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FLUORESCENT MOLECULAR PROBES III. 2′,7′-BIS-(3-CARBOXYPROPYL)-5-(AND-6)-CARBOXYFLUORESCEIN (BCPCF): A NEW POLAR DUAL-EXCITATION AND DUAL-EMISSION PH INDICATOR WITH A PKA OF 7.0

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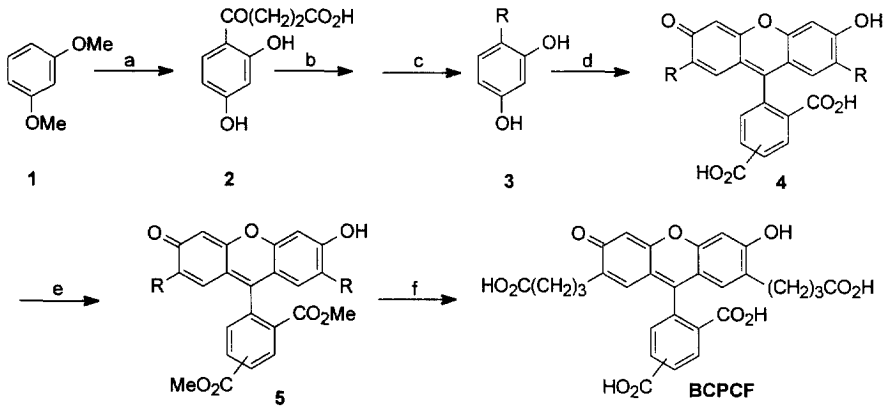
Abstract: 2′,7′-Bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF) was prepared and characterized as a new polar dual-excitation and dual-emission pH indicator with a pKa of 7.0.

Intracellular pH plays an important role in many cellular events such as cell growth,[1] calcium regulation,[2] endocytosis,[3] chemotaxis,[4] cell adhesion,[5] and other cellular processes.[1] Both invasive methods such as microelectrodes, and noninvasive techniques, such as NMR and fluorescence spectroscopy, have been used to measure intracellular pH. Among them, fluorescence spectroscopy provides greater sensitivity and convenience than other methods. 1,4-Dihydroxyphthalonitrile (1,4-DHPN),[6] 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS),[7] 5-(and -6)-carboxyfluorescein,[8] 4′,5′-dimethyl-5-(and -6)-carboxyfluorescein,[9] 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF),[10] carboxyseminaphthofluorescein (SNAFL®) dyes, and carboxyseminaphthorhodafluor (SNARF®) dyes have been used as fluorescent pH-sensitive probes.[11] BCECF has a pKa of ~7.0, which makes spectra of the compound sensitive to pH changes near the physiological range. Four to five negative charges of BCECF at physiological pH retard leakage of the compound from cells. Additionally, the dual-excitation fluorescence properties of BCECF with an isosbestic point at 439 nm, makes it a useful excitation-ratiometric pH indicator. These advantages have made BCECF the most widely used fluorescent indicator for measuring intracellular pH under physiological conditions.[12] However, there are still some disadvantages with BCECF, such as its weak absorption at the isosbestic point of the excitation spectrum and its lack of pH-dependent dual emission. This has encouraged us to develop a new fluorescent pH indicator to overcome the disadvantages of BCECF. Herein, we report the synthesis and spectral properties of 2′,7′-bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF).

BCPCF was synthesized from commercially available 1,3-dimethoxybenzene as shown in Scheme 1. Friedel–Crafts acylation of 1,3-dimethoxybenzene and in situ simultaneous demethylation of the acylated product gave compound **2**. Compound **2** was esterified to yield a ketone ester in 90% yield. The carbonyl

group of the ketone ester was quantitatively hydrogenated in AcOH at 90 °C to give resorcinol **3**. The condensation of resorcinol **3** with trimellitic acid in methanesulfonic acid afforded the fluorescein derivative **4**. However this crude BCPCF was not easily purified. Alternatively, crude **4** was first esterified, and the resulting ester was readily purified on a silica gel column. The purified compound **5** was then converted into BCPCF nearly quantitatively by hydrolysis with base. All structures in Scheme 1 are consistent with their ^{1}H NMR spectra.[13]



Scheme 1. Synthesis of BCPCF (R = $(CH_2)_3CO_2Me$). (a) succinic anhydride, $AlCl_3$, 1,2-dichloroethane, reflux, yield: 40%; (b) MeOH, H_2SO_4, reflux; yield: 90%; (c) H_2/10% Pd/C, AcOH, 90 °C, yield: 100%; (d) trimellitic acid, methanesulfonic acid, 80 °C, yield: 68%; (e) MeOH, H_2SO_4, reflux, yield: 88%; (f) NaOH, MeOH/H_2O, yield: 90%.

As shown in Figure 1, BCPCF exhibits a pH-dependent wavelength shift with isosbestic points in both the excitation and emission spectra. The excitation and emission maxima were 505 nm with an isosbestic point of 454 nm and 527 nm with an isosbestic point of 504 nm, respectively. Its fluorescence quantum yield was 0.83 in 0.1 M NaOH. Its pKa was determined to be 7.0. These properties are compared with those of BCECF in Table 1.

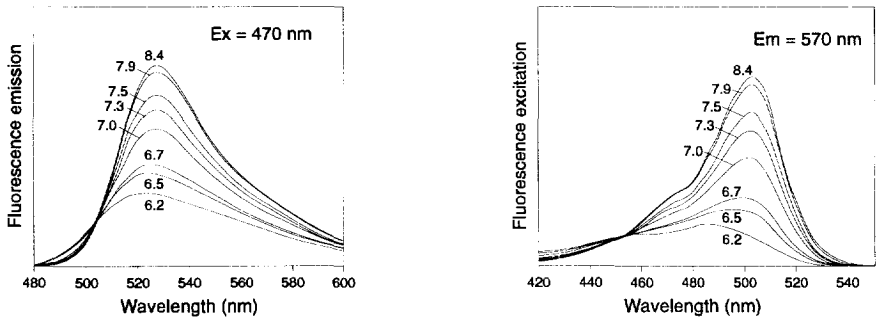


Fig 1. Excitation and emission spectra of BCPCF in 50 mM potassium phosphate-buffered solution at various pH.

Table 1. Spectral and photophysical properties of BCPCF and BCECF

Compound	λ^{Ex}_{iso} (nm)	λ^{Ex}_{max} (nm)	λ^{Em}_{iso} (nm)	λ^{Em}_{max} (nm)	Φ^{*}	pKa
BCPCF	454	505	504	527	0.83	7.0
BCECF	439	505	none	527	0.84	7.0

* All the fluorescence quantum yields were determined in 0.1 M NaOH using fluorescein (Φ = 0.92) as reference.

Structurally, both BCPCF and BCECF are carboxyfluorescein derivatives differing only in the length of the side chain of positions 2′ and 7′. The similarity in structure leads to similar pKa values, negative charges and fluorescence quantum yields, as seen from Table 1. As with BCECF, the dual excitation spectrum of BCPCF with an isosbestic point at 454 nm should make the compound a good excitation-ratiometric pH indicator. As has previously been discussed,[12] ratiometric imaging makes intracellular pH determination essentially independent of several variable factors, including dye concentration, path length, cellular leakage and photobleaching rate. Intracellular pH measurements with BCECF are made by determining the pH-dependent ratio of emission intensity (at 535 nm) when the dye is excited at 505 nm versus the emission intensity when excited at its isosbestic point of 439 nm. Because the BCECF absorption at 439 nm is quite weak, the denominator wavelength is often increased to ~450 nm to improve the signal-to-noise characteristic for ratio imaging applications.[14] This disadvantage has been overcome by the new pH indicator BCPCF. As shown above, the isosbestic point of BCPCF is red-shifted to 454 nm and has an increased molar absorptivity compared to that of BCECF. The longer wavelength and higher molar absorptivity at the isosbestic point of the BCPCF makes it a better excitation-ratiometric pH indicator than BCECF.

Furthermore, we noted that the slight structural difference between BCECF and BCPCF makes the emission spectrum of BCPCF significantly different from that of BCECF (Fig. 1). BCECF only exhibits a single emission peak at different pH values, but BCPCF has a dual emission at different pH values with an isosbestic point at 504 nm. This dual emission property of BCPCF should make BCPCF a useful emission-ratiometric pH indicator, in addition to its utility for dual excitation measurements.

In summary, BCPCF was prepared and characterized as a new polar pH indicator that not only exhibits a better pH-dependent dual excitation than BCECF, but also a strong pH-dependent dual emission that is lacking in BCECF. These novel spectral properties of BCPCF make the compound useful as both an excitation- and emission-ratiometric pH indicator.

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13. For example, BCPCF: ^{1}H NMR (CD_3OD) δ: 8.61 (s), 8.35 (d), 8.29 (d), 8.09 (d), 7.72 (s), 7.30 (d), 6.62 (d, 2H), 6.45 (d, 2H), 2.42(t, 4H), 2.10 (t, 4H), 1.69 (m, 4H).
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