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Synthesis and Photophysical Properties of New Fluorinated Benzo[*c*]xanthene Dyes as Intracellular pH Indicators

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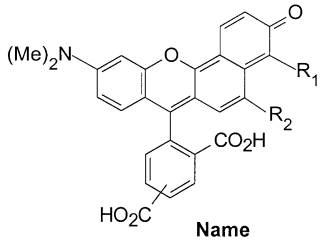
Abstract—Two new fluorinated benzo[*c*]xanthene dyes were synthesized by reaction of fluorinated 1,6-dihydroxynaphthalenes with 2,4- (and 2,5)-dicarboxy-3′-dimethylamino-2′-hydroxybenzophenone. The two critical fluorinated 1,6-dihydroxynaphthalene intermediates were prepared via a regioselective route. The fluorinated benzo[*c*]xanthene dyes exhibit desired lower pK_a values (6.4 and 7.2, respectively) than their parent compound (pK_a = 7.5) while the pH-dependent dual-emission characteristics are well retained. Their cell-permeable esters have been prepared for intracellular applications.

Intracellular pH plays an important modulating role in many cellular events, including cell growth, calcium regulation, enzymatic activity, receptor-mediated signal transduction, ion transport, endocytosis, chemotaxis, cell adhesion and other cellular processes.[1–8] pH-Sensitive fluorescent dyes have been widely applied to monitor changes in intracellular pH in recent years. Imaging techniques that use fluorescent pH indicators also allow researchers to investigate these processes with much greater spatial resolution and sampling density that can be achieved using other technologies such as microelectrode.[9] The most popular pH-sensitive probes include 1,4-dihydroxyphthalonitrile (1,4-DHPN),[10] 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS),[11] 5- (and 6)-carboxyfluorescein,[12] 5- (and 6)-carboxy-4′,5′-dimethyl fluorescein,[13] 2′,7′-bis-(2-carboxyethyl)-5- (and 6)-carboxyfluorescein (BCECF),[14] 2′,7′-bis-(2-carboxypropyl)-5- (and 6)-carboxyfluorescein (BCPCF),[15] carboxyseminaphthofluorescein (carboxy SNAFL) and carboxyseminaphthorhodafluor-1 (carboxy SNARF-1) dyes.[16] Among these pH sensitive dyes, carboxy SNARF-1 indicator has both dual emission and dual excitation with excitability by 488 or 514 nm spectral lines of the argon-ion laser. These advantages have made carboxy SNARF-1 exceptionally suitable for both confocal laser scanning microscopy and flow cytometry. However, the relatively high pK_a (~7.5) of carboxy SNARF-1 has limited its cellular applications. This has motivated us to modify the existing carboxy SNARF-1 indicator by fluorinating its benzo[*c*]xanthene ring system. Herein, we report the synthesis and photophysical properties of the newly synthesized carboxy SNARF-4F and carboxy SNARF-5F indicators (Fig. 1).

We have tried two approaches to synthesize fluorinated carboxy SNARF dyes. One is the direct fluorination of carboxy SNARF-1. The other approach is the condensation of 2,4- (and 2,5)-dicarboxy-3′-dimethylamino-2′-hydroxybenzophenone with fluorinated 1,6-dihydroxynaphthalenes that were prepared by the regiospecific routes, as described below. There are several active positions on carboxy SNARF-1 aromatic moiety that are readily available for direct electrophilic fluorination; however, these routes gave a mixture of fluorinated products that were difficult to separate. Thus, we decided to develop the regiospecific synthesis of 5-fluoro-



Name	R_1	R_2
Carboxy SNARF-1	H	H
Carboxy SNARF-4F	F	H
Carboxy SNARF-5F	H	F

Figure 1. The structures of carboxy SNARF-1, carboxy SNARF-4F, and carboxy SNARF-5F indicators.

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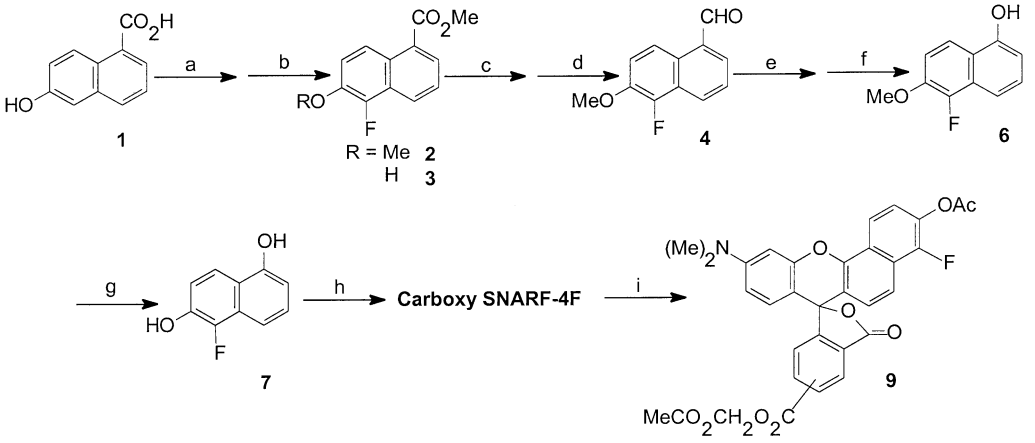
and 4-fluoro-1,6-dihydroxynaphthalenes as key intermediates to achieve an effective synthesis of carboxy SNARF-4F and carboxy SNARF-5F.

Carboxy SNARF-4F was synthesized from commercially available 6-hydroxy-1-naphthoic acid as shown in Scheme 1. Reaction of 6-hydroxy-1-naphthoic acid with MeI/K_2CO_3 in THF followed by fluorination with SelectFluor [1-chloromethyl-4-fluoro-1,4-diazabicyclo [2.2.2]-octane bis(tetrafluoroborate)][17] gave a mixture of compounds **2** and **3** with some unreacted starting material. Interestingly, SelectFluor can demethylate compound **2** to give compound **3** under the reported reaction conditions. Reduction of compound **2** with BH_3·THF gave alcohol **4** in high yield. Oxidation of alcohol **4** with PCC gave aldehyde **5**. Baeyer–Villiger oxidation of aldehyde **5** with *m*-CPBA followed by basic hydrolysis yielded compound **6**. Treatment of compound **6** with BBr_3 gave the desired intermediate 5-fluoro-1,6-dihydroxynaphthalene **7**. Condensation of **7** with 2,4- (and 2,5)-dicarboxy-3′-dimethylamino-2′-hydroxybenzophenone **8**, which was prepared by reaction of 3-dimethylaminophenol with trimellitic acid in toluene, afforded the desired carboxy SNARF-4F dye. The carboxy SNARF-4F was purified via the conversion to the corresponding acetate **9** on a silica gel column, which was then converted back into carboxy SNARF-4F nearly quantitatively by basic hydrolysis.

Carboxy SNARF-5F was synthesized from commercially available 1,6-dihydroxynaphthalene rather than 1,6-dimethoxynaphthalene. As discussed above, the demethylation (by SelectFluor) gave a very messy mixture. Fluorination of 1,6-dihydroxynaphthalene with SelectFluor gave a mixture of the desired product and other fluorinated by-products, along with some unreacted starting material. The reaction mixture was difficult to be purified. The mixture was methylated with MeI/K_2CO_3, and the resulting ether mixture was purified on a silica gel column to give pure 4-fluoro-1,6-dimethoxynaphthalene **10**. Demethylation of compound **10** with HBr in H_2O/AcOH yielded 4-fluoro-1,6-dihydroxynaphthalene, which was used to make carboxy SNARF-5F, utilizing the method analogous to prepare carboxy SNARF-4F. Finally, both carboxy SNARF-4F and carboxy SNARF-5F were converted to their corre-



Scheme 1. Synthesis of carboxy SNARF-4F dye. Reaction conditions: (a) MeI, K_2CO_3, THF, reflux, yield: 96%; (b) SelectFluor, CH_3CN, 0 °C, yield: for **2**: 60%, for **3**: 15%; (c) BH_3·THF, reflux, yield: 96%; (d) PCC, CH_2Cl_2, rt, yield: 81%; (e) *m*-CPBA, CH_2Cl_2, reflux, yield: 39%; (f) LiOH, MeOH/H_2O, rt, yield: 95%; (g) BBr_3, CH_2Cl_2, rt, yield: 93%; (h) (1) **8**, $ZnCl_2$, 160 °C; (2) Ac_2O, NEt_3, THF, reflux; (3) NH_3·H_2O, MeOH, rt; yield: 60%; (i) (1) Ac_2O, NEt_3, THF, reflux; (2) $CH_3CO_2CH_2Br$, NEt(Pr-*i*)$_2$, THF, rt; yield 31%.

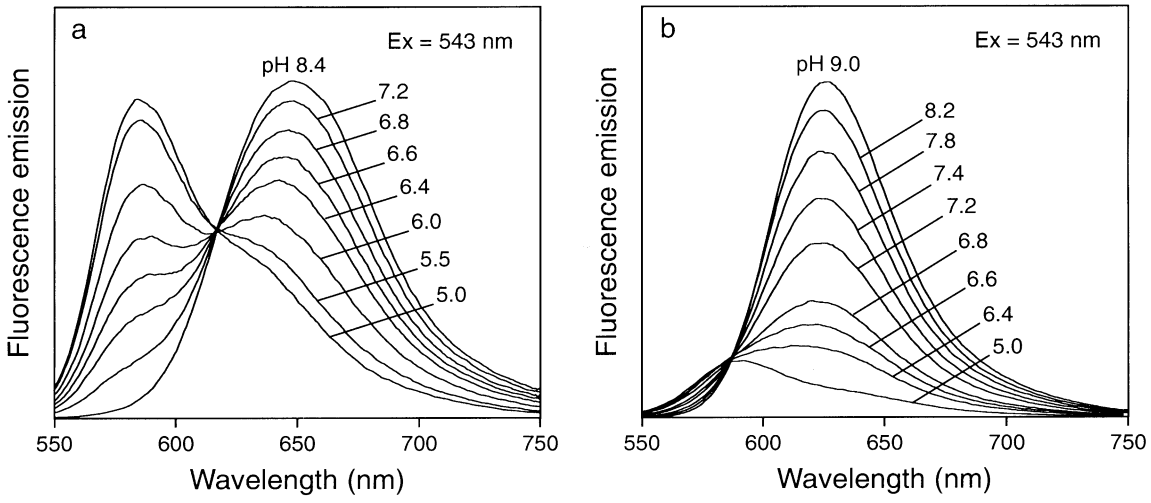


Figure 2. Fluorescence emission spectra of (a) carboxy SNARF-4F and (b) carboxy SNARF-5F in buffer solution at various pH values.

sponding cell-permeable esters. All compounds were confirmed with ^{1}H and ^{19}F NMR spectra.[18]

As shown in Figure 2, carboxy SNARF-4F exhibits a pH-dependent wavelength shift with the emission maxima at 587 and 650 nm, and with an isosbestic point at 618 nm. Its pK_a was determined to be 6.4 by the fluorescence emission spectra. Similarly, carboxy SNARF-5F also retains the pH-dependent dual-emssion characteristics, with maxima of 575 and 628 nm. Its pK_a was determined to be 7.2 by the fluorescence emission spectra. As discussed above, the high pK_a value of 7.5 of carboxy SNARF-1 makes it less suitable for measuring the cytosolic pH of most cell lines (pH $\sim$6.8–7.4). The new fluorinated carboxy SNARF-4F and carboxy SNARF-5F apparently have an improved pK_a. The low pK_a values of 6.4 for carboxy SNARF-4F and 7.2 for carboxy SNARF-5F will make these indicators exceptionally suitable for pH measurement in the range from about 6.0 to 7.5 with confocal laser scanning microscopy and flow cytometry.

In summary, carboxy SNARF-4F and carboxy SNARF-5F were synthesized and characterized as new dual-emission pH indicators with pK_a values of 6.4 and 7.2. The two key intermediates, 5-fluoro-1,6-dihydroxynaphthalene and 4-fluoro-1,6-dihydroxynaphthalene can also be used to prepare other fluorescent probes.

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18. **Carboxy SNARF-4F**: ^{1}H NMR (CD_3OD) δ 8.82 (s), 8.54 (m), 8.39 (m), 8.00 (s), 7.45 (d), 7.26 (m), 7.00 (m), 6.57 (d), 3.15 (s); ^{19}F NMR ϕ 126.7, 127.1. **Carboxy SNARF-5F**: ^{1}H NMR (CD_3OD) δ 8.90 (s), 8.49 (m), 8.40 (s), 8.02 (s), 7.82 (d), 7.55 (d), 7.44 (m), 7.22 (m), 7.11 (m), 3.40 (s); ^{19}F NMR ϕ 150.4.