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Nonclassical SNAPFL Analog as a Cy5 Resonance Energy Transfer Partner

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Abstract

FRET is one of the most powerful tools used to explore protein function and identify new small molecules for drug development. We have synthesized a new SNAPFL analog (1) which exhibits a large Stokes shift (80 nm) (abs. 542 nm, em. 622 nm) with good quantum yield. Due to a large area

of overlap between the emission spectrum of 1 and the excitation spectrum of Cy5, we investigated the potential use of these two fluorophores in FRET-based experiments. Using an estrogen receptor (ER) site-specifically labeled with Cy5, we monitored the FRET signal change upon binding of an ER ligand, cyclofenil, that had been tethered to compound 1. The energy transfer observed between these two fluorophores could be blocked with addition of excess unlabeled estrogen, which proves the dependence of the FRET transfer on fluorophore proximity. This new SNAPFL analog not only exhibits a large Stokes shift making it generally useful for FRET experiments, but it also produces a spectral pattern that specifically supports its use as a new FRET partner for the well-known dye Cy5.

FRET is one of the most widely used spectroscopic tools in drug discovery, elucidation of gene¹ and protein function (protein conformational change), examination of protein interactions with ligands, genes, or other proteins, and use in high throughput assays.^{2, 3, 4} 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 whose excitation spectrum is 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 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. Recently, both Urano⁵ and Peterson⁶ 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. The commercial SNAPFL is provided as a mixture of the 5- and 6-carboxylic acid isomers. Therefore, we designed new synthetic route to prepare

unsymmetrical SNAPFL analog **1** as a single isomer; our replacement of the carboxyl in SNAFL 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 analog dye **1** ((3,10-dihydroxy-benz[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 analog **1** (3,10-dihydroxy-benz[c]xantene-7-yl-(4-carboxy-2-methylbenzene), the precursor **10** (3,10-di-[(t-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.⁷ Addition of 2,4-dimethoxyphenyl magnesium bromide to aldehyde **6** afforded alcohol **7** in 96% yield, and subsequent oxidation with MnO₂ (3 eq.) 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 150 °C. Both phenolic hydroxyls in compound **9** were protected as tbutyldimethylsilyl ethers by reacting this bisphenol with TBDMSCl and imidazole in DMF at rt.

Xanthene moiety **10** was coupled with Grignard reagent **13**, which was generated from 4-iodo-3-methylbenzoic acid, according to a modified Knochel method,⁸ followed by treatment with an aqueous 1N HCl solution to provide SNAPFL analog **1** in 64% yield.

This fluorophore, intended for use as a FRET partner with Cy5, was conjugated with mono aminoethyl cyclofenil **14**,^{9, 10} a non-steroidal antagonist of the estrogen receptor (ER), which is a ligand-modulated transcription factor.

The binding affinity of compound **2** for both estrogen receptor subtypes, ER α and ER β , was evaluated using a radiometric binding assay whereby test compounds compete with a tritiated ligand estradiol for binding to the receptor. Binding affinities for compound **2**, expressed relative to the binding of estradiol set at 100%, were determined to be $3.3 \pm 0.5\%$ for ER α and $28 \pm 3.2\%$ for ER β .¹¹ These percentages correspond to a K_i of approximately 6 nM for the tethered compound binding to ER α , based on a K_d of 0.2 nM for estradiol binding to ER α .

The relative quantum yield (SI) ($\Phi = 0.24$) was measured using TMR as a standard in phosphate buffer at pH 8.5, according to previously described protocols.¹² 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).^{6, 13}

To evaluate the possible use of this new fluorophore in FRET experiments, we site-specifically labeled the ligand binding domain of ER α at cysteine 417 with a thiol-reactive Cy5 conjugate.¹⁴

As shown in Figure 1B, compound **1** has a large Stokes shift (80 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 estrogen conjugate **2** at 520 nm instead of at its λ_{max} , 542 nm.

Incubation of ER α -Cy5 with **2** for 8 hrs at 4 °C (1 nM ER α , 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 μ M, energy transfer between the dyes disappeared (Figure 3, red line vs. green line), as expected.

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 bleedthrough 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, Em. 567 nm; 7% bleedthrough at 543 nm), Alexa Fluor 568 (Ex. 578, Em 603 nm; 18% bleedthrough at 578 nm), and Alexa Fluor 594 (Ex. 590 nm, Em. 618 nm; 30% bleedthrough at 590 nm), Rhodamine RedX (Ex. 572 nm, Em. 592 nm; 20% bleedthrough at 572 nm) and Cy3 (Ex. 553 nm, Em 565 nm, 9% bleedthrough at 553 nm) (see Figure 2 in supporting Information). The SNAPFL fluorophore analog described here, in contrast to many of the abovementioned commonly used fluorophores in this spectral range, is excited at a shorter wavelength that 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 valuable new fluorophore and a potential FRET partner for the well-known fluorescent dye Cy5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- (1). Shapiro A, Rivin O, Gao N, Hajec L. *Anal Biochem* 2005;347:254. [PubMed: 16266678]
- (2). Kajihara D, Abe R, Iijima I, Komiyama C, Sisido M, Hoshida T. *Nat. Methods* 2006;3:923. [PubMed: 17060916]
- (3). Gipmans BN, Adams SR, Ellisman MH, Tsien RY. *Science* 2006;312:517.
- (4). Vogel SS, Thaler C, Koushik SV. *Sci STKE* 2006;(331):re2. [PubMed: 16622184]
- (5). Urano Y, Kamiya M, Kanda K, Ueno T, Hirose K, Nagano T. *J. Am. Chem. Soc* 2005;127:4888. [PubMed: 15796553]
- (6). Mottram LF, Boonyarattanakalin S, Kovel RE, Peterson BR. *Org. Lett* 2006;8:581. [PubMed: 16468716]
- (7). (i) Banerjee DK, Chatterjee S, Pillai CN, Bhatt MV. *J. Am. Chem. Soc* 1956;78:3767. (ii) Coelho PJ, Salvador MA, Oliveira MM, Carvalho LM. *Synlett* 2004;(6):1015. (iii) Bilger C, Demerseman P, Buisson J-P, Royer R, Gayral P, Fourniat J. *Eur. J. Med. Chem* 1987;22:213.
- (8). Kopp F, Wunderlich S, Knochel P. *Chem. Commun* 2007;(20):2075.
- (9). Kim SH, Katzenellenbogen JA. *Angew. Chem. Int. Ed* 2006;45:7243.
- (10). Nettles KW, Bruning JB, Gil G, Nowak J, Sharma SK, Hahn J,B, Kulp K, Hochberg RB, Zhou H, Katzenellenbogen JA, Katzenellenbogen BS, Kim Y, Joachimiak A, Greene GL. *Nat. Chem. Biol* 2008;4:241. [PubMed: 18344977]

- (11). Carlson KE, Choi I, Katzenellenbogen BS, Katzenellenbogen JA. *Biochemistry* 1997;36:14897. [PubMed: 9398213]
- (12). Anstead GM, Hwang K-J, Katzenellenbogen JA. *Photochemistry and Photobiology* 1993;57:616. [PubMed: 8506389]
- (13). Olmstead J III. *J. Phys. Chem* 1979;83:2581.
- (14). Tamrazi A, Katzenellenbogen JA. *Methods Enzymol* 2003;364:37. [PubMed: 14631838]

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 μM). 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).

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

Figure 3.

FRET experiment between **2** and Cy5-ER α . Emission spectrum of **2** alone (4 nM in pH 8.5 Tris buffer, **black line**), Cy5-ER α alone (1 nM in pH 8.5 Tris buffer, blue line), pre-incubated Cy5-ER α and **2** (1 nM ER α and 4 nM **2** in the same buffer, green line), and pre-incubated 1nM Cy5- ER α and 4 nM **2** in the presence of excess 1 μ M E₂ (red line). The excitation was fixed at 520 nm.

Scheme 1.

Synthetic scheme to prepare for benz[c]xanthen-7-one (10)

Scheme 2.

Synthesis of SNAPFL analog (1) and Cyclofenil-conjugated SNAPFL (2)