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Convenient One-Step Synthesis of 5-Carboxy-Seminaphthofluoresceins

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ABSTRACT

The one-step synthesis and characterization of a series of regioisomerically pure 5-carboxy-seminaphthofluoresceins (5-carboxy-SNAFLs) is reported. The optical properties were determined in aqueous buffer at around biological pH, and highly pH sensitive, large Stokes-shift fluorophores with emission in the deep-red to near-infrared region were identified.

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1. Introduction

The development and characterization of functional red and near-infrared-emitting (NIR) organic dyes with available anchoring sites is of great current interest especially in fields such as biomedicine, biochemistry, and soft-matter science.¹⁻⁴ Due to the low cost, reliability, and low invasiveness of fluorescent techniques in, for example, diagnostics and imaging, there is an ongoing search for new and better dyes. The importance placed on red-NIR dyes in this context is primarily due to the near-absence of biological autofluorescence in this spectral range, and the large penetration depth of NIR light in biological tissue.⁵⁻⁷

5- and 6-carboxy-fluorescein are well-known functional dyes, with strongly pH sensitive optical properties and anchoring sites for target conjugation, and are suitable for a wide range of biological and materials science applications.^{8,9} The long history of their chemical modification of these dyes has broadened the scope of their use, however a fundamental limitation is the relatively high-energy emission, centered at approximately 520 nm. Developing robust approaches to substantially lowering the emission energy of this otherwise excellent class of dyes has thus been a topic of significant interest.⁵⁻¹⁵ One such approach has been to extend the fluorescein scaffold with additional benzoid rings to one or both sides, yielding seminaphthofluorescein (SNAFL) or naphthofluorescein respectively (Fig. 1). These represent fluorescein analogs of the well-known structurally

related seminaphthorhodafluors (SNARF) rhodamine derivatives (see Fig. 1).

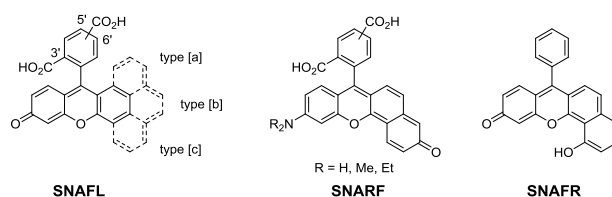


Figure 1. Structure of type [a], [b] and [c] seminaphthofluorescein (SNAFL), seminaphthorhodafluor (SNARF) and seminaphthofluorene (SNAFR).

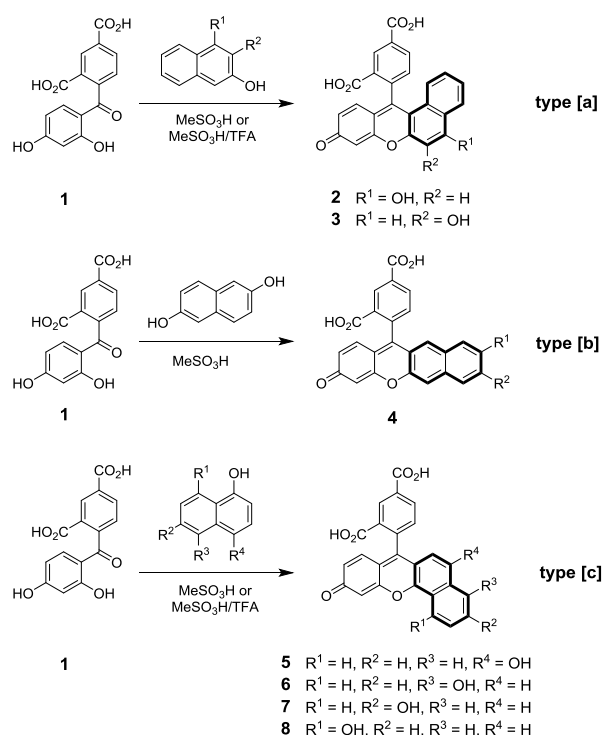
Furthermore, as a class of dyes they show tunable wavelengths, with emission ranging from the deep-red to the near-infrared region.⁵⁻¹³ This range of emission properties appear due to its dependence on both the geometry of the conjugated system and the position of the hydroxy group on the naphthalene moiety.^{5-7, 13} The benzene unit can in general be positioned in three different ways termed type [a], [b] and [c] (Fig. 1). The importance of the benzannulation position has been studied both by semiempirical calculations and by synthesizing a large range of regioisomers of closely related seminaphthofluorene (SNAFR) derivatives.¹³ The structure of carboxy-SNAFLs diverges from SNAFRs by two additional carboxylic acids at the 3' and 5' or 6'

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positions (Fig. 1). SNAFL derivatives thus lack the obvious linking sites provided by the 5' or 6' carboxylic acids. In general the synthesis of seminaphtho- and naphthofluorescein requires multiple steps and, in the cases where a carboxylic acid is present at the 5' or 6' positions, mixtures of the two regioisomers are formed.¹⁶ The fluorescence quantum yields are typically low in aqueous solutions (< 9%). In most cases an increase in quantum yield, Stokes shifts and maximum absorption/emission peak is observed in organic solvents.⁶

In the present work we report the synthesis and optical properties of a number of 5-carboxy-SNAFL derivatives. The general synthetic approach uses the 5-carboxy-fluorescein scaffold and extends it by a benzene unit, enabling the use of well-known chemistry from 5-carboxy-fluorescein to synthesize the newly developed dyes. Depending on the direction of benzannulation, and in particular the position of the naphthol, the compounds exhibit deep-red to NIR emission with large Stokes shifts and pK_a values around 8.

2. Results and Discussion



Scheme 1. Synthesis of type [a], [b] and [c] 5-carboxy-SNAFLs **2-8**.

The synthetic approach described herein is based on our previous work, where we reported the condensation between 2- and 4-(2,4-dihydroxybenzoyl)isophthalic acid and a number of different resorcinol derivatives.^{9,17} The reaction between electron-rich dihydroxynaphthalene (naphthalene-1,3-diol) was found to proceed in a high yield and could easily be purified by precipitation to yield 5-carboxy-SNAFL-**2**.

To further investigate the scope of the reaction, the condensation between 4-(2,4-dihydroxybenzoyl)isophthalic acid (**1**) in methanesulfonic acid (or a 1:1 mixture with TFA), and the 10 regioisomers of dihydroxynaphthalene (Scheme 1 and Table 1) was examined. Through this procedure, 7 new benzannulated 5-carboxy-SNAFLs (**2-8**) were produced.^{18,19} To the best of our knowledge this constitutes the largest set of type [c] isomers of SNAFL (**5-8**) with the hydroxyl group located at 4 different positions.⁶ 2,7-Dihydroxynaphthalene gave a 1:1 mixture of regioisomeric 5-carboxy-SNAFLs (LCMS, not shown), however, the isomers were not separated. Only trace amounts of the desired product were observed using 1,2- and 1,7-dihydroxynaphthalene (LCMS).

Table 1. Yields, solvents and reaction times.

5-Carboxy-SNAFL	Yield (%)	Solvent	Time (h)
2 ^a	79	MeSO ₃ H	1 week ^c
3	29	MeSO ₃ H/TFA	24
4	80	MeSO ₃ H	12
5	74	MeSO ₃ H/TFA	20
6	75	MeSO ₃ H	3
7	79	MeSO ₃ H/TFA	24
8	59 ^b	MeSO ₃ H/TFA	2

^aRef. 9. ^bBased on limiting dihydroxynaphthalene. ^cNot tested using TFA.

X-ray quality crystals of carboxy-SNAFL-**5** co-crystallized with acetic acid were obtained (Fig. 2). The conjugated system is twisted so that the additional benzoid moiety is tilted an angle of 9.88(1)° with respect to the central ring. The angle between the plane of the central ring and the phthalic acid ring is 79.91(6)°.

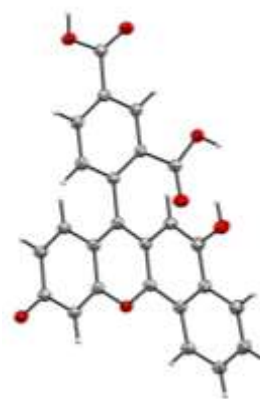


Figure 2. Single crystal X-ray structure of carboxy-SNAFL-**5** (co-crystallizes with one acetic acid molecule which has been omitted for clarity).

One of the great strengths of fluorescein dyes is their potential application in biological sensing and imaging. It is thus of primary importance to characterize the SNAFL derivatives in aqueous buffer near biological pH. The photophysical properties of the dyes in a standard aqueous phosphate/citric acid buffer containing 1% DMSO were evaluated and their optical properties summarized in Table 2, where the properties of 5-carboxy-SNAFL-**2**, are provided for comparison.

In direct analogy to carboxy-fluoresceins, the carboxy-SNAFL dyes exist in one of two forms around biological pH: a “naphthol” form, and the corresponding naphtholate, called “oxyanion” in the following. The relative population of these two species at a given pH is strongly dependent on the structure of the dye. The absorption spectra of both naphthol and oxyanion species for all dyes are shown in Fig. 3.

In mildly acidic buffer, the naphthalene orientation and hydroxyl substitution pattern appears to have only a modest influence on the spectral properties. Under these conditions the main absorption band appears with moderate oscillator strength at approximately 520 nm. The vibrational progression is clearly resolved, and overall the spectral structure is similar to what would be expected from rigid aromatic molecules, *e.g.* xanthenes. The spectral similarity between these species is presumably a result of identical overall extent of the π conjugated system, and that the hydroxyl group only weakly perturbs the electronic structure.

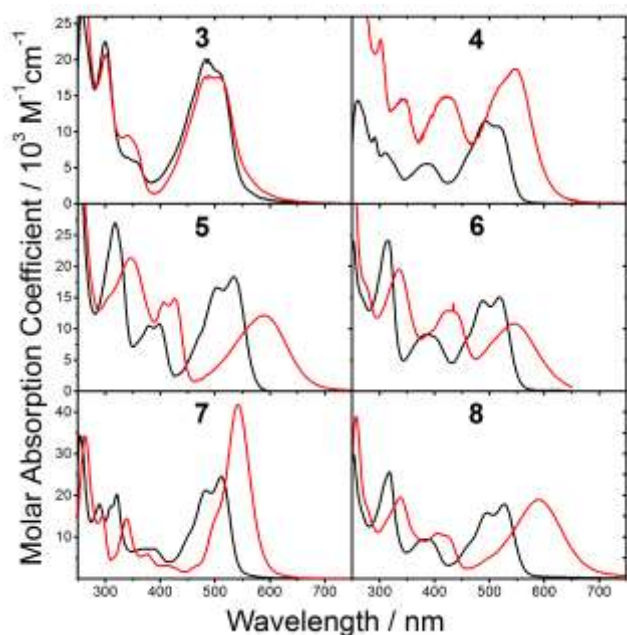


Figure 3. Absorption spectra in aqueous buffer containing 1% DMSO at pH 6 (black) and pH 11 (red).

As the pH is increased, the naphthol moiety is deprotonated, resulting in a red-shift of the absorption band, a loss of vibrational structure, and an increase in oscillator strength. This behavior is similar to that observed for carboxy-fluorescein derivatives. Clear isobestic points are observed during the pH titration of the dyes, implying a simple two-species equilibrium over the pH range from approximately 5 to 10. We note that at very low pH the dyes exist largely as a colorless, likely lactone form, again analogous to carboxy-fluoresceins.

The extent of deprotonation at a given pH appears to depend on whether the dye can support a charge-resonance between the two oxygen substituents. In both **7** and **8** such charge-resonances are possible, effectively stabilizing the deprotonated form, which is reflected in a relatively low pK_a value. In contrast, **4** has a pK_a value of approximately 9, while it does not appear to be possible to deprotonate **3** in aqueous media as partial decomposition of the dye to a colorless product starts around pH 13. Transitions to charge-resonant states generally appear red-shifted, with the magnitude of the shift being related to the length of the resonance system involved. This is exemplified by, for example, the spectral behavior of cyanines. As **8** has a longer conjugated resonant system than **7**, a stronger red-shift is predicted, in agreement with the spectra in Fig. 3.

Table 2. Optical properties of 5-carboxy-SNAFLs **3-8**.

SNAFL	$\lambda_{\max}^{\text{abs}}$ (nm)	ϵ_{\max}	$\lambda_{\max}^{\text{abs}}$ (nm)	ϵ_{\max}	$\lambda_{\max}^{\text{em}}$ (nm)	Φ	$\lambda_{\max}^{\text{em}}$ (nm)	Φ	pK_a
	Naphthol ^d	($M^{-1} \text{cm}^{-1}$)	Oxyanion ^e	($M^{-1} \text{cm}^{-1}$)	Naphthol ^d		Oxyanion ^e		
2 ^a	508	-	-	-	550	0.11	-	-	11
3	508	20300	- ^c	- ^c	- ^b	-	- ^b	-	$\gg 10$ ^c
4	517	20000	548	32500	- ^b	-	- ^b	-	9.1
5	534	17100	590	12200	595	0.02	- ^b	-	8.5
6	518	16200	545	11000	- ^b	-	- ^b	-	8.5
7	512	24500	538	43000	553	0.06	632	0.11	8.0
8	527	18000	590	19200	600	<0.01	748	~ 0.01	7.9

All samples in aqueous phosphate/citric acid buffer containing 1% DMSO. ^aData from Ref. 9. ^bEmission too weak to determine. ^cNot measurable in aqueous buffer. ^dMeasured at pH 6. ^eMeasured at pH 11.

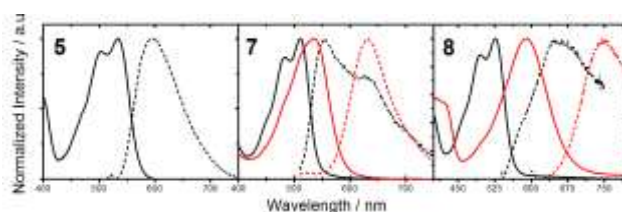


Figure 4. Normalized absorption (full line) and emission (dotted line) of **5**, **7** and **8** in aqueous buffer at pH 6 (black) and pH 11 (red). See text for details.

The emission properties of the dyes investigated herein are surprisingly diverse. Neither **3**, nor **4**, nor **6** fluoresce appreciably in aqueous solution, regardless of pH, which is unexpected, considering the close structural analogy to the highly efficient carboxy-fluorescein fluorophores. The dye **5** and the charge-resonant species **7** and **8** are emissive in aqueous buffer, however, with **5** and **7** being moderately strong red emitters and **8** being a weak near-infrared emitter. The normalized absorption and emission spectra of these species are shown in Fig. 4. Compound **5** emits under acidic and neutral conditions, but is essentially non-emitting in alkaline media. Both charge-resonant species show substantially higher quantum yields and large Stokes shift in alkaline solution, however, the overall yield of **7** is in excess of 10 times larger than that of **8** regardless of environment. We note that **7** appear to be a rather strong excited-state proton donor. As a result, there is a noticeable contribution to the spectrum at approximately 630 nm from the oxyanion form even at relatively low pH.

3. Conclusion

The synthesis, structure, and optical properties of a series of 5'-carboxy SNAFL dyes. The synthetic approach is quite flexible, and may be generalized to yield a broad range of "fluorescein-type" dyes with diverse optical properties. The SNAFL dyes are water soluble and can be conjugated to a variety of targets using standard amide-coupling protocols or by subsequent formation of the *N*-hydroxysuccinimide ester. Of the SNAFL species investigated, the charge-resonant compounds **7** and **8** prove most interesting due to reasonably efficient fluorescence. Dye **7** in particular displayed emission of comparable strength to many red/NIR emitting dyes, and may find use in biological sensing due to its relatively low pK_a and strong optical response to pH. The emission of **8** is comparatively weak, and in practice only appreciable in a weakly alkaline environment. The NIR emission and large Stokes shift of the oxyanion form however results in a dye that is highly resistant to autofluorescence background and scattering. It might therefore, despite its relatively low emission yield, prove useful in applications where background rejection rather than excitation/emission power is the major concern.

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X-ray crystallographic data, synthesis procedures, and NMR spectra.

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- Hammershøj P.; Clausen, M. H. PCT Int. Appl. (2015), WO 2015169854 A1 2015111
- General Procedure for the Synthesis of 5-Carboxy-SNAFLs.** 4-(2,4-Dihydroxybenzoyl)isophthalic acid (**1**) (500 mg, 1.65 mmol) and the appropriate dihydroxynaphthalene (275 mg, 1.75 mmol) were dissolved in methanesulfonic acid (10 mL) or a mixture of TFA (5 mL) and methanesulfonic acid (5 mL), and stirred at room temperature overnight. The reaction was quenched with H₂O (25 mL) and the resulting dark purple precipitate collected by centrifugation. After decantation, the precipitate was dissolved in NaOH(aq) (2 M, 15 mL) and precipitated with HCl(aq) (2 M, 20 mL). After decantation, the precipitate was washed with H₂O (2 x 35 mL), dissolved in EtOH (10 mL) and precipitated with H₂O. After decantation and washing with H₂O (2 x 35 mL), the compound was dried *in vacuo* yielding a dark purple powder. If required, further purification by silica gel dry column vacuum chromatography (2% AcOH in CH₂Cl₂/MeOH with 5% increments) was performed by dissolution of the compound in MeOH and NaOH(aq) (12 M, 2 drops) and evaporation onto Celite *in vacuo*.
- Analytical data for a typical 5-carboxy-SNAFL (3).** Starting from 2,3-dihydroxynaphthalene (54 mg, 331 μmol) and 4-(2,4-dihydroxybenzoyl)isophthalic acid (**1**) (100 mg, 331 μmol). Purification by chromatography was necessary. Yield: 41 mg, 29%; Mp: 205–208 °C; ¹H NMR (400 MHz, acetone-*d*₆) δ 9.15 (broad s, D₂O exchangeable OH), 9.10 (broad s, D₂O exchangeable OH), 8.68 (dd, *J* = 1.5, 0.6 Hz, 1H), 8.34 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.76 (d, *J* = 8.0 Hz, 1H), 7.52 (s, 1H), 7.42 (dd, *J* = 8.0, 0.6 Hz, 1H), 7.28 (ddd, *J* = 8.0, 5.9, 2.0 Hz, 1H), 7.11 – 6.98 (m, 2H), 6.97 – 6.91 (m, 1H), 6.71–6.70 (m, 2H); ¹³C NMR (101 MHz, Acetone) δ 170.16, 167.15, 160.90, 160.65, 151.59, 146.64, 144.39, 138.34, 134.42, 133.53, 130.49, 129.63, 129.10, 128.07, 127.31, 126.48, 126.22, 125.84, 125.01, 115.19, 114.98, 112.53, 110.79, 103.97, 85.12; MS (ESI) *m/z* [M + H]⁺ calcd for C₂₅H₁₄O₇⁺ 427.4, found 427.1. HR-MS (ESI): *m/z* [M + H]⁺ calcd for C₂₅H₁₄O₇⁺ 427.0812, found 427.0825.