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Water-Soluble NIR Absorbing and Emitting Indolizine Cyanine and Indolizine Squaraine Dyes for Biological Imaging

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Abstract

Organic dyes that absorb and emit in the near-infrared (NIR) region are potentially noninvasive, high-resolution, and rapid biological imaging materials. Indolizine donor-based cyanine and squaraine dyes with water-solubilizing sulfonate groups were targeted in this study due to strong absorptions and emissions in the NIR region. As previously observed for nonwater-soluble derivatives, the indolizine group with water-solubilizing groups retains a substantial shift toward longer wavelengths for both absorption and emission with squaraines and cyanines relative to classically researched indoline donor analogues. Very high quantum yields (as much as 58%) have been observed with absorption and emission >700 nm in fetal bovine serum. Photostability studies, cell culture cytotoxicity, and cell uptake specificity profiles were all studied for these dyes, demonstrating exceptional biological imaging suitability.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.9b03108>.

Photostability studies; molar absorptivity and normalized emission of ICG in several solvents; electronic absorption studies; fluorescence studies; ^1H NMR and ^{13}C { ^1H } NMR for the prepared compounds; and solubility studies (PDF)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.joc.9b03108>

The authors declare no competing financial interest.

INTRODUCTION

Near-infrared (NIR) emissive materials are intensely researched due to a plentiful number of practical applications, including biological imaging,^{1–8} photodynamic therapy,^{1,9–12} telecommunications,^{13–15} secure displays, which can be coupled to night vision technologies,^{16–18} and many other innovative areas of research.^{5,19–21} NIR biological imaging agents use low-energy photons in a spectral region where tissue is readily penetrated at a maximal depth optically.^{4,10,22,23} The desirable properties of biological imaging dyes include (1) absorbing and emitting light in the “therapeutic window” ranging from 700 to 1400 nm, (2) a significant Stokes shift to enable high-precision imaging, (3) efficient absorption and emission of photons through both a high molar absorptivity and quantum yield, and (4) water solubility. While there are many exciting dye design prospects being pursued in the literature,² exceptional NIR emissive materials are still needed for practical biological imaging since current dyes fall short of meeting all of the desired criteria simultaneously.

Among NIR emissive dyes, squaraines^{21,24} and cyanines^{19,25} are ubiquitous.^{1,3,26} Previous research has frequently investigated indoline donor-based cyanine and squaraine dyes with indocyanine green (ICG) having been awarded FDA approval several decades ago.^{27,28} Both of these classes of dyes need design strategies that allow access to longer wavelength NIR photons in aqueous environments.² In fact, the indoline squaraine absorbs outside the therapeutic window, which renders it impractical for use as a noninvasive biological imaging agent.²⁹ To deepen the NIR absorbing properties of these dye classes, designs capable of extending the π -system through a conjugated donor (such as indolizine) are attractive.^{29–32} Recent photophysical studies using proaromatic indolizine donors in place of indoline donors show red-shifting of the absorption and emission profiles of these dyes firmly into the therapeutic window in nonaqueous environments (**C5** and **SQ** compared to ICG and indoline squaraine, respectively; Figure 1).^{29–32} These studies indicated that in the case of phenyl-indolizine derivatives, the phenyl group does not play a significant role in tuning the absorption or emission energy values of the NIR indolizine dyes.^{29,30} This enables the use of the phenyl group to append water-soluble functionality without perturbing the core

chromophore properties of the dyes, permitting the probing of the photophysical properties of the dyes in aqueous environments. Sulfonate-substituted dyes, **SO₃C5** and **SO₃SQ**, were targeted given the water-solubilizing properties of sulfonate groups and FDA approval for human use by way of ICG (Figure 1). This work aims to compare the photophysical properties of **SO₃C5**, **SO₃SQ**, and benchmark ICG in a biologically relevant medium.

SYNTHESIS

The target dyes were synthesized in two steps from previously reported indolizine phenol 1 (Scheme 1).²⁹ First, 1 was alkylated with butane sultone (2) in the presence of base to give sulfonate indolizine 3. Interestingly, this reaction required a careful selection of solvent and base to avoid significant side product formation as a carbon alkylated product (see Table S1 and Figure S1). Intermediate 3 is common to both **SO₃C5** and **SO₃SQ** target dyes. The target sulfonate indolizine cyanine dye (**SO₃C5**) is synthesized by reacting 3 with the methine bridge linker 4 in the presence of perchloric acid and acetic anhydride at room temperature similar to previously reported conditions in the literature.³⁰ The target sulfonate indolizine squaraine (**SO₃SQ**) is synthesized via an electrophilic aromatic substitution/condensation reaction between sulfonate indolizine 3 and squaric acid (5). Importantly, during optimization of the condensation reaction, it was found that the addition of small amounts of water led to an *increase* in product formation. Ultimately, running the reaction in nondried laboratory-grade methanol gave the highest yields (Table S2). Both target dyes are formed in an approximately 50% overall yield in this route over two steps.

RESULTS AND DISCUSSION

With **SO₃C5** and **SO₃SQ** in hand, absorption and emission studies were undertaken for comparison to ICG as a benchmark reference dye (Figures 2, S2 and S3; Table 1). Dimethyl sulfoxide (DMSO) and MeOH were also selected as solvents, which allows for the comparison of **SO₃C5** and **SO₃SQ** to the previously reported dyes **C5** and **SQ**, respectively (see Table S3 for solubility data).^{29,30} H₂O and fetal bovine serum (FBS) were selected as well due to the biological relevance and for a convenient analysis of how these dyes will behave with biological matrix elements in place, as has been previously reported in the literature.^{4,33,34} The absorption profiles of both dyes in DMSO and MeOH are not significantly changed relative to the previously published dyes with no sulfonate groups.^{29,30} The absorption curves in H₂O and FBS are similar to the curves observed in DMSO and MeOH for **SO₃SQ**; however, the absorption curve of **SO₃C5** in water deviates shape significantly, which is likely due to aggregation in water (Figure S4). Upon changing concentrations, no significant evidence of aggregate disruption could be observed down to concentrations near the detection limit of the spectrometer (Figure S5). Notably, the evidence of aggregation of **SO₃SQ** (modest amounts) and **ICG** (significant amounts) in H₂O at concentrations of 10^{-5} M is apparent at higher-energy wavelengths when the absorption curves are overlaid with curves at 10^{-6} M in H₂O, which appear to have disrupted aggregate features (Figures S6 and S7).³⁵ It should be noted that while all three dyes were observed to form aggregates in H₂O via dynamic light scattering analysis at 5×10^{-6} M (Figure S8), **SO₃C5** is the only dye that shows a significant impact of this aggregative behavior from the absorption profile. Interestingly, upon dissolving **SO₃C5** in FBS, the original curve shape

observed in DMSO and MeOH is regained with a notable red shift of the $\lambda_{\max}$ value by about 40 nm (Figure 2).

FBS is commonly used in photophysical studies for NIR dyes intended for biological use as the albumin proteins help to better separate dye molecules from one another as is dramatically demonstrated here. The disruption of aggregation under biologically relevant conditions is encouraging as it leads to the minimization of thermal relaxations resulting in increased quantum yields. For comparison, the curve shape of ICG retains the same profile in all four solvents (Figure S3). The molar absorptivities are similar for **SO₃C5** and **SO₃SQ** in MeOH and DMSO at approximately 110 000–150 000 M⁻¹ cm⁻¹ (Figure 2 and Table 1). In H₂O and FBS, the **SO₃C5** molar absorptivities drop substantially to ~60 000 M⁻¹ cm⁻¹ or less. For **SO₃SQ** in H₂O or FBS, the molar absorptivities remain significantly higher at 113 000–93 000 M⁻¹ cm⁻¹. The molar absorptivity values and order of $\lambda_{\max}$ values follow the same trend for **SO₃SQ** and ICG with each of the four solvents. **SO₃C5** deviates from this trend with obvious aggregation in water-based solutions. Compared to ICG, the $\lambda_{\max}$ of **SO₃C5** is shifted toward lower energy values (~15 nm), and the $\lambda_{\max}$ of **SO₃SQ** is shifted toward higher energy by about 100 nm (0.21 eV, Table 1).

Fluorescence spectroscopy was probed with each of the dyes to understand the excited-state behavior. Prior reports on **C5** and **SQ** in organic solvents such as dichloromethane have shown Stokes shifts of 43 and 50 nm, respectively (taken as the difference between the $\lambda_{\max}^{\text{abs}}$ and $\lambda_{\max}^{\text{emis}}$).^{29–31} The Stokes shifts of both sulfonate dyes decreased to 26 nm (400 cm⁻¹) in all solvents where single molecule behavior is likely occurring. As the magnitude of the Stokes shift is directly related to the reorganization energy of the dye, the decrease in the Stokes shift demonstrates that there is smaller reorganization energy of **SO₃SQ** and **SO₃C5** relative to the nonsulfonate-substituted dyes. Stokes shifts for **SO₃C5** could not be determined in FBS and H₂O due to the presence of multiple species due to aggregation, leading to apparent emission energies higher than the lowest energy features of the combined absorption spectrum of all monomer and aggregate states. This suggests that in aqueous solvents, the higher-energy absorption feature of **SO₃C5** corresponds to the observed emission, while the lower-energy absorption feature is either weakly emissive or has an emission beyond the InGaAs fluorimeter detection limit.

Quantum yields (Φ) for each dye were calculated as relative values with respect to ICG in DMSO at 11.0% as the benchmarking system (Table 1).³⁶ The quantum yields for ICG in water, MeOH, and FBS were calculated with respect to ICG in DMSO. Previously reported quantum yields for **C5** in organic solvents ranged from 2 to 3.6%;^{30,31} however, **SO₃C5** shows Φ values below 1% in all three protic solvents examined. In DMSO, a Φ of 2% is observed, which is similar to the values observed for the parent **C5** structure. The Φ data for **SO₃SQ** is exceptionally intriguing. In water or methanol, the Φ of **SO₃SQ** is <1%, which is not uncommon in the NIR spectral region. Upon changing the solvent environment to DMSO, a significantly increased Φ value of 8.6% is observed, which can be rationalized as the protic environments promoting non-radiative decay pathways leading to low Φ values in water and methanol. However, **SO₃SQ** in FBS gives a 58% quantum yield, which is a number that is truly remarkable in the field of NIR dyes and encourages further studies with this material. The dramatic enhancement of Φ values in the complex biologically relevant

environment of FBS has been previously observed in the literature.³³ By comparison, the benchmark dye ICG shows a much lower 9% Φ in FBS.

An important metric for biological imaging materials is the molecular brightness (MB), which balances the contributions of ϵ and Φ through the equation $MB = \epsilon \times \Phi$. MB is a good indicator of the amount of dye needed to give comparable fluorescence signal intensities among dyes with varying molar absorptivities and quantum yields. The MB values for **SO₃C5** are quite low in all solvents at $2180 \text{ M}^{-1} \text{ cm}^{-1}$ when compared with ICG at a maximum value of $23\,000 \text{ M}^{-1} \text{ cm}^{-1}$ in DMSO (Table 1). Notably, the observed MB value for ICG is low in H₂O at $780 \text{ M}^{-1} \text{ cm}^{-1}$ but remains high in the remaining solvents examined ($>9000 \text{ M}^{-1} \text{ cm}^{-1}$). **SO₃SQ** shows an MB value of $>65\,000 \text{ M}^{-1} \text{ cm}^{-1}$ in FBS, which is dramatically larger than the substantial $23\,000 \text{ M}^{-1} \text{ cm}^{-1}$ value for ICG and is likely one of the largest values in this spectral region known in the literature. This indicates that significantly less dye ($\sim 3\times$ less) would be needed with **SO₃SQ** to generate the same amount of signal as ICG.

A key desirable property of biological imaging dyes is prolonged photostability.³⁷ To probe this, **SO₃C5**, **SO₃SQ**, and ICG were dissolved in water under an ambient atmosphere at a concentration of $1 \times 10^{-6} \text{ M}$ and irradiated with a solar-simulated light-emitting diode (LED) spectrum from 400 to 1100 nm with white light irradiation (Figures 3 and S9–S11). Under these conditions, ICG has a half-life of approximately 35 min with complete consumption by 80 min. Both **SO₃C5** and **SO₃SQ** show a higher photostability than ICG with half-lives of 50 and 100 min, respectively. The higher photostability of **SO₃SQ** can be attributed, in part, to the squaraine structure being devoid of double-substituted alkenes, which are known to undergo various degradation pathways with ICG.³⁸ In MeOH, **SO₃SQ** and ICG are exceptionally stable with half-lives $>24 \text{ h}$ (Figures S12–S15). **SO₃C5** is notably less stable than ICG or **SO₃SQ** in MeOH where the dye appears to be deaggregated, which suggests that the aggregate states of **SO₃C5** in water could impart some photostability since this dye is more stable than ICG in water. Overall, **SO₃SQ** has a likely record quantum yield in this spectral region, a high molar absorptivity, an exceptional molecular brightness, and the highest photostability measured in this study in H₂O.

Finally, the cytotoxicity and cellular uptake of **SO₃C5** and **SO₃SQ** were examined with human (HEK293) and *Drosophila* (S2) tissue culture cells (Figure 4). HEK cells are a human cell line grown at physiological conditions found in mammals. Performance and cytotoxicity of the dyes in HEK cells inform in vivo biocompatibility for clinical and veterinary settings for use in diagnostic or therapeutic technologies. In contrast, S2 cells grow at a lower temperature under normal atmospheric conditions and are derived from invertebrates (fruit fly embryos). Dye behavior in these cells is a proxy for understanding the environmental impact of these compounds. Both **SO₃C5** and **SO₃SQ** show $< 25\%$ HEK cell death at or below 2 mM concentrations. At high dye loadings (10 mM), cell death is near 30%, which indicates that these dyes are relatively benign toward a human cell line. A similar trend is observed with the cytotoxicity studies of the S2 cell line, although 2 mM **SO₃C5** and 10 mM **SO₃SQ** show $>50\%$ cell death. S2 cells are more phagocytically active compared to HEK293 cells, which may explain greater sensitivity to the dyes. This suggests that low-to-moderate concentrations of the dyes are not toxic to animal cells. **SO₃C5** and

SO₃SQ cell uptake studies were undertaken with LysoTracker by comparing the fluorescence overlap of each NIR dye with LysoTracker. With these studies, the **SO₃C5** dye shows a Pearson's correlation coefficient of 0.9, indicating that HEK cells largely uptake this dye into the cell lysosomes. A similarly high Pearson's correlation coefficient is observed for **SO₃SQ** and S2 cells at 0.7. This shows that nonfavorable interactions of the dye with cell organelles are minimized.

CONCLUSIONS

The need for biological imaging dyes that absorb and emit strongly in the NIR region is of paramount importance to society since this could enable noninvasive, nontoxic, and rapid imaging of medical patients and biological specimens. Indolizine donor-based cyanine and squaraine dyes with attached sulfonate groups were synthesized to provide a direct comparison in biological medium to benchmark ICG. The increased donor strength of the indolizine compared to that of the indoline allowed for these dyes to have a more red-shifted absorption and emission than their indoline counterparts. **SO₃SQ** demonstrated a remarkable quantum yield of 58.3% (MB of > 65 000) in FBS, which is notable in that this is representative of the optical properties inside of a biological medium. **SO₃C5** shows significant concentration-independent aggregation effects in water, while **SO₃SQ** shows minimal aggregation in water. Prolonged irradiation studies demonstrate that both of the indolizine dyes have a significantly higher photostability than ICG in water. Cell culture studies exhibited high specificity for lysosomal uptake with overlap coefficients of 0.9 and 0.7 for **SO₃C5** and **SO₃SQ**, respectively, as well as low levels of cytotoxicity. The high molecular brightness, simple synthesis, prolonged photostability, and low toxicity of **SO₃SQ** show that it is an attractive potential NIR biological imaging material with very high metrics significantly surpassing the benchmark ICG over a range of tests.

EXPERIMENTAL SECTION

All commercially obtained reagents and solvents were used as received without further purification. All heating was done in an oil bath. Thin-layer chromatography (TLC) was conducted with Merck KGaA TLC silica gel 60 RP-18 F₂₅₄S glass-backed plates and visualized with UV. Flash column chromatography was performed using a CombiFlash Rf + system. RediSep cartridges were charged with silica gel from RediSep Rf reversed-phase C18, 40–63 μ m (230–400 mesh). ¹H and ¹³C {¹H} NMR spectra were recorded on a Bruker Ascend-300 (300 MHz) spectrometer and are reported in ppm using solvent as an internal standard (DMSO-*d*₆ at 2.50 ppm and CD₃OD-*d*₄ at 3.31 ppm). Data are reported as s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, b = broad, ap = apparent, dd = doublet of doublets, dt = doublet of triplets; coupling constant(s) are in Hz; integration. UV–vis–NIR spectra were measured¹³ with a Cary 5000 UV–vis–NIR spectrometer. For photostability studies, samples were irradiated with a Class AAA Rated G2V pico LED solar simulator providing a spectral range from 400 to 1100 nm at 100 mW cm⁻². High-resolution mass spectrometry (HRMS) spectra were obtained with a quadrupole time-of-flight (QTOF) HRMS utilizing nanospray ionization. The mass analyzer was set to the 200–2000 Da range. Infrared spectra were recorded with an Agilent Cary 660 attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrometer. All emission data was

obtained using a Horiba PTI fluorimeter. Excitation wavelengths were achieved by passing white light through a dual-grating system. Photons were collected through a photomultiplier tube. The relative quantum yields were obtained using this equation

$$\phi_{\text{sample}} = \phi_{\text{standard}} \times \frac{E_{\text{sample}}}{E_{\text{standard}}} \times \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \frac{\eta_{\text{sample}}^2}{\eta_{\text{standard}}^2}$$

For the equation above, E is the sum of emission intensities and A is the maximum absorbance, η is the refractive index of the solvent used, and Φ denotes the quantum yield. The standard used to obtain the relative quantum yields was indocyanine green (ICG) with a quantum yield of 11% in DMSO.³⁶

Cell Imaging.

HEK293 cells were grown in standard conditions (37 °C, 5% CO₂) in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% FBS. S2 cell cultures were maintained in S2 media with 10% FBS at 25 °C. Dye cytotoxicity was determined with a CyQUANT LDH Cytotoxicity Assay Kit™ Invitrogen using a BioTek Synergy™ H1 microplate reader. Cells were imaged with a Zeiss LSM 510 META laser scanning confocal microscope following a 24 h exposure to dyes. Lysosome was visualized with a LysoTracker Green DND-26 (Invitrogen) following the manufacturer's protocols.

Cesium 4-(4-(1-Methylindolizin-2-yl)phenoxy)butane-1-sulfonate (3).

To a flame-dried round-bottom flask equipped with a stir bar and reflux condenser that had been purged with N₂ for 10 min were added 4-(1-methylindolizin-2-yl)phenol (1) (1.00 g, 4.48 mmol),²⁹ Cs₂CO₃ (3.65 g, 11.20 mmol), and 1,4-butane sultone (2) (1.15 mL, 11.20 mmol). The reaction mixture was dissolved in tetrahydrofuran (THF) (140 mL) and heated to 80 °C in an oil bath for 16 h. The reaction mixture was cooled down to room temperature and yielded a light brown precipitate. Ethyl acetate was added (100 mL) to further precipitate the product, which was subsequently filtered to yield the crude material. The crude mixture was purified by recrystallization in water to yield a pure golden brown metallic in appearance solid (1.87 g, 85%). ¹H NMR (300 MHz, DMSO-*d*₆; Figure S16) δ 8.13 (d, J = 6.9 Hz, 1H), 7.63 (s, 1H), 7.42 (d, J = 8.7 Hz, 2H) 7.37 (d, J = 9.1 Hz, 1H), 6.99 (d, J = 8.7 Hz, 2H), 6.60 (dd, J = 8.1, 7.2 Hz, 1H), 6.45 (dt, J = 7.1, 1.2 Hz, 1H), 3.98 (t, J = 5.8 Hz, 2H), 2.46 (t, J = 7.1 Hz, 2H), 2.34 (s, 3H), 1.85–1.65 (m, 4H). ¹³C {¹H} NMR (300 MHz, DMSO-*d*₆; Figure S17) δ 157.3, 130.2, 129.1, 127.8, 127.8, 125.2, 117.0, 115.6, 114.6, 109.8, 109.7, 104.2, 67.3, 51.1, 28.1, 22.0, 9.7. HRMS m/z calculated for C₁₉H₂₀NO₄S [M–Cs][–]: 358.1119, found 358.1128. IR (neat, cm^{–1}) 3413, 3310, 3064, 3048, 2931, 2860, 1675, 1611, 1535, 1515. Melting point (dec.): 226–232 °C.

Cesium 4-(4-(1-Methyl-3-((1*E*,3*E*)-5-((*Z*)-1-methyl-2-(4-(4-sulfonatobutoxy)phenyl)-3*H*-indolizin-4-ium-3-ylidene)-penta-1,3-dien-1-yl)indolizin-2-yl)phenoxy)butane-1-sulfonate (SO₃C5).

To a round-bottom flask equipped with a stir bar was added cesium 4-(4-(1-methylindolizin-2-yl)phenoxy)butane-1-sulfonate (3) (0.50 g, 1.02 mmol) to acetic

anhydride (10.2 mL) followed by perchloric acid (0.088 mL, 1.02 mmol) and *N*-[5-(phenylamino)-2,4-pentadienylidene]aniline monohydrochloride (4) (0.15 g, 0.51 mmol). The mixture was then sonicated and stirred until all of the starting material appeared to have been consumed in this first phase of the reaction (about 10 min). When the starting material was no longer visible, the reaction was allowed to stir for another 10 min before the addition of triethylamine (0.17 mL, 1.22 mmol). The reaction mixture quickly turned green and was allowed to stir for 2 h before diethyl ether was added to precipitate the crude product. The precipitate was filtered and rinsed once more with diethyl ether, collecting the solids. The solids were purified via reversed-phase column chromatography beginning with 100% H₂O and gradually transitioning to 40:60 ethanol/H₂O. The water was removed by blowing air over the surface of the solution to avoid heating. The pure product was yielded as a dark green solid (250 mg, 53%). ¹H NMR (300 MHz, CD₃OD-*d*₄; Figure S18) δ 8.89 (d, *J* = 6.8 Hz, 2H), 7.73 (d, *J* = 8.6 Hz, 2H), 7.66–7.54 (m, 4H), 7.42–7.22 (m, 7H), 7.12 (d, *J* = 8.5 Hz, 4H), 6.57 (t, *J* = 13.1 Hz, 2H), 4.16 (t, *J* = 5.9 Hz, 4H), 2.95 (t, *J* = 7.0 Hz, 4H), 2.20 (s, 6H), 2.14–1.94 (m, 8H). ¹³C {¹H}NMR (300 MHz, CD₃OD-*d*₄; Figure S19) δ 168.4, 160.9, 144.4, 140.1, 135.6, 132.6, 131.3, 129.7, 127.4, 126.2, 122.5, 121.2, 119.4, 118.0, 116.1, 68.9, 52.4, 29.5, 23.1, 9.3. HRMS *m/z* calculated for C₄₃H₄₄CsN₂O₈S₂ [M + H]⁺: 913.1588, found 913.1606. IR (neat, cm⁻¹): 3375 (br), 3099, 3070, 3033, 2917, 2861, 1651, 1611, 1521, 1508. UV–vis–NIR (DMSO) λ_{max} = 801 nm; UV–vis–NIR (MeOH) λ_{max} = 791 nm; UV–vis–NIR (H₂O) λ_{max} = 726 nm; UV–vis–NIR (FBS) λ_{max} = 842 nm. Melting point (dec.): 211–217 °C.

Cesium (Z)-4-(4-(1-Methyl-3-(3-(1-methyl-2-(4-(4-sulfonatobutoxy)phenyl)-3*H*-indolizin-4-ium-3-ylidene)-2-oxido-4-oxocyclobut-1-en-1-yl)indolizin-2-yl)phenoxy)-butane-1-sulfonate (SO₃SQ).

To a pressure flask equipped with a stir bar were added cesium 4-(4-(1-methylindolizin-2-yl)phenoxy)-butane-1-sulfonate (3) (0.100 g, 0.204 mmol) and 3,4-dihydroxy-1,2-cyclobutanedione (5) (0.012 g, 0.102 mmol). The reagents were dissolved in 10 mL methanol and degassed with N₂ for 10 min. The reaction mixture was then heated to 100 °C in an oil bath for 6 h. The reaction mixture was then concentrated, and the solids were further purified via reversed-phase column chromatography beginning with 100% H₂O and gradually transitioning to 40:60 ethanol/H₂O. The water was removed by blowing air over the surface of the solution as the product appeared to be sensitive to heat. The pure product was yielded following this step as a metallic red solid in appearance (65 mg, 60%). ¹H NMR (300 MHz, CD₃OD-*d*₄; Figure S20) δ 9.76 (d, *J* = 6.9 Hz, 2H), 7.57 (d, *J* = 8.7 Hz, 2H), 7.37 (dd, *J* = 6.8, 1.0 Hz, 2H), 7.23 (d, *J* = 8.6 Hz, 4H), 7.04–6.95 (m, 6H), 4.13 (t, *J* = 6.0 Hz, 4H), 2.95 (t, *J* = 7.0 Hz, 4H), 2.21 (s, 6H), 2.12–1.83 (m, 8H). ¹³C {¹H} NMR (300 MHz, CD₃OD-*d*₄; Figure S21) δ 177.8, 166.1, 160.1, 143.2, 138.6, 134.9, 132.9, 128.9, 127.7, 121.0, 119.5, 118.4, 116.1, 114.8, 68.7, 52.5, 29.6, 23.1, 9.5. HRMS *m/z* calculated for C₄₂H₃₉N₂O₁₀S₂ [M–2Cs + 2H][–]: 795.2052, found 795.2050. IR (neat, cm⁻¹): 3398 (br), 3068, 3038, 2919, 2864, 1733, 1600, 1570, 1523. UV–vis–NIR (DMSO) λ_{max} = 720 nm; UV–vis–NIR (MeOH) λ_{max} = 704 nm; UV–vis–NIR (H₂O) λ_{max} = 699 nm; UV–vis–NIR (FBS) λ_{max} = 719 nm. Melting point: 240–244 °C.

Photostability Studies.

All photostability studies were conducted at a concentration of 1×10^{-6} M to maintain consistency and deter any aggregative effects throughout the photodecomposition process. The samples were irradiated with a white LED lamp providing a spectral range from 400 to 1100 nm. The experiment was allowed to take place under ambient conditions without any further precautions to remove oxygen from the system. UV-vis-NIR was used to periodically measure the decline in absorption with respect to the λ_{max} . The photostability in methanol was conducted over a period of 24 h, while the photostability in water was conducted over a period of just 6 h as the photodecomposition was observed within a shorter time frame.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Current work demonstrating water solubility of indolizine cyanine and indolizine squaraine dyes using sulfonate groups.

Figure 2. Vis–NIR region molar absorptivity and normalized fluorescence emission of $\text{SO}_3\text{C5}$ (top) and SO_3SQ (bottom) in DMSO, MeOH, H_2O , and FBS at $5 \times 10^{-6} \text{ M}$.

Figure 3.
Photostability of SO₃C5, SO₃SQ, and ICG in water.

Figure 4.

Interaction of $\text{SO}_3\text{C5}$ and SO_3SQ dyes with human (HEK) and *Drosophila* (S2) cells. (A–D) Percent cytotoxicity for each combination as determined by the LDH assay. *X*-axis values are mM. Error bars represent standard error, and letter denotes significance groups determined by Tukey's HSD ($p < 0.05$). (E, F) Confocal images of live HEK (E) and S2 (F) cells after exposure to dye and LysoTracker. Left panels show the merging of bright field, lysotracker (green), and dye (red). Dye fluorescence alone is shown in the right panel (E', F'). Pearson's correlation coefficient (noted as the overlap coefficient) shows colocalization between the dye and LysoTracker.

Scheme 1.
Synthetic Route to SO₃C5 and SO₃SQ

Table 1.
Optical Properties of SO₃C5, SO₃SQ, and ICG in Water, Fetal Bovine Serum (FBS), Methanol, and DMSO

dye	solvent	abs. max. (nm)	emis. max. (nm)	stokes shift (nm)eV(cm ⁻¹)	ϵ (M ⁻¹ cm ⁻¹)	Φ (%)	MB ($e \times \Phi$)
SO ₃ C5	DMSO	801	821	20 0.04 304	109 000	2.0	2180
	MeOH	791	817	26 0.05 403	139 000	0.3	420
	FBS	842	808		61 000	0.1	60
	water	726	808		40 000	0.05	20
SO ₃ SQ	DMSO	720	734	14 0.03 265	145 000	8.6	12 500
	MeOH	704	719	15 0.04 296	156 000	0.8	1250
	FBS	719	730	11 0.03 210	113 000	58.3	65 900
	water	698	716	18 0.04 360	93 000	0.3	280
ICG	DMSO	794	825	31 0.06 473	211 000	11.0	23 200
	MeOH	784	819	35 0.07 545	230 000	4.3	9900
	FBS	798	827	29 0.05 439	174 000	9.3	16 200
	water	779	812	33 0.06 522	157 000	0.5	780