluorescence QY and, correspondingly, their *in vivo* applicability.^{19,20} We found that our fluorophores display extinction coefficient values in

serum to be greater than $200,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Table 1S) probably because of the rigidity associated with the electrostatic interaction between the ammonium cation and the partially negative central oxygen atoms (Figure 3). Common intramolecular salt bridge distances are less than $4 \text{ \AA}$ as ionic interactions are no longer effective at longer distances. Observing the energy-minimized structures and measuring the point-to-point distances of compounds **10** and **17**, we can qualitatively see this interaction occurring. Density functional theory (DFT) calculations suggest that the quaternary ammonium arms fold back onto the central core of the electrophilic chain to within $4 \text{ \AA}$. In Figure 3, we show these intramolecular distances and the electrostatic maps associated with both the symmetric and asymmetric fluorophores. All distances for compounds' quaternary ammonium nitrogen and corresponding central oxygen are less than $3.7 \text{ \AA}$ with representative compounds **10** and **17** shown as examples. This adds rigidity and stability by locking the conformational state of the squaraine fluorophore, which minimizes the relaxation pathways that compete with fluorescence. We suspect that this rigidization of the conjugated system of the compounds owes to the increase in QY. We noted, however, that bromine incorporation reduced the overall QY, especially in serum. This could be because of a heavy atom stabilization of the triplet state or increased hydrophobicity and diminishment of fluorescence because of micro-aggregation.

Finally, the paramount issue associated with squaraine dyes is their inherent instability. It was reported by Johnson *et al.*²¹ that the squaraine rotaxane compound was well-demonstrated as the stable NIR probe compared to the commercial and widely used Cy5. To determine the stability of our squaraine fluorophores bearing an internal salt bridge, each compound was incubated in FBS at 37°C and irradiated using a high energy light for 72 h. The optical half-life recognized by absorbance reaching half of the original value for each of the compounds **9** and **16** was not reached after 72 h; however, the industry standard, Cy5²¹ (which is a more stringent test as it is inherently more stable than previously known squaraine dyes), was depleted of $>75\%$ of the absorption measurements after the same timeframe. We noted an increase in stability of the symmetric version over the asymmetric compound, however, the difference was not substantial as even the asymmetric compound is more stable than Cy5.

Next, we examined the effect of surface charges on the biodistribution and clearance of squaraines *in vivo*. To this end, we injected 25 nmol of the individual compounds **9**, **16**, or **22** into 25 g of CD-1 mice and monitored their behaviors using the intraoperative FLARE imaging system up to 4 h (Figure 4). Two positively (+2) charged compound **9** ($\log D = -5.15$, pH 7.4) was cleared through both renal and hepatobiliary routes with an overall distribution in most tissues and organs because of the strong positive charges (Figure 4A).^{22,23} Interestingly, +1 charged compound **16** with increased hydrophobicity ($\log D = -1.46$, pH 7.4) and +1/-1 charged compound **22** ($\log D = 0.12$, pH 7.4) showed a major uptake in the liver but minimum to no signals in the kidneys and bladder, indicating predominant excretion through the hepatobiliary route (Figure 4B). Histologically, kidney tissue showed the NIR fluorescence signal for compound **9** in the cortex but not in the medulla after injection of a squaraine dye (Figure 4C), indicating little renal clearance. Consistently, liver tissue showed a diffuse signal in hepatocytes, indicating the uptake and clearance of squaraine dyes through the hepatobiliary route (Figure 4C). Compounds **16**

and **22** show decreased uptake in the chest wall and head/neck area, which suggests that we could reproducibly control the biodistribution and clearance of squaraines *in vivo* by introducing different surface charges to the backbone molecule as the appropriate.

CONCLUSIONS

To conclude, we have synthesized and evaluated the ability of an intramolecular salt bridge to protect the central oxocyclobutenolate ring of squaraine fluorophores and increase the molar absorptivity and QY in serum owing to increased rigidity associated with the locked structure. The asymmetric scaffold was modified to include a hexanoic acid moiety optimized for biological-labeling experiments, which require high extinction coefficients and QY in the NIR. The biodistribution and clearance of squaraines *in vivo* were achieved by introducing different surface charges to the backbone of the compounds. These findings offer a facile solution to squaraine stability, while finally increasing their *in vivo* appeal.

EXPERIMENTAL SECTION

All starting reagents were obtained commercially from Sigma-Aldrich or Matrix Scientific and were used without purification. Nuclear magnetic resonance experiments were performed on a Bruker AVANCE 400 MHz spectrometer and processed using TopSpin 3.1. Chemical shift values are reported versus tetramethylsilane as an internal standard purchased in the commercially obtained deuterated solvents (Cambridge Isotope Laboratory). The reactions were followed using silica gel 60 F₂₅₄ thin-layer chromatography plates (Merck EMD Millipore, Darmstadt, Germany). Open column chromatography was utilized for the purification of all final compounds using 60–200 μ , 60A, classic column silica gel (Dynamic Adsorbents, Norcross, GA). UV–vis/NIR absorption spectra were recorded on a Varian Cary 50 spectrophotometer. High-resolution accurate mass spectroscopy (HRMS) spectra were obtained either at the Georgia State University Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer or utilizing a Waters Micromass LCT TOF ES + Premier Mass Spectrometer. Liquid chromatography utilized a Waters 2487 single-wavelength absorption detector with wavelengths set between 640 and 700 nm depending on the particular photophysical properties. The column used in LC was a Waters Delta-Pak 5 μ M 100 A 3.9 $\times$ 150 mm reversed-phase C₁₈ column. Evaporative light-scattering detection analyzes trace impurities that cannot be observed by alternate methods; SEDEX 75 ELSD was utilized in tandem with liquid chromatography to confirm purity (>95% as determined by LC-ELSD-MS). The precursors and the final compounds were purified using column chromatography, and the purity of all synthesized compounds was confirmed with ¹HNMR, ¹³CNMR, and ESI-HRMS. These analytical methods used to determine the purity of compounds to be 95%.

2,3,3-Trimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium Bromide (5).

(2.52 g, 61%); mp 197–199 °C. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.58 (s, 6H), 2.41 (m, 2H), 2.99 (s, 3H), 3.17 (s, 9H), 3.98 (t, $J = 7.6$ Hz, 2H), 4.57 (t, $J = 7.6$ Hz, 2H), 7.45 (m, 2H), 7.86 (d, $J = 5.2$ Hz, 1H), 8.19 (d, $J = 5.2$ Hz, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 21.3, 22.0, 25.4, 44.6, 52.5, 61.9, 64.9, 115.6, 123.5, 129.0, 129.4, 140.9, 141.8, 197.8. HRMS (ESI): calcd for $[\text{C}_{17}\text{H}_{28}\text{N}_2]^+$, m/z 260.2174; found, m/z 260.2177.

5-Fluoro-2,3,3-trimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium Bromide (6).

It is obtained in 90% yield; mp 237–240 °C; ^1H NMR (400 MHz, D_2O): δ 1.56 (s, 6H), 2.47 (t, $J = 8.8$ Hz, 2H), 3.17 (s, 9H), 3.65 (tt, $J = 8.4$ Hz, $J = 8.4$ Hz, 2H), 4.53 (t, $J = 8.0$ Hz, 2H), 7.34 (t, $J = 9.2$ Hz, 1H), 7.82 (dd, $J = 8.8$ Hz, $J = 4.0$ Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 12.2, 20.0, 20.7, 43.3, 52.1, 53.2, 61.5, 115.9, 123.3, 128.8, 134.3, 138.2, 142.3, 197.9. HRMS (ESI): calcd for $[\text{C}_{17}\text{H}_{27}\text{FN}_2]^+$, m/z 278.2194; found, m/z 278.2182.

5-Chloro-2,3,3-trimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium Bromide (7).

It is obtained in 82% yield; mp 215–217 °C; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.65 (s, 6H), 2.56 (t, $J = 8.0$ Hz, 2H), 3.25 (s, 9H), 3.73 (tt, $J = 8.0$ Hz, $J = 8.0$ Hz, 2H), 4.64 (t, $J = 8.0$ Hz, 2H), 4.80 (s, 3H), 7.69 (dd, $J = 8.8$ Hz, $J = 2.0$ Hz, 1H), 7.84–7.92 (m, 2H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 12.2, 20.0, 20.7, 43.3, 52.1, 53.2, 61.5, 115.9, 123.3, 128.8, 134.3, 138.2, 142.3, 197.9. HRMS (ESI): calcd for $[\text{C}_{17}\text{H}_{27}\text{ClN}_2]^{2+}$, (m/z) 294.1956; found, m/z 294.1910.

5-Bromo-2,3,3-trimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium Bromide (8).

It is obtained in 81% yield; mp 225–227 °C; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.57 (s, 6H), 2.27 (s, 9H), 3.02 (s, 3H), 3.83 (t, $J = 8.0$ Hz, 2H), 4.57 (t, $J = 8.0$ Hz, 2H), 7.61 (m, 2H), 7.88 (t, $J = 5.6$ Hz, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 15.2, 22.0, 25.5, 30.6, 52.3, 54.3, 63.9, 115.6, 123.5, 128.8, 129.3, 140.9, 141.7, 197.7. HRMS (ESI): calcd for $[\text{C}_{17}\text{H}_{27}\text{BrN}_2]^{2+}$, (m/z) 338.1320; found, 338.1305.

General Procedure for the Synthesis of Symmetric Squaric Acid Fluorophores.

A mixture of the corresponding indolenine salt (1.0 mol equiv) and squaric acid (0.5 mol equiv) and quinoline (5.0 mol equiv) was refluxed in a mixture of *n*-butanol and benzene (40 mL, 1:1) with azeotropic distillation using a dean stark apparatus for 15 h. The reaction mixture was monitored by vis/NIR spectrophotometry to observe the completion of the reaction and by measuring the new wavelength (~640 nm in EtOH) of the formed NIR dyes. The solvent was removed under reduced pressure. The residue was stirred in ethyl acetate (100 mL). The precipitated product was filtered, washed with ethyl acetate and acetone, and dried under vacuum. The crude material was dissolved in methanol (10 mL) and poured into ethyl acetate (100 mL). The precipitated product was filtered and dried under vacuum. This precipitation procedure was repeated once more, and the obtained pure product was dried under vacuum.

Mono((Z)-4-((3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yl) methylene)-2-((E)-(3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene) methyl)-3-oxocyclobut-1-enolate) Tribromide (9).—It is obtained in 70% yield; mp > 260 °C ^1H NMR (400 MHz, DMSO): δ 1.71 (s, 6H, CH₃), 2.15 (s, 2H, CH₂), 3.12 (s, 9H, CH₃), 3.57–3.61 (s, 2H, CH₂), 4.16 (s, 2H, CH₂), 5.86 (s, 1H, CH), 7.20 (t, J = 7.2 Hz, 1H, ArH), 7.38 (t, J = 8.0 Hz, 1H, ArH), 7.46 (d, J = 8.0 Hz, 1H, ArH), 7.55 (d, J = 7.2 Hz, 1H, ArH); ^{13}C NMR (100 MHz, DMSO): δ 21.0, 27.1, 49.3, 53.0, 63.2, 86.8, 110.8, 122.7, 124.3, 128.4, 141.8, 142.4, 169.6, 180.4, 181.0. HRMS (m/z): for C₃₈H₅₂N₄O₂²⁺ calcd, 298.3046; found, 298.3036.

Mono((Z)-4-((5-fluoro-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yl)methylene)-2-((E)-(5-fluoro-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-enolate) Tribromide (10).—It is obtained in 64% yield; mp > 260 °C

¹H NMR (400 MHz, DMSO): δ 1.71 (s, 12H, CH₃), 2.13 (s, 4H, CH₂), 3.10 (s, 18H, CH₃), 3.55 (s, 4H, CH₂), 4.14 (s, 4H, CH₂), 5.83 (s, 2H, CH), 7.21–7.25 (s, 2H, Ar-H), 7.46 (brs, 2H, Ar-H), 7.53–7.54 (m, 2H, ArH); ¹³C NMR (100 MHz, DMSO): δ 20.7, 26.5, 40.8, 49.5, 53.3, 63.3, 86.4, 110.4, 110.6, 111.7, 111.8, 115.0, 115.2, 138.1, 143.8, 143.9, 159.0, 161.4, 170.9, 175.8, 182.7. HRMS (*m/z*): for C₃₈H₅₀F₂N₄O₂²⁺ calcd, 316.2046; found, 316.2065.

Mono((Z)-4-((5-chloro-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yl)methylene)-2-((E)-(5-chloro-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-enolate) Tribromide (11).—It is obtained in 75% yield; mp > 260 °C ^1H NMR (400 MHz, DMSO): δ 1.71 (s, 12H, CH_3), 2.13 (s, 4H, CH_2), 3.10 (s, 18H, CH_3), 3.54 (s, 4H, CH_2), 4.15 (s, 4H, CH_2), 5.86 (s, 2H, CH), 7.43–7.49 (s, 4H, Ar-H), 7.70 (s, 2H, Ar-H); ^{13}C NMR (100 MHz, DMSO): δ 21.0, 26.9, 49.0, 49.5, 53.1, 63.3, 87.3, 112.2, 123.1, 128.3, 128.8, 141.4, 143.9, 169.6, 180.9, 181.2. HRMS (m/z): for $\text{C}_{38}\text{H}_{50}\text{Cl}_2\text{N}_4\text{O}_2^{2+}$ calcd, 332.1634; found, 316.1629.

Mono((Z)-4-((5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-2-yl)methylene)-2-((E)-(5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-enolate) Tribromide (12).—It is obtained in 62% yield; mp > 260 °C ^1H NMR (400 MHz, DMSO): δ 1.70 (s, 12H, CH₃), 2.12 (s, 4H, CH₂), 3.09 (s, 18H, CH₃), 3.54 (s, 4H, CH₂), 4.14 (s, 4H, CH₂), 5.85 (s, 2H, CH), 7.43–7.55 (s, 4H, Ar-H), 7.80 (s, 2H, Ar-H); ^{13}C NMR (100 MHz, DMSO): δ 20.9, 26.9, 49.5, 53.0, 63.1, 87.3, 112.8, 116.5, 125.9, 131.2, 141.8, 144.2, 159.4, 162.3, 169.3, 180.9. HRMS (m/z): for C₃₈H₅₀Br₂N₄O₂²⁺ calcd, 377.2035; found, 378.2039.

Synthesis of (Z)-2-Hydroxy-3-oxo-4-((1,3,3-trimethyl-3H-indol-1-ium-2-yl)methylene)cyclobut-1-enolate iodide (15) and (Z)-4-((1-(5-Carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-2-hydroxy-3-oxocyclobut-1-en-1-olate (21).—Individual salt (4.0 mol), 3,4-diisopropoxycyclobutane-1,2-dione (4.0 mol), and triethylamine (1.0 mL) were heated in EtOH for 3 h. After the reaction was completed, the reaction was cooled, and the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography (CH₂Cl₂/MeOH). Compound (1.0 mol) was dissolved in EtOH (10 mL), and NaOH (40%, 0.5 mL) was added. The mixture was heated at 70 °C for 30 min. After the reaction was completed, the mixture was cooled, and 2 N HCl (5 mL) was added. The product was purified by silica gel column chromatography (MeOH/DCM 1:9). These intermediates were used in the next steps without characterization.

General Procedure for the Synthesis of Asymmetric Squaric Acid Fluorophores.

A mixture of the corresponding indolenine salt **5–8** (1.0 mol equiv) and mono squaric acid-modified compound **15** or the corresponding indolenine salt **5** (1.0 mol equiv), compound **21** (1.0 mol equiv) and quinoline (5.0 mol equiv) were refluxed in a mixture of *n*-butanol and benzene (40 mL, 1:1) with azeotropic distillation using the dean stark apparatus for 12 h. The reaction mixture was monitored by vis/NIR spectrophotometry to observe the completion of the reaction and by measuring the new wavelength (~630 nm in EtOH) of the formed NIR dyes. The solvent was removed under reduced pressure. The residue was stirred in ethyl acetate (100 mL). The precipitated product was filtered, washed with ethyl acetate and acetone, and dried under vacuum. The crude material was dissolved in methanol (10 mL) and poured into ethyl acetate (100 mL). The precipitated product was filtered and dried under vacuum. This precipitation procedure was repeated once more, and the obtained pure product was dried under vacuum.

Mono((Z)-2-((E)-(3,3-dimethyl-1-(3-(trimethylammonio)propyl)-indolin-2-ylidene)methyl))-3-oxo-4-((1,3,3-trimethyl-3H-indol-1-ium-2-yl)methylene)cyclobut-1-enolate Mono Bromide Mono Iodide (16).—It is obtained in 37% yield; mp > 198–200

$^{\circ}\text{C}$ ^1H NMR (400 MHz, MeOD): δ 1.76–1.77 (m, 12H, CH_3), 2.34 (brs, 2H, CH_2), 3.22 (s, 9H, CH_3), 3.63–3.67 (m, 5H, CH_3 & CH_2), 4.23 (brs, 2H, CH_2), 5.89 (s, 1H, CH), 5.99 (s, 1H, CH), 7.21–7.26 (m, 2H, Ar-H), 7.32–7.43 (m, 4H, Ar-H), 7.46–7.50 (m, 2H, Ar-H), ^{13}C NMR (100 MHz, MeOD): δ 20.6, 25.6, 26.1, 30.3, 40.0, 49.0, 49.4, 52.5, 52.8, 62.3, 63.3, 109.8, 110.3, 121.9, 122.0, 123.9, 124.6, 128.0, 129.2, 130.0, 135.2, 141.3, 141.8, 142.6, 144.6, 147.5, 182.7. HRMS (m/z): for $\text{C}_{33}\text{H}_{40}\text{N}_3\text{O}_2^+$ calcd, 510.8955; found, 510.8995.

Mono((Z)-2-((E)-(5-fluoro-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxo-4-((1,3,3-trimethyl-3H-indol-1-ium-2-yl)methylene)cyclobut-1-eno-late) Mono Bromide Monoiodide (17).—It is obtained in 43% yield; ^1H NMR (400 MHz, MeOD): δ 1.76 (s, 12H, CH₃), 2.32 (s, 2H, CH₂), 3.22 (s, 9H, CH₃), 3.62–3.70 (m, 5H, CH₃ & CH₂), 4.20 (s, 2H, CH₂), 5.85 (s, 1H, CH), 5.99 (s, 1H, CH), 7.13 (t, J = 8.0 Hz, 1H, ArH), 7.25–7.29 (m, 2H, Ar-H), 7.32–7.36 (m, 2H, Ar-H), 7.40–7.43 (m, 1H, Ar-H), 7.50 (t, J = 7.6 Hz, 1H, ArH); ^{13}C NMR (100 MHz, MeOD): δ 20.5, 25.5, 26.1, 30.3, 40.1, 52.5, 63.5, 85.3, 86.2, 109.8, 110.1, 110.2, 110.3, 114.0, 114.3, 121.8, 124.7, 127.9, 138.2, 141.9, 142.7, 169.0, 173.4, 173.9, 177.8, 183.0. HRMS (m/z) for C₃₃H₃₉FN₃O₂⁺ calcd, 528.3021, found 528.2086.

Mono((Z)-2-((E)-(5-chloro-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxo-4-((1,3,3-trimethyl-3H-indol-1-ium-2-yl)methylene)cyclobut-1-eno-late) Mono Bromide Monoiodide (18).—It is obtained in 31% yield; ^1H NMR (400 MHz, MeOD): δ 1.75 (s, 12H, CH₃), 2.30–2.31 (m, 2H, CH₂), 3.23 (s, 9H, CH₃), 3.63–3.65 (m, 2H, CH₂), 3.71 (s, 3H, CH₃), 4.17–4.21 (m, 2H, CH₂), 5.84 (s, 1H, CH), 6.01 (s, 1H, CH), 7.26–7.36 (m, 4H, ArH), 7.40–7.42 (m, 1H, Ar-H), 7.44–7.46 (m, 1H, Ar-H), 7.49–7.51 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, MeOD): δ 20.4, 25.5, 26.2, 30.4, 40.0, 49.7, 52.5, 63.5, 85.5, 86.6, 110.5, 121.9, 122.5, 124.9, 127.8, 128.0, 128.8, 141.0, 142.0, 142.6, 143.1, 168.0, 173.9, 183.0. HRMS (m/z): for C₃₃H₃₉ClN₃O₂⁺ calcd, 543.1150; found, 543.1108.

Mono((Z)-2-((E)-(5-bromo-3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxo-4-((1,3,3-trimethyl-3H-indol-1-ium-2-yl)methylene)cyclobut-1-eno-late) Mono Bromide Monoiodide (19).—It is obtained in 36% yield; mp 204–206 °C ^1H NMR (400 MHz, MeOD): δ 1.75–1.76 (m, 12H, CH_3), 2.31 (s, 2H, CH_2), 3.23 (s, 9H, CH_3), 3.71–3.72 (m, 5H, CH_3 & CH_2), 4.17 (s, 2H, CH_2), 5.84 (s, 1H, CH), 6.01 (s, 1H, CH), 7.28–7.32 (m, 2H, Ar-H), 7.35–7.37 (m, 1H, Ar-H), 7.40–7.43 (m, 1H, Ar-H), 7.49–7.51 (m, 2H, Ar-H), 7.58–7.61 (m, 1H, Ar-H); ^{13}C NMR (100 MHz, MeOD): δ 20.4, 25.5, 26.1, 30.4, 39.9, 49.6, 52.5, 63.3, 85.5, 86.6, 110.6, 111.1, 116.0, 121.9, 124.9, 125.4, 128.0, 130.8, 141.4, 142.0, 142.5, 173.6, 178.2, 182.9. HRMS (m/z) for $\text{C}_{33}\text{H}_{39}\text{BrN}_3\text{O}_2^+$ calcd, 588.6220; found, 588.6233.

(Z)-2-((E)-(1-(5-Carboxypentyl)-3,3-dimethylindolin-2-ylidene)-methyl)-3-oxo-4-((1,3,3-trimethyl-3H-indol-1-ium-2-yl)methylene)-cyclobut-1-enolate, Iodide (22).—It is obtained in 43% yield; mp 166–168

$^{\circ}\text{C}$ ^1H NMR (400 MHz, CDCl_3): δ 1.70–1.91 (m, 18H, CH_3 & CH_2), 2.45 (s, 2H, CH_2), 3.58 (s, 3H, CH_3), 4.04 (s, 2H, CH_2), 5.89 (s, 1H, CH), 6.08 (s, 1H, CH), 6.97–7.02 (m, 2H, Ar-H), 7.16–7.17 (m, 2H, Ar-H), 7.28–7.38 (m, 4H, Ar-H); ^{13}C NMR (100 MHz, CDCl_3): δ 23.8, 25.7, 26.8, 26.9, 34.3, 43.6, 49.1, 49.2, 86.5, 87.1, 109.3, 109.5, 122.1, 122.2, 123.8, 123.9, 127.8, 142.1, 142.2, 142.9, 170.7, 170.9, 176.7, 177.6, 183.4. HRMS (m/z): for $\text{C}_{38}\text{H}_{48}\text{N}_3\text{O}_4$ calcd, 610.3675; found 610.3635.

Optical Property Analyses.

All optical measurements were performed in various solvents, including EtOH, DMSO, PBS (pH 7.4), and at 37 $^{\circ}\text{C}$ in 100% FBS buffered with 50 mM HEPES, pH 7.4. Absorbance and fluorescence emission spectra of the series of NIR fluorophores were measured using the Varian Cary 50 absorbance spectrophotometer (190–1100 nm) and Shimadzu RF-5301PC spectrofluorometer (350–1000 nm). For fluorescence QY measurements, rhodamine 800 in absolute EtOH (QY = 28%) was used as a calibration standard, under conditions of matched absorbance at 620 nm.

Stability Studies.

The stability experiments were performed to determine the photo bleaching and heat decomposition of the fluorophores in serum. We determined this by measuring the absorbance at the wavelength of maximum absorption over 72 h time period. The light condition involved a cuvette containing individual contrast agent (0.01 mm in FBS) and was affixed 250 mm away from a 15W F15T8 broad spectrum bulb being irradiated using a portable lamp at room temperature. Similarly, the heat was based on measurements conducted in sealed centrifuge tubes at physiological temperatures (37 $^{\circ}\text{C}$) for the indicated time periods, and measurements were taken intermittently.

Molecular Modeling Analyses.

In silico calculations of chemical structures were performed using the geometric conformation of the lowest energy, and distances were calculated Spartan DFT calculations at the B3LYP level.

Animal Models and Intraoperative Imaging.

Animals were housed in an AAALAC-certified facility, and all procedures were performed according to the approved institutional protocol by Massachusetts General Hospital (protocol #2016N000136). CD-1 mice (Charles river weight, 19–21 g; age, 6 weeks; male) were purchased from Charles River Laboratories (Wilmington, MA) and were given free access to food and water. Mice were fed with using chlorophyll-free mouse chow (VWR international) 5 days prior to imaging study to minimize autofluorescence. To investigate the biodistribution of each squaraine fluorophore, mice were imaged using the FLARE imaging system.^{19,20} The objective lens was positioned at 25 cm from the operative field. NIR fluorescence and bright-field images were simultaneously obtained using a 660 nm excitation laser at a power of 2 mW/cm² through a 710 $\pm$ 25 nm emission filter and white light (400–650 nm; 40,000 lux). To accurately confirm the biodistribution of these fluorophores, camera exposure time was adjusted appropriately. Intraoperative fluorescent

imaging was performed under isoflurane anesthesia. Four hours prior to imaging, 25 nmol (250 μ M, 100 μ L) of each squaraine fluorophore was systemically injected *via* retro-orbital sinus under isoflurane anesthesia. Upon imaging, the limbs were taped, and the skin from the pubic bone to the neck was incised in the middle. Then, the salivary gland was exposed. After taking images of the salivary gland, it was split between the left lobe and the right lobe, and the muscle in front of the trachea was incised to expose the thyroid gland for fluorescent images. Animals were sacrificed after time-course NIR fluorescence imaging for *ex vivo* biodistribution study. The abdominal wall was incised, and then, the chest wall and diaphragm were incised to thoracotomy. The excretion route of the fluorophore was identified from the abdominal image. After *in vivo* imaging, the major organs including heart, lung, liver, pancreas, spleen, kidney, duodenum, intestine, and muscle were excised. NIR signals from individual resected tissues were imaged using the FLARE imaging system.

Quantitative Analysis of NIR Signals.

The fluorescence intensity of a region of interest (ROI) over each organ/muscle was quantified using ImageJ version 1.52p. The SBR was calculated as the SBR = target signal/muscle signal.

NIR Fluorescence Microscopy.

Major organs were harvested 4 h postintravenous injection of squaraine dyes, trimmed, and embedded in the Tissue-Tek OCT compound (Fisher Scientific). Frozen sections of 10 μ m thickness were cut with a cryostat until the ROI was reached (Leica, Germany). After fluorescence images were acquired on a fluorescence microscope, the sections were stained with H&E. Fluorescence and bright-field imaging were conducted using a Nikon TE2000 microscope (Nikon, Melville, NY) equipped with a custom filter set for NIR imaging (Chroma Technology, Brattleboro, VT). Images were acquired on an Orca-AG CCD camera (Hamamatsu, Bridgewater, NJ) with a custom filter set (Chroma, Brattleboro, VT) composed of a 650 ± 22 nm excitation filter, a 675 nm dichroic mirror, and a 710 ± 25 nm emission filters (Chroma Technology).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(top) General overview of covalently labeling a biomolecule with a NIR-dye (bottom) commonly used fluorophores with benefits, in green, and negatives, in red, associated with their *in vivo* use.

Figure 2.

(left) Absorption and fluorescence spectra for compounds **9–12**, **16–19**, and **22** in FBS supplemented with HEPES buffer at pH = 7.4 and warmed to physiological temperature (37 °C). (right) Absorption-based stability in light and under heat (37 °C) in FBS buffer for compounds **9** and **16**, and industry-standard Cy5 as representative examples.

Figure 3.

(left) DFT calculations (Wavefunction, Spartan'10, B3LYP 6-311++G**) confirmed the presence of a salt bridge between the quaternary ammonium cation and the delocalized negative charge across the central oxygens in both the symmetric and asymmetric compounds; (right) schematic cartoon showing the locked nature of the double bonds, which is thought to contribute to improved absorptivity, QY, and stability.

Figure 4. Biodistribution and clearance of compounds **9**, **16**, and **22**. Each fluorophore (25 nmol) was injected intravenously into 25 g of CD-1 mice 4 h prior to imaging and resection. (A) Intraoperative NIR fluorescence imaging of mice and resected organs 4 h postinjection. Scale bars = 5 mm; (B) quantitative analysis of the signal-to-background ratio (SBR) for major organs against muscle at 4 h postinjection. (C) Histological evaluation of biodistribution of compound **9** in major organs. Hematoxylin and eosin (H&E) and NIR fluorescence images of the resected kidney and liver 4 h postintravenous injection are shown. Abbreviations used are: Bl, bladder; BD, bile duct; Du, duodenum; Ga, gallbladder (arrowhead); He, heart; In, intestine; Ki, kidneys; Li, liver; Lu, lungs; Mu, muscle; Pa, pancreas; and Sp, spleen. Scale bars = 200 μ m.

Scheme 1.
Synthesis of Squaraine Dyes 9–12 Containing Quaternary Ammonium Propyl Bromide

Scheme 2.
Final Asymmetric Compound 16–19 Preparation

Scheme 3.
Final Asymmetric Compound **22** Preparation