

Nonclassical SNAPFL Analogue as a  
Cy5 Resonance Energy Transfer Partner

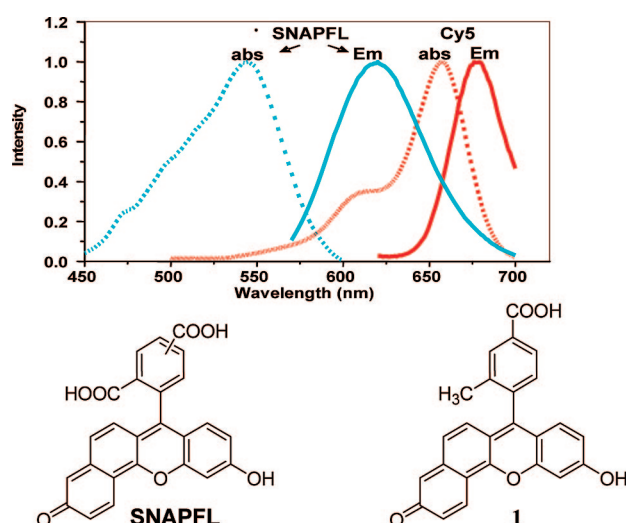
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## ABSTRACT



We have synthesized a new SNAPFL analogue (1) that exhibits a large Stokes shift (78 nm) (abs. 542 nm, em. 620 nm) and a good quantum yield. Because of the large overlap between the emission spectrum of 1 and the absorption spectrum of Cy5, 1 functions well as a fluorescence donor to Cy5 and has been used in FRET-based experiments using estrogen receptor site-specifically labeled with Cy5 and a receptor ligand conjugated to SNAPFL.

FRET is one of the most widely used spectroscopic tools in drug discovery, elucidation of gene<sup>1</sup> and protein function (protein conformational change), examination of protein interactions with ligands, genes, or other proteins, and use in high-throughput assays.<sup>2–4</sup> Fluorophores that are typically used for FRET studies include those of the cyanine and Alexa classes, tetramethylrhodamine (TMR), and fluorescein; each of these, however, has a Stokes shift less than 30 nm and a

broad excitation and emission range (approximately 150 nm). Consequently, many possible FRET pairs that provide ample overlap of the donor emission and acceptor excitation spectra cannot be optimally used because the broad acceptor excitation spectrum (150 nm) results in the *direct* excitation of the acceptor and subsequent undesired increase in acceptor emission. This necessitates a more complicated calculation to obtain an accurate determination of the energy transfer and opens the results to systematic errors due to experimental variability. To minimize this direct excitation of the acceptor, a donor with an excitation spectrum sufficiently separated from the acceptor excitation can be chosen, but oftentimes this results in poor FRET overlap between the donor emission and acceptor excitation. To improve this situation, we have

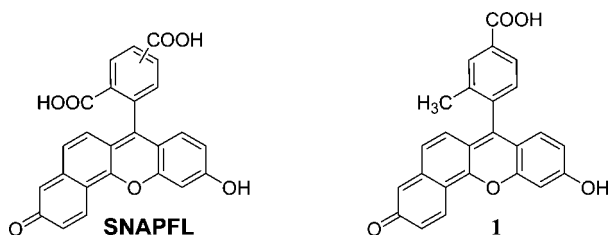
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sought a new dye system having a large Stokes shift that would allow efficient energy transfer between the donor and acceptor with minimal direct excitation of the acceptor fluorophore; this would result in a more direct and accurate measurement of the energy transfer between the fluorophores.

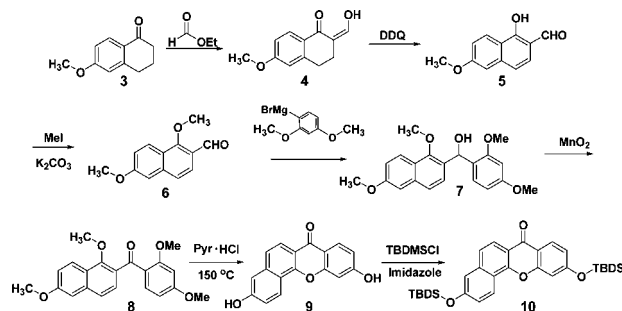


Upon searching through a variety of organic dyes to find a better FRET pair for the Cy5 dye (ex. 653 nm, em. 665 nm), we discovered the unsymmetrical SNAPFL (Semi Naphthalene Fluorescein) fluorophore, which exhibits a large Stokes shift and has a good emission overlap with the excitation spectrum of Cy5. Commercial SNAPFL is provided as a mixture of the 5- and 6-carboxylic acid isomers. Recently, both Urano<sup>5</sup> and Peterson<sup>6</sup> reported that synthesis of a novel type of fluorescein with a methyl at the 2 position of the phenyl ring instead of a carboxy afforded a fluorophore with a quantum yield and photophysical properties very similar to fluorescein itself. Therefore, we designed a new synthetic route to prepare the unsymmetrical SNAPFL analog **1** as a single isomer; our replacement of the carboxyl in SNAPFL with a methyl group in **1** also eliminated a charged group that can sometimes be troublesome in biological applications that require cell uptake.

Here, we describe the synthesis of a new SNAPFL analogue dye **1** ((3,10-dihydroxybenz[*c*]xantene-7-yl)-4-carboxy-2-methylbenzene), characterization of its physical properties, conjugation of **1** with a nonsteroidal estrogen receptor ligand, and investigation of its potential as a FRET partner for Cy5 using a biological model system for ligand-protein interactions.

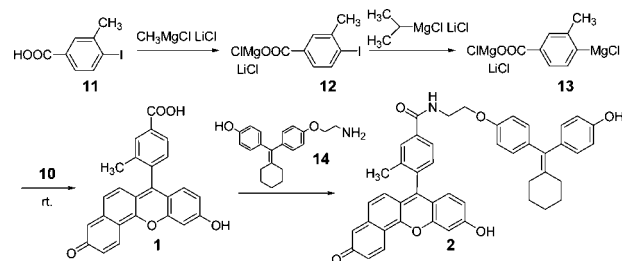
To synthesize SNAPFL analogue **1** (3,10-dihydroxybenz[*c*]xantene-7-yl-(4-carboxy-2-methylbenzene), the precursor **10** (3,10-di[(*tert*-butyl-dimethyl)silyloxy]benz[*c*]xanthene-7-one) was prepared as shown (Scheme 1). 2-Formyl-1,6-dimethoxy-naphthalene **6** was synthesized according to previously described methods.<sup>7</sup> Addition of 2,4-dimethoxyphenyl magnesium bromide to aldehyde **6** afforded alcohol **7** in 96% yield, and subsequent oxidation with MnO<sub>2</sub> (3 equiv) in methylene chloride gave ketone **8** quantitatively. Xanthene moiety **9** was obtained quantitatively by the simultaneous demethylation and cyclization of **8** with pyridinium hydrogen chloride salt over 180 °C. Both phenolic

**Scheme 1.** Synthetic Scheme To Prepare for Benz[*c*]xanthene-7-one (**10**)



hydroxyls in compound **9** were protected as *tert*-butyldimethylsilyl ethers by reacting this bisphenol with TBDMSCl and imidazole in DMF at rt. Xanthene moiety **10** was coupled with Grignard reagent **13**, generated from 4-iodo-3-methylbenzoic acid according to a modified Knochel method,<sup>8</sup> followed by treatment with an aqueous 1 N HCl solution to provide SNAPFL analog **1** in 64% yield (Scheme 2).

**Scheme 2.** Synthesis of SNAPFL Analogue (**1**) and Cyclofenil-Conjugated SNAPFL (**2**)



This fluorophore, intended for use as a FRET partner with Cy5, was conjugated with monoaminoethyl cyclofenil **14**,<sup>9,10</sup> a nonsteroidal antagonist of the estrogen receptor (ER), which is a ligand-modulated transcription factor. After cyclofenil conjugation with SNAPFL, the  $\lambda_{\text{max}}$  for emission shifted from 620 to 624 nm, and the emission intensity decreased 15% compared to SNAPFL. Interestingly, upon binding of the ligand-SNAPFL conjugate to unlabeled ER, the emission intensity was almost fully recovered with essentially no change in emission wavelength ( $\lambda_{\text{max}}$  624 nm).

The binding affinity of compound **2** for both estrogen receptor subtypes, ER $\alpha$  and ER $\beta$ , was evaluated using a radiometric binding assay whereby test compounds compete with a tritiated ligand estradiol for binding to the receptor.

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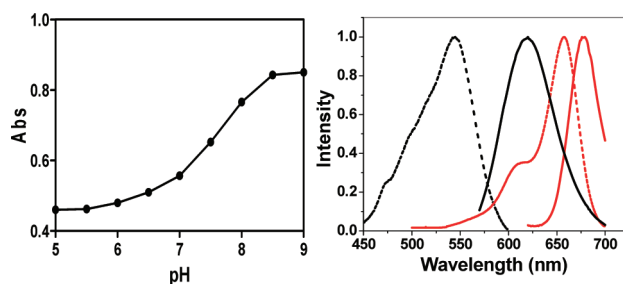
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Binding affinities for compound **2**, expressed relative to that of estradiol set at 100%, were determined to be  $3.3 \pm 0.5\%$  for ER $\alpha$  and  $28 \pm 3.2\%$  for ER $\beta$ .<sup>11</sup> These percentages correspond to a  $K_i$  of approximately 6 nM for the tethered compound binding to ER $\alpha$ , based on a  $K_d$  of 0.2 nM for estradiol binding to ER $\alpha$ .

The relative quantum yield (SI) ( $\Phi = 0.21$ ) was measured using rhodamine B as a standard in phosphate buffer at pH 9.0, according to previously described protocols.<sup>12</sup> The  $pK_a$  value was calculated by measuring the absorbance of **1** with varying pH. As shown in Figure 1A, the  $pK_a$  of **1** (7.5) is a full log unit less acidic than fluorescein (6.5).<sup>6,13</sup>

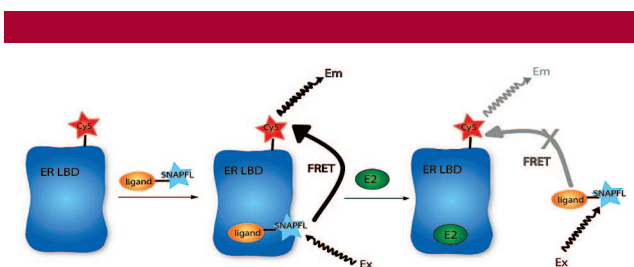


**Figure 1.** Characterization of compound **1**. (A) Plot of pH versus compound **1** absorbance allowed calculation of the  $pK_a$  of compound **1** (7.5). Different pH sample solutions were prepared using a 0.1 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer and diluting the stock dye solution 1:10 into buffer (final dye concentration = 10  $\mu\text{M}$ ). (B) Absorption spectrum of **1** (blue broken line) and Cy5 (red broken line) and emission spectrum of **1** (blue solid line) and Cy5 (red solid line). Extinction coefficients for  $\lambda_{542}$  at pH 9, 8, 7, 6, and 5 are 58300, 46500, 26800, 18000, and 16500  $\text{cm}^{-1} \text{M}^{-1}$ , respectively.

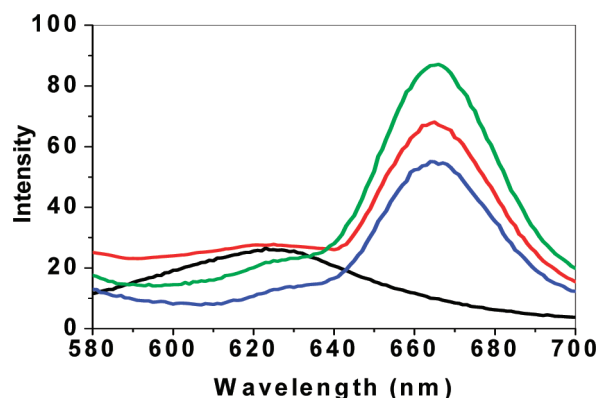
To evaluate the possible use of this new fluorophore in FRET experiments, we site-specifically labeled the ligand binding domain of ER $\alpha$  at cysteine 417 with a thiol-reactive Cy5 conjugate.<sup>14</sup> Based on the quantum yield of SNAPFL, the Forster radius for Cy5 at pH 9.0, estimated by the formula  $R_0 = [(8.8 \times 10^{23})\kappa^2 n^{-4} QY_D J(\lambda)]^{1/6} \text{ \AA}$ ,<sup>15</sup> was  $R_0 = 62.5 \text{ \AA}$ .

As shown in Figure 1B, compound **1** has a large Stokes shift (78 nm), and its emission nicely overlaps with the excitation spectrum of Cy5 around 620 nm. To minimize any direct excitation of Cy5, we excited the cyclofenil conjugate **2** at 520 nm instead of at its  $\lambda_{\text{max}}$ , 542 nm.

Incubation of ER $\alpha$ -Cy5 with **2** for 1 h at 4  $^\circ\text{C}$  (1 nM ER $\alpha$ , 4 nM compound **2**, 50 mM Tris buffer, pH 8.5) resulted in a 70% increase in Cy5 emission intensity, with a concomitant decrease in the emission intensity of **2** by 23% (see Figure 2 (schematic) and Figure 3 (data), green line vs black and blue lines). When **2** was blocked from binding to the estrogen receptor by addition of competing ligand estradiol at 1  $\mu\text{M}$ , energy transfer between the dyes disappeared, as expected (Figure 3, red line vs green line). For this experiment, a 1 h incubation usually decreased the FRET signal by 80%; we extended the incubation to 8 h to ensure complete ligand



**Figure 2.** Schematic of SNAPFL-Cy5 FRET. Cy5-labeled ER (left) binds cyclofenil-SNAPFL (**2**) and FRET is observed (middle). When excess estradiol out-competes the ligand-fluorophore (right), FRET signal is decreased.



**Figure 3.** FRET experiment between **2** and Cy5-ER $\alpha$ . Emission spectrum of **2** alone (4 nM in pH 8.5 Tris buffer, black line), Cy5-ER $\alpha$  alone (1 nM in pH 8.5 Tris buffer, blue line), preincubated Cy5-ER $\alpha$  and **2** (1 nM ER $\alpha$  and 4 nM **2** in the same buffer, green line), and preincubated 1 nM Cy5-ER $\alpha$  and 4 nM **2** in the presence of excess 1  $\mu\text{M}$  E<sub>2</sub> (red line). The excitation was fixed at 520 nm.

exchange. The loss of FRET is evident by the decrease in acceptor (Cy5) emission intensity at 665 nm and the increase in donor (SNAPFL) emission intensity at 624 nm. The fluorescence remaining after estradiol has been added (red curve) represents not FRET but background fluorescence from Cy5 and SNAPFL in the absence of FRET; it is, essentially, the sum of the blue curve and the black curve, respectively.

The wavelength used to excite the SNAPFL fluorophore **2**, 520 nm, results in very little direct excitation of the Cy5 FRET partner. In fact, this bleed-through energy resulting from direct excitation comprises only 3% of the total intensity of Cy5 emission at 649 nm (when excited through a FRET transfer) and is therefore negligible for most experiments. In contrast, other commonly used fluorophores having smaller Stokes shifts must be excited at longer wavelengths, resulting in a higher percentage of Cy5 emission due to direct excitation instead of FRET transfer. For example, the following commonly used FRET partners for Cy5 produce varying degrees of this direct excitation: TMR (ex. 543 nm,

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em. 567 nm; 7% bleed-through at 543 nm), Alexa Fluor 568 (ex. 578, em. 603 nm; 18% bleed-through at 578 nm), and Alexa Fluor 594 (ex. 590 nm, em. 618 nm; 30% bleed-through at 590 nm), Rhodamine RedX (ex. 572 nm, em. 592 nm; 20% bleed-through at 572 nm) and Cy3 (ex. 553 nm, em. 565 nm, 9% bleed-through at 553 nm) (see Figure 2 in the Supporting Information). The SNAPFL fluorophore analogue described here, in contrast to many of the above-mentioned commonly used fluorophores in this spectral range, is excited at a shorter wavelength, which minimizes direct Cy5 excitation in FRET experiments and, therefore provides a truer measure of the energy transfer between two molecules.

This new SNAPFL dye, which is characterized by a large Stokes shift and a pH-dependent absorbance spectrum, is a

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valuable new fluorophore and a potential FRET partner for the well-known fluorescent dye Cy5.

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**Supporting Information Available:** Procedures for the synthesis of SNAPFL (**1**) and spectrometric characterization of intermediates (**5–10**), **2**, and **1**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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