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Journal Title: Bioconjugate Chemistry
Volume: Volume 19, Number 12
Publisher: American Chemical Society | 2008-12-01, Pages 2559-2567
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1021/bc800415t
Permanent URL: https://pid.emory.edu/ark:/25593/s1477

Final published version: http://dx.doi.org/10.1021/bc800415t

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Accessed September 15, 2017 8:32 AM EDT
Amine-Reactive Fluorene Probes: Synthesis, Optical Characterization, Bioconjugation, and Two-Photon Fluorescence Imaging

Alma R. Morales, Katherine J. Schafer-Hales, Adam I. Marcus, and Kevin D. Belfield

With the increasing demand for confocal and two-photon fluorescence imaging, the availability of reactive probes that possess high two-photon absorbitivity, high fluorescence quantum yield, and high photostability is of paramount importance. To address the demand for better-performing probes, we prepared two-photon absorbing amine-reactive fluorenyl-based probes 2-(9,9-bis(2-((2-methoxyethoxy)ethyl)-2-isothiocyanato-9H-fluoren-7-yl)benzothiazole (1) and 2-(4-(2-(9,9-bis(2-((2-ethoxyethoxy)ethyl)-2-isothiocyanato-9H-fluoren-7-yl)vinyl)phenyl)benzothiazole (2), incorporating the isothiocyanate as a reactive linker. Probe design was augmented by integrating high optical nonlinearities, increased hydrophilicity, and coupling with reactive functional groups for specific targeting of biomolecules, assuring a better impact on two-photon fluorescence microscopy (2PFM) imaging. The isothiocyanate (NCS) derivatives were conjugated with cyclic peptide RGDfK and Reelin protein. The study of the chemical and photophysical properties of the new labeling reagents, as well as the conjugates, is described. The conjugates displayed high chemical stability and photostability. The NCS derivatives had low fluorescence quantum yields, while their bioconjugates exhibited high fluorescence quantum yields, essentially “lighting up” after conjugation. Conventional and 2PFM imaging and fluorescence lifetime imaging (FLIM) of HeLa, NT2, and H1299 cells, incubated with two-photon absorbing amine-reactive probe (1), RGDfK-dye conjugate (7), and Reelin-dye conjugate (6), was demonstrated.

INTRODUCTION

In the past decade, two-photon fluorescence microscopy (2PFM) has provided several advantages in biological research, including high three-dimensional (3D) spatial resolution as a result of the inherent nonlinear dependence of two-photon fluorescence (2PF) on the illumination intensity (1–4). In this technique, the excitation volume is limited to the focal plane, minimizing out-of-focus excitation, fluorescence, photobleaching, and photodamage. Near-IR excitation used in 2PFM (780 nm) enables deeper imaging into optically thick tissue (∼50 cm4···s) and improves tissue viability (6). The inherently low 2PF signal in contrast to one-photon fluorescence (1PF) is due to the small two-photon absorption (2PA) cross sections (∼10−50 cm4·s) of the fluorescent probes. This is the motivating factor in the continuing efforts to synthesize fluorescence markers with large 2PA cross sections. A steady increase in the biomedical applications of 2PFM has uncovered the lack of efficient two-photon absorbing fluorescence probes with high specificity. In order to be truly useful for such applications, it is necessary to have not only an imaging component which undergoes strong two-photon absorption (2PA) at wavelengths greater than 700 nm, but also a targeting component which binds the fluorescent probe selectively to the target tissue or organelle.

Currently, only a limited number of 2PA fluorophores, specifically tailored for direct labeling of biomolecules for two-photon induced fluorescence imaging studies, have been reported (7–11). The most common of these are fluorophores designed for the labeling of lysine residues on proteins with amine-reactive compounds bearing functional groups capable of forming a stable linkage with these biomolecules. Representative labeling functionalities include isothiocyanates and succinimidyl esters for coupling with primary and secondary amines.

Our previous success with fluorene dyes in the field of 2PA (13–19) encouraged us to further investigate this class of chromophores as two-photon excitable fluorescent labels. Here, we present the synthesis of novel two-photon excitable fluorescent labeling reagents, along with their peptide and bioconjugate analogues. Our strategy consists of building 2PA chromophores containing the isothiocyanate functionality, −N=C=S, capable of forming a stable linkage with biomolecules containing −NH2 amino groups. The chromophores are based on fluorene derivatives containing the benzothiazole motif as an electron-acceptor group. The π-electron conjugation length was increased via the incorporation of a styryl group directly connected to the fluorenyl π-bridge construct.

EXPERIMENTAL SECTION

Materials and Methods. The cyclic peptide (Arg-Gly-Asp-D-Phe-Lys) c(RGDfK) was purchased from Peptides International, Inc. All other chemicals and reagents were purchased from Aldrich or Acros Organics and used as received unless otherwise specified. 2-(7-Nitrofluoren-2-yl)benzothiazole (A), 2-iodo-7-nitrofluorene (D), and 2-(4-vinylphenyl)benzothiazole (F) were prepared as described previously (13, 14, 19).

1H and 13C NMR spectra were recorded in CDCl3 on a Varian 300 NMR spectrometer (300 MHz for 1H, referenced to TMS at δ = 0.0 ppm and 75 MHz for 13C, referenced to CDCl3 at δ = 77.0 ppm). FT-IR spectra were recorded on a Perkin-Elmer spectrophotometer model PE-1300 F0241. Elemental analyses...
were performed at Atlantic Microlab, Inc., Norcross, GA. High-resolution mass spectrometry (HR-MS) analysis was performed in the Department of Chemistry, University of Florida, Gainesville, FL.

General Synthetic Procedure for Preparation of 2-(9,9-Di-(2-methoxyethoxy)-ethyl)-7-nitrofluoren-2-yl-benzothiazole (B) via Alkylation. 2-(7-Nitrofluoren-2-yl)-benzothiazole (A) (2.18 g, 6.33 mmol), 1-bromo-2-(2-methoxyethoxy)ethane (2.77 g, 15.13 mmol), and KI (0.1 g, 0.65 mmol) were placed into anhydrous DMSO at room temperature. To the stirred solution, freshly powdered KOH (1.43 g, 25.49 mmol) was slowly added, turning the yellow reaction mixture dark green. Reaction progress was monitored by TLC hexanes/EtOAc (65:35). The reaction mixture was poured into distilled water and its organic components extracted with CH2Cl2 and dried over MgSO4. Removal of solvent provided a dark orange oil, which was purified via silica gel column chromatography using the eluent system above. Compound B was obtained as a yellow solid (2.5 g, 51% yield, mp 78–79 °C). 1H NMR (300 MHz, CDCl3) δ: 8.33 (s, 1H, ArH), 8.30 (d, 1H), 8.22 (s, 1H), 8.15 (d, 1H), 8.11 (d, 1H), 7.94, (d, 1H), 7.88 (d, 1H), 7.86 (d, 1H), 7.52 (t, 1H), 7.41 (t, 1H), 3.22 (m, 4H, OCH2), 3.21 (m, 6H, OCH3), 3.15, (m, 4H, OCH2), 2.90 and 2.82 (m, 4H, OCH2), 2.55, (t, 4H, CH2). 13C NMR (75 MHz, CDCl3): 72.02, 70.01, 69.47, 66.72, 66.47 (OCH3), 51.83 (C9), 39.36 (CH2). Anal. Calcd. for C30H32N2O4S:C, H, N, 65.73; H, 5.98; N, 4.96.

Synthesis of 7-Benzothiazol-2-yl-9,9-di-(2-(methoxyethoxy)-ethyl)fluoren-2-amine (C). 2-(9,9-Di-(2-methoxyethoxy)-ethyl)-7-nitrofluoren-2-yl-benzothiazole, B, 0.218 g, 0.44 mmol was dissolved in a mixture of EtOH/THF (1:1) under N2. This mixture was stirred for 12 h. Upon completion, the reaction was passed through a silica gel plug washed with diethyl ether and hexane/EtOAc (1:1), and concentrated, providing a yellow oil. Purification was accomplished by column chromatography on silica gel, eluting with diethyl ether/MeOH (9:1) to provide a pure product C, 65.67% yield, mp 69–71 °C.

Synthesis of 2-Iodo-9,9-di-(2-(methoxyethoxy)-ethyl)-7-nitrofluorene (E) via the General Alkylation Procedure (above). Compound B was obtained as a yellow oil, which solidified into a waxy solid (0.88 g, 42% yield). 1H NMR (300 MHz, CDCl3) δ: 8.26 (s, 1H), 8.24 (d, 1H), 7.82 (s, 1H), 7.67 (d, 1H), 7.73 (1H), 7.51 (d, 1H), 3.37 (q, 4H, OCH2), 3.29 (t, 4H, OCH2), 3.15 (t, 4H, OCH2), 2.85 (m, 4H, OCH2), 2.47 (m, 4H, CH2), 1.13 (t, 6H, CH3) 13C NMR (75 MHz, CDCl3): 152.90, 150.12, 147.50 (CNO2), 145.83, 137.74, 136.99, 133.12, 123.65, 122.69, 120.05, 119.0, 95.52, 70.14, 69.60, 66.75, 66.62 (OCH2), 52.24 (C9), 39.20 (CH2), 15.07 (CH3). Anal. Calcd. for C33H31INO5S: C, 52.73; H, 5.66; N, 2.46. Found: C, 52.36; H, 5.60; N, 2.47.

Nitro compound G (0.32 g, 0.48 mmol) was dissolved in 8 mL of 1:1 (v/v) EtOH/THF at room temperature. To this was added 0.02 g of 10% Pd/C. The reaction mixture was then heated to 70 °C under Ar to which hydrazine hydrate (0.09 g, 2.97 mmol) was added dropwise via syringe over 20 min. The reaction mixture was stirred for 18 h at 70 °C, cooled to room temperature, and concentrated, affording a yellow oil. Purification was accomplished by column chromatography using hexane/THF/MeOH (3:1:1), resulting in 0.27 g of yellow solid (90% yield). This intermediate was not further characterized and used directly in the next step for oxidative lithiation.

Synthesis of Amine-Reactive Fluorene Probe (1). Compound C, (0.6 g, 1.15 mmol) was dissolved in CHCl3 to whichaq CaCO3 (0.26 g, 2.11 mmol) was added at 0 °C. Thiophosgene (0.12 mL, 1.5 mmol) was then added dropwise with vigorous stirring (20). After 1 h, the starting material was completely consumed, as determined by TLC (9:1 diethyl ether/MeOH), achieving near-quantitative conversion. After an additional 30 min, 10% HCl was added until no gas generation was observed. The reaction mixture was poured into H2O, extracted with CH2Cl2, dried over MgSO4, and, upon filtration and concentration, resulted in an orange oil. The crude product was purified by column chromatography on silica gel eluting with diethyl ether/MeOH, (90:10), followed by hexane/EtOAc (60:40), affording 0.65 g of a yellow solid (94% yield, mp 70–71 °C). The FT-IR spectrum revealed a characteristically strong −NCS stretch at 2111 cm−1, while no signal from the −NH2 group (ca. 3600 cm−1) was observed. 1H NMR (300 MHz, CDCl3) δ: 8.15 (s, 1H), 8.09 (dd, 2H), 7.93 (d, 1H), 7.75 (q, 2H), 7.50 (t, 1H), 7.42 (m, 2H), 7.24 (d, 1H), 3.21 (m, 10H, OCH2, OCH3), 3.13 (m, 4H, OCH2), 2.85 (m, 4H, OCH2), 2.48 (m, 4H, CH2).

Synthesis of Amine-Reactive Fluorene Probe (2). 9,9-Bis(2-(ethoxyethoxy)ethyl)-7-(4-(benzothiazol-2-yl)styryl)-fluoren-2-amine H (0.21 g, 0.33 mmol) was dissolved in CHCl3 (8 mL)
to which aq CaCO₃ (0.11 g, 2.11 mmol) was added. Thiophos- 
gene (0.04 mL, 0.56 mmol) was added dropwise at 0 °C 
with vigorous stirring. After 2.5 h, the starting material 
was completely consumed, as determined by TLC (silica, 1:1 hexane/ 
THF). 10% HCl (5 mL) was added until no gas generation was 
observed. The reaction mixture was poured into H₂O, extracted 
with CH₂Cl₂, dried over MgSO₄, and, upon filtration and 
concentration, resulted in an orange oil. The crude product was 
purified by column chromatography on silica gel eluting with hexane/ 
THF (1:1), followed by hexane/THF (3:5:1), affording 0.15 g of a yellow solid (64% yield, mp 73–74 °C). FT-IR 
analysis revealed a characteristically strong ~NCS stretch at 
2111 cm⁻¹. ¹H NMR (300 MHz, CDCl₃) δ: 8.08, (t, 3H), 7.89 
(d, 1H), 7.62 (q, 4H), 7.51 (t, 2H), 7.40 (t, 2H), 7.28 (m, 4H), 
3.39 (m, 8H, OCH₂), 3.21, (d, 4H, OCH₂), 2.80 (m, 4H, OCH₂), 
2.43 (bs, 4H, CH₂), and 1.13 (t, 6H, CH3). 13C NMR (75 MHz, 
CDCl₃) δ: 180.72, 168.37 (Ar–N), 157.19, 154.13, 
150.59, 143.62, 140.75, 138.74, 134.96, 131.63, 129.54, 128.59, 
127.24, 126.60, 125.98, 123.26, 122.89, 122.21, 120.83, 71.78, 
69.84, 67.14 (OCH₃), 58.70, 55.71, 52.00 (C9), 49.55, 44.02, 
32.33, 28.47, 25.60, 23.81. HRMS-ESI theoretical 
formula of 5% CO₂ and 37 °C incubation chamber was used to maintain a humidified atmo-
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**Scheme 1. Preparation of the Isothiocyanate Amine-Reactive Tag I**

![Diagram](image)

**General Synthetic Procedure for Reactive Probe Adduct via Addition Reaction (3).** A mixture of amine-reactive probe 2 (0.12 g, 0.214 mmol) and n-butylamine (0.23 mL) was stirred 
at room temperature for 2 h. The excess n-butylamine was removed in vacuo and the residue purified by column chromato-
graphy on silica gel eluting first, with EtOAc/THF (95:5), followed by diethyl ether/EtOAc (80:20), affording 0.082 g of 
pale yellow solid (63% yield, mp 128–129 °C). ¹H NMR (300 MHz, CDCl₃) δ: 8.11 (s, 1H), 8.10 (t, 2H), 8.04 (d, 1H), 7.92 
(q, 2H), 7.79 (t, 2H), 7.72 (t, 1H), 7.50 (d, 1H), 6.8 (s, 1H, 
NH), 3.67 (m, 16H, OCH₃, OCH₂, CH₂), 2.10 (m, 4H, OCH₂), 
1.54 (m, 4H, CH₂), 1.36 (m, 2H, CH₂), 1.15 (m, 2H, CH₂), 
0.91 (t, 3H). ¹³C NMR (75 MHz, CDCl₃) δ: 180.72, 168.37 
(Ar–C=N), 155.84, 154.22, 150.56, 149.31, 139.47, 
138.85, 138.77, 136.36 (N=CH=CH₂), 134.78, 134.58, 132.26, 
129.80, 129.55, 127.56, 126.63, 126.41, 125.99, 124.81, 122.77, 
121.25, 120.72, 120.58, 120.27, 120.05, 69.91, 69.42, 66.66, 
66.40 (OCH₂), 51.32 (C9), 39.55 (CH₂), 15.02 (CH₃). Anal. 
Calcd. for C₁₂H₁₂N₂O₄S₂: C, 71.27%; H, 6.13%; N, 4.05%. 
Found: C, 71.06%; H, 6.28%; N, 4.01%. 

**Preparation of Cyclic Peptide Conjugate (7).** Cyclic peptide 
RGDK (10 mg, 0.016 mmol) was dissolved in DMSO (0.75 mL), 
and then amine-reactive dye I (9.6 mg, 0.017 mmol) was added 
slowly to the solution. The clear solution was stirred at room 
temperature that gradually changed to a fluorescent yellow 
coloration. After 5 h, the starting material was completely 
consumed as determined by TLC (silica gel, diethyl ether). 
DMSO was removed by washing with diethyl ether several 
times, producing 16 mg of yellow solid (90% yield, mp 
265–266 °C). ¹H NMR (300 MHz, CDCl₃) δ: 9.85 (s, 1H, 
COOH). ¹³C NMR (75 MHz, CDCl₃) δ: 180.78, 174.52, 172.47, 
171.52, 171.23, 170.91, 168.19 (Ar=C=N), 157.19, 154.13, 
150.59, 143.62, 140.75, 138.74, 134.96, 131.63, 129.54, 128.59, 
127.24, 126.60, 125.98, 123.26, 122.89, 122.21, 120.83, 71.78, 
69.84, 67.14 (OCH₃), 58.70, 55.71, 52.00 (C9), 49.55, 44.02, 
32.33, 28.47, 25.60, 23.81. HRMS-ESI theoretical m/z [M + 
H]+ = 1164.49, found 1164.49; theoretical m/z [M + Na]+ = 
1186.38, found 1186.48.

**NT2 Cell Culture.** The NT-2/D1 cells were plated at a density 
of 5 × 10⁴ per 75 cm² in a tissue culture treated flask (Corning). 
Cell culture media was Dulbecco’s modified Eagle’s medium with F-12 (DMEM/F12, Invitrogen), supplemented with 10% 
heat-inactivated fetal bovine serum (Atlanta Biologics). An 
incubation chamber was used to maintain a humidified atmo-
sphere of 5% CO₂ and 37 °C. NT2 cells were passed twice a week with trypsin/EDTA (Invitrogen) treatment. 

**HeLa Cells Incubated with Amine-Reactive Probe (1).** HeLa 
cells were plated onto 4-well glass chamber slides. Stock 
solutions of fluorophore I dissolved into DMSO were prepared as either 10 or 5 μM solutions. Diluted solutions in complete 
growth medium were then freshly prepared and placed over the 
cells for either a 1 or 5 h period. All cells were washed with 
PBS (3–5×) and fixed in a 3.7% formaldehyde solution for 5 
min at room temperature.

**Reelin conjugate 6 diluted in NT2 cell media was added to NT2 cells and incubated for 3 h. After incubation, cells were washed once**
with PBS followed by addition of fresh media. Cells were fixed prior to viewing using 4% paraformaldehyde.

**Spectroscopic Measurements.** Steady-state absorption and fluorescence emission spectra of compounds 1–3 were investigated in DMSO in concentrations on the order of 10⁻⁶ M at room temperature in 1 × 1 cm quartz cuvettes using an Agilent 8453 spectrophotometer and PTI Quantamaster spectrofluorimeter, respectively. All solvents and solutions used in these experiments were checked for spurious emission in the region of interest. Fluorescence quantum yields, $Q$, were measured for

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**Scheme 2. Preparation of the Isothiocyanate Amine-Reactive Tag 2 with Extended Conjugation Length**

**Scheme 3. Preparation of the Model Adducts 3 and 4 and Bioconjugates 5 and 6 with the Amine-Reactive Fluorenyl Reagents 1 and 2**

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**Figure 1.** Normalized absorption spectra and steady-state fluorescence emission spectrum of the amine-reactive probe 1 (---), dye adduct 3 (···), and Reelin bioconjugate 5 (−). Absorption spectrum of the free Reelin protein in PBS is shown for reference.

**Figure 2.** Normalized UV—visible absorbance and fluorescence emission spectra of the amine-reactive probe 1 (−−) and cyclic peptide conjugate 7 (−−) in DMSO $\lambda_{max}^{abs} = 355$ nm, $\lambda_{max}^{em} = 460$ nm, $Q_y = 0.92$. 
all compounds by a standard method (21), relative to Rhodamine 6G in ethanol ($Q = 0.94$) (22).

**Two-Photon Absorption Properties.** The two-photon fluorescence (2PF) spectra and two-photon absorption (2PA) cross sections of all molecules studied were determined by employing a two-photon induced fluorescence method (23, 24), using a tunable femtosecond Ti:sapphire laser (Mira 900-F, 220 fs pulse width, 76 MHz repetition rate, Coherent, USA) as the excitation source. After passing through a round, continuously variable neutral density filter that was used to control the laser irradiance, the femtosecond NIR laser beam was focused into a $1 \times 1$ cm quartz cuvette containing sample solutions by a plano-convex lens ($f = 50$ mm, Thorlabs, USA). The excitation laser was adjusted to be as close to the wall as possible in order to reduce reabsorption effects. The upconverted fluorescence was first collected by an objective lens (20×, NA = 0.50, Newport, USA) at a direction perpendicular to the pump beam, then focused by a large beam collimator (F810SMA-543, Thorlabs, USA) into a multimode fiber (400 µm core, Ocean Optics, USA), and, finally, delivered to a fiber optic spectrometer (SD2000, Ocean Optics, USA), which was used to record the upconverted fluorescence spectra. The two-photon fluorescence spectra recorded by the spectrometer were used without further linearity correction. This technique was confirmed by measuring the 2PA cross section of a well-characterized fluorene-based 2PA fluorophore (13, 25).

**One-Photon Fluorescence Imaging.** An inverted microscope (Olympus IX70) equipped with a QImaging cooled CCD (Model Retiga EXi) was used for conventional fluorescence imaging, where the output of a filtered 100 W mercury lamp was used as the excitation source. A customized filter cube (Ex 377/50, DM 409; Em 460/50) was used for fluorescence imaging.

**Two-Photon Fluorescence Microscopic Imaging and Two-Photon Fluorescence Lifetime Imaging.** Two-photon fluorescence microscopic imaging (2PFM) and two-photon fluorescence lifetime imaging (2P-FLIM) were performed on a modified Olympus Fluoview FV300 microscope system coupled with the tunable Coherent Mira 900F Ti:sapphire laser and a compact FLIM system from PicoQuant, Germany. Output from the femtosecond NIR laser (tuned to 760 nm, 220 fs pulse width, 76 MHz repetition rate) was used as the two-photon excitation source for both 2PM and 2P-FLIM experiment. The fluorescence collected by a $40 \times$ microscopic objective (UPLANAP 40×, NA = 0.85, Olympus) was reflected by a dichroic beam splitter.
and then focused into a multimode fiber by a microscope objective (20 ×, N.A. 0.4, Newport). A beam reducer, consisting of a plano-convex lens and a plano-concave lens, was used to reduce the fluorescence beam diameter in front of the objective. The output fluorescence was delivered to an avalanche photodiode (APD) detector (PicoQuant, Germany). A broad band-pass filter (D500/200m, Chroma) was placed in front of the APD detector. Data acquisition and analysis were done with a combination of a stand-alone time-correlated single photon counting (TCSPC) module TimeHarp 300 and software package SymPhoTime, both from PicoQuant, Germany.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Amine-Reactive Fluorenyl Dyes 1 and 2. The reactivity of the isothiocyanate group –N=C=S is well-documented, yielding thioureas upon reaction with amines. The molecular structures of the target isothiocyanate fluorophores share some similarity with our previously described two-photon fluorescent chromophores. In the 9-position of these fluorophores, two identical oligo(ethylene glycol) chains were used to impart hydrophilicity and provide better solubility. In the 7-position, we opted for the benzothiazole moiety as an electron-withdrawing group (Schemes 1 and 2).

A key intermediate in the synthesis of the isothiocyanate derivative 1 was amine C. This was achieved via the quantitative reduction of nitro derivative B using hydrazine hydrate and 10% Pd/C in a 1:1 mixture of EtOH/THF at 70 °C. The isothiocyanate reactive group was then obtained by reaction of amine C with thiophosgene. The target functionalized chromophore 1 was obtained in 94% yield.

For isothiocyanate derivative 2, the conjugation length was increased via the addition of a polarizable π-system (styril) between the fluorenyl moiety and the benzothiazole acceptor group. The route began with the synthesis of 2-(4-(2-(9,9-bis(2-(2-ethoxyethoxy)ethyl)-2-nitro-fluoren-7-yl)vinyl)phenyl)benzothiazole (G) via an efficient Pd-catalyzed Heck coupling reaction between 9,9-bis(2-(2-ethoxyethoxy)ethyl)-2-ido-7-nitrofluorene (E) and 2-(4-vinylphenyl)benzothiazole (F), fol-
lowing the synthetic pathway shown in Scheme 2. The FT-IR spectra of the isolated compounds revealed characteristically strong $\text{NCS}^-$ stretching at 2111 cm$^{-1}$, whereas no signal at ca. 3600 cm$^{-1}$ from the $\text{NH}_2$ group was observed. Structures of all new compounds have been confirmed by $^1\text{H}$ and $^{13}$C NMR and C, H, N analysis, with the exception of oxidatively labile amine $\text{H}$, which was used immediately after being formed.

**Conjugation with $n$-Butylamine and Reelin, and Their Optical Properties.** To test the feasibility of the conjugation of fluorene isothiocyanate with biopolymers, we first used a simple reaction of $n$-butylamine with the amine-reactive probes 1 and 2 as model reactions (Scheme 3). Furthermore, preparation of the model adducts (3 and 4) allowed for facile single- and two-photon spectroscopic characterization, which more closely resembles the bioconjugate than that of the amine-reactive probe (conjugate precursor). The reaction of the amine-reactive dye with $n$-butylamine was fast, with completion in $\sim$40 min at room temperature. The expected thiourea group was evident in the $^1\text{H}$ NMR spectrum.

To show the potency of the isothiocyanate-functionalized chromophore in two-photon-based biological applications, the protein Reelin was conjugated with amine-reactive probes 1 and 2 (Scheme 3). Reelin is a large extracellular matrix glycoprotein, important in guiding neural stem cells in the central nervous system in normal development (26, 27). While much attention is focused on Reelin’s role during corticogenesis, the existence of Reelin expression in several neural population in the adult brain may point to other important functions for this protein. Consequently, attaching a 2PA fluorophore to Reelin enhances the possibility of visualizing cellular events that involve Reelin by using 2PFM.

Conjugation of Reelin with 1 and 2 was performed according to standard methods (27) in PBS buffer by dissolving the fluorenyl isothiocyanate in DMSO immediately prior to addition into a stirred Reelin carbonate buffer solution (pH 9.5). The reaction mixture was stirred for 3 h at ambient temperature. The bioconjugate was then separated from the unbound fluorophore by filtration through a gel chromatography column, enabling the separation of the bioconjugate in PBS solution (pH 7.2). The bioconjugate was collected in several fractions that were then identified and characterized spectrophotometrically. The concentration of the reactive dye solution was varied such that a 1:10 and a 1:5 mol ratio of protein to reactive dye were prepared to establish a degree of labeling (DOL) for the probe. Hence, a 1:10 and a 1:5 mol ratio of protein to probe allowed for an estimated DOL ranging from 2.2 to 3.4.

The normalized UV–visible absorption and steady-state fluorescence emission spectra of amine-reactive probe 1, dye adduct 3 in DMSO, and Reelin bioconjugate 5 in PBS (buffer pH 7.2) are shown in Figure 1. For reference, the absorption spectrum of the free Reelin protein in PBS solution is also shown. The Reelin bioconjugate exhibited absorption peaks corresponding to that of the Reelin protein in the shorter wavelength range ($\lambda_{\text{max}} = 280$ nm), as well as that of the fluorescent dye in the longer absorption range ($\lambda_{\text{max}} = 357$ and 375 nm), and exhibited an emission maximum at 455 nm. We observe that the absorption spectrum of the Reelin bioconjugate 5 showed only some broadening compared to the absorption spectrum of the unbound fluorophore 1. The fluorescence quantum yield of the NCS-containing amine-reactive probe in DMSO was 0.02, nearly nonfluorescent, while that of the dye adduct 3 in DMSO increased significantly to 0.72, indicating that the fluorescence of the reactive tag is restored (turns on) upon conjugation. The increase in conjugation of the $\pi$-system from compound 1 to compound 2 is reflected in an increase of its fluorescence quantum yield (0.85).

**RGD Conjugation and Spectroscopic Characteristics of the Cyclic Peptide Conjugate (7).** Integrins are a family of heterodimeric transmembrane glycoproteins that play an important role in mediating cell–cell and cell–matrix interactions...
(29, 30). Integrins recognize short peptide sequences found in the extracellular matrix and on the cell surface. Particularly, the sequence Arg-Gly-Asp (RGD) is recognized by a number of integrins (29). Numerous studies have assessed the potential of $\alpha_\text{v}\beta_3$ as a target for tumor imaging agents (30). Along with the derivatives of the RGD-series, c(RGDfK) is often used for the delivery of therapeutics, because the lysine residue (K) makes it an ideal building block for further chemical conjugation reactions. To show the efficacy of the amine-reactive probe to be used as a two-photon fluorescence marker for optical tumor imaging, the RGDfK peptide was conjugated with amine-reactive probe 1 in DMSO at room temperature (Scheme 4). High-resolution mass spectrometric analysis confirmed the molecular structure of oligopeptide conjugate 7.

The linear absorption and emission spectra of cyclic peptide-conjugate 7 in DMSO are shown in Figure 2. Conjugate 7 displayed absorption peak at 352 nm, while the emission maximum shifted from 400 nm for 1 to 460 nm for 7. In addition, the fluorescence quantum yield of 7 increased to 0.9 after conjugation.

**Two-Photon Absorption Properties.** 2PA cross sections were obtained by the upconversion fluorescence method using a femtosecond Ti:sapphire laser as the excitation source. The 2PF measurements of the amine-reactive probe 2, model adduct 4, and cyclic peptide bioconjugate 7 were performed in DMSO ($1.6 \times 10^{-3}$ M) and exhibited a 2PA cross section of $\sim 30$ GM at 740 nm. With its fluorescence quantum yield of $\sim 0.7$–0.9, the two-photon action cross section for the model adduct is $\sim 20$ GM, reasonable enough to be used for bioimaging. These values are higher than those for common commercial dyes (0.16 GM for DAPI, 1 GM for cascade blue) used currently as blue fluorescence dyes in 2PFM (23).

**Single- and Two-Photon Fluorescence and FLIM Cell Imaging.** The utility of amine-reactive tag 1 as a 2PA biological marker was demonstrated by incubation with HeLa cells. Cells were incubated with a solution of $10^{-6}$ mol L$^{-1}$ of chromophore, and after 1 and 5 h incubation times, images were taken with a modified Olympus Fluoview FV300 microscope system. Strong fluorescence was observed after 5 h of incubation, with a homogeneous coloration of the cytoplasm region. Differential interference contrast (DIC) and epifluorescence microscopic images of the stained cells are shown in Figure 3a,b. The low quantum yield of this probe (0.02) was an important characteristic, because after incubation, it appeared to have reacted spontaneously with a protein in HeLa cells, generating a bioconjugate with significantly enhanced fluorescence (Qy = 0.7), improving detection by single- and two-photon fluorescence microscopy imaging (Figure 3b). 2PFM images of the same amine-reactive fluorene 1 stained cells were collected on a modified Olympus Fluoview FV300 microscope system combined with a tunable Coherent Mira 900F Ti:sapphire laser. Two-photon induced fluorescence was observed predominantly from the cytoplasmic region, consistent with the images collected from epifluorescence imaging (Figure 3c).

DIC and epifluorescence microscope images of NT2 neuron fixed cells incubated with Reelin conjugate 6 (DOL = 3.4) were collected on a modified Olympus Fluoview FV300 microscope (Figure 4a,b). The resulting optical images clearly showed that successful uptake was achieved. 2PFM images and fluorescence lifetime imaging microscopy (FLIM) were performed on the same cells. Though select areas of the cells were chosen for lifetime analysis, the area selected was representative of the dye distribution within the cytoplasm, resulting in a homogeneous monoexponential fluorescence decay (Figure 4c and 5). The average measured was on the order of 1.5 ns.

The efficacy of cyclic peptide-conjugate 7 was evaluated by incubation with H1299 lung tumor line cells (Figures 6 and 7). Additionally, fluorescence was observed predominantly from the cytoplasmic region of the cells, with the nucleus clearly outlined. Two-photon induced fluorescence was observed predominantly from the cytoplasmic region, consistent with the images collected from single fluorescence images (Figure 6c). Some differences were observed in the probe lifetime. This is a subject of further investigation.

**CONCLUSIONS**

The data presented in this paper confirm that our strategy of introducing the $\text{NCS}$ functionality into the aromatic skeleton of suitably derivatized fluorenes, by means of commercial thioisophene, proven both useful and versatile, facilitating the preparation of a variety of fluorescent fluorene isothiocyanates (amine-reactive probes). The fluorene isothiocyanate derivatives, whose synthesis and conjugation to a protein and oligopeptide are reported in this paper, represent an improvement over commercial isothiocyanate probes. Our results demonstrate the potential of these fluorene-based amine-reactive probes as fluorescent markers, owing to their good optical properties, chemical and optical stability, ease of color tunability, and reactive functionality for biomolecule conjugation. Demonstration of 2PM images of HeLa cells incubated with a well-
characterized 2PA fluorophore lends credence to our efforts to further refine fluorene-based derivatives for bioimaging applications. Performance of second-generation fluorene-based fluorophores for aqueous compatibility and integrated with additional specific biomolecule-reactive functionalities and vectors is currently being investigated.

ACKNOWLEDGMENT

The authors wish to acknowledge support from the National Institutes of Health (1 R15 EB008588-01), the U.S. Civilian Research and Development Foundation (UKB2-2923-KV-07), and the National Science Foundation (ECS-0524533). We also wish to acknowledge Dr. Zhen-Li Huang for assistance in two-photon fluorescence imaging.

LITERATURE CITED