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Synthesis of Novel Fluorinated Coumarins: Excellent UV–Light Excitable Fluorescent Dyes

Wei-Chuan Sun,*[,1] Kyle R. Gee, Richard P. Haugland

Molecular Probes, Inc. 4849 Pitchford Ave. Eugene, Oregon 97402-9165

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Abstract: Two new fluorinated fluorescent dyes, 6,8-difluoro-7-hydroxy-4-methylcoumarin (Marina Blue™) and 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue™), exhibit excellent photophysical properties among a series of novel fluorinated 7-hydroxycoumarins. Most of these fluorinated coumarins have quantum yields (0.63 to 0.89) equal to or higher than that of the parent compound (0.63), which, in combination with their lower pK_as and higher photostability, make them superior fluorescent dyes for use as reporter molecules in biological systems.

Introduction:

The performance required from fluorescent dyes, especially for use in intense illumination produced in most fluorescence instrumentation,[2] has induced many chemists to synthesize new molecules possessing improved photophysical properties such as high quantum efficiency, photostability, and low pK_a.[3] Among these compounds, 7-hydroxy-4-methylcoumarin (β-methylumbelliferone, **1**) and 3-carboxy-7--hydroxycoumarin (3-carboxyumbelliferone, **2**) and their derivatives constitute an important class of UV–light excitable fluorescent dyes. They have been widely used in the preparation of fluorescent dye–protein conjugates and as the basis of fluorogenic enzyme substrates. For example, the phosphate monoester of **1** has been extensively used to detect the enzymes alkaline and acid phosphatase.[4] β-Methylumbelliferyl *p*-guanidinobenzoate has been used to determine concentrations of the serine proteases trypsin,[5] thrombin and factor Xa.[6] Additionally, numerous fluorogenic glycosidase substrates have been prepared based on β-methylumbelliferone, including the β-methylumbelliferyl galactopyranoside[7] (MUG), and glucuronide.[8]

Despite their widespread use in biological assays, 7-hydroxycoumarins and their conjugates are not fully deprotonated (and therefore not maximally fluorescent) unless the dye is in an environment having a pH of 10 or higher.[9] The sensitivity of assays using 7-hydroxycoumarin–based conjugates and enzyme substrates therefore decreases at lower pH, where some or all of the assay components are incompatible with basic pH levels. The sensitivity of continuous assays for glycosidases and acid phosphatases is especially limited, as these enzymes have optimal turnover rates at or below pH 7. Similarly, many protein conjugates of 7-hydroxycoumarin are unstable or not useful with respect to the basic conditions required to obtain maximal fluorescence of the label.

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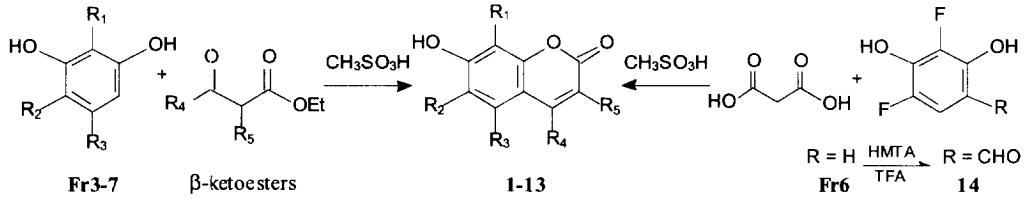
Results and Discussion:

In our search for substitutes that improve upon the properties of 7-hydroxycoumarins, we chose to examine the effect of fluorination on the photophysical properties of these fluorescent dyes. While several fluorinated coumarins have been described in the literature,[10] fluorinated versions of the widely used 7-hydroxycoumarins have not been reported. We now wish to report our findings on a series of novel fluorinated coumarins.

The first synthesis of 7-hydroxy-4-methylcoumarin **1** was reported by von Pechmann and Duisberg by the condensation of resorcinol with ethyl acetoacetate in strongly acidic media.[11] To prepare a family of fluorinated umbelliferones we utilized a regiospecific synthesis of fluorinated resorcinols as building blocks.[12] These fluororesorcinols (**Fr3–7**) were condensed with various β-ketoesters[13] in methanesulfonic acid at room temperature to give 7-hydroxycoumarin derivatives (**3–8**) in 45–80% yields.[14]



Fr3-7 β-ketoesters **1-13** **Fr6** **14**

Table 1. Photophysical properties of Coumarins.

Compd	R_1	R_2	R_3	R_4	R_5	$\varepsilon \times 10^{-3}$	Abs/Em[a]	pK_a	Φ_F[b]	Bleaching[c]
1	H	H	H	CH_3	H	17.0	360/450	7.8	0.63	22%
3	F	H	H	CH_3	H	17.4	359/465	6.4	0.63	17%
4	H	F	H	CH_3	H	17.8	360/440	6.4	0.64	20%
5	H	H	F	CH_3	H	18.7	354/456	5.9	0.78	12%
6	F	F	H	CH_3	H	17.5	358/455	4.7	0.89	5%
7	F	F	F	CH_3	H	15.8	359/459	4.2	0.54	nd[d]
8	F	F	H	CH_3	CH_2CO_2H	18.7	362/459	4.7	0.89	nd
9	H	H	H	CF_3	H	16.3	385/501	7.3	0.34	31%
10	H	F	H	CF_3	H	16.2	385/491	5.7	0.44	18%
11	F	F	H	CF_3	H	16.1	384/504	4.0	0.23	7%
2	H	H	H	H	CO_2H	36.7	386/448	7.5	0.70	nd
12	F	F	H	H	CO_2H	29.5	400/447	3.7	0.75	nd
13	F	F	H	H	CO_2Et	30.0	405/450	3.8	0.78	nd

a) in nm (10^{-9} m). b) Quantum yield, determined in 0.1 M phosphate buffer at pH 10. c) Percent decrease in fluorescence intensity after 33 min of illumination in a fluorometer at the wavelength of maximum absorption. d) not determined.

The wavelength of maximum absorption (Abs), fluorescence emission maximum (Em), fluorescence quantum yield (Φ_F), photostability, pK_a, and extinction coefficient (ε) of each dye is shown in Table 1. For comparison, the photophysical properties of **1**, **2** and 7-hydroxy-4-trifluoromethylcoumarin **9** are also included. In general, fluorination of the phenolic ring had only minor effects on the absorption and fluorescence wavelengths. As expected, the pK_as of all the new fluorinated coumarins were lower than those of the parent compounds, which should result in a significant signal enhancement for applications in biological systems that are conducted at a near–neutral pH as reporter molecules. Another advantage was that the quantum yields of all the new fluorinated coumarins with a 4-methyl group (**3–6**) were higher than those of the parent compounds. In contrast, the new fluorinated coumarins with a 4-trifluoromethyl substituent (**10**, **11**) have lower quantum yields than the parent compound. Fluorination on the phenolic ring of 7-hydroxycoumarins resulted in compounds with improved photostability (**3–6** vs. **1**; **10** and **11** vs. **9**). Preparation of a carboxylic acid–containing analog (**12**) of **6** allows for conjugation to biomolecules via reactive esters.

3-Carboxy-7-hydroxycoumarin **2** has previously been prepared from β-resorcylaldehyde and diethylmalonate in H_2SO_4.[15] In order to make fluorinated 3-carboxy-7-hydroxycoumarin derivatives, a fluorinated β-resorcylaldehyde was needed. We chose to examine the formylation of 2,4-difluororesorcinol (**Fr6**) because **6** gave the best performance among fluorinated derivatives of **1**. Subjection of **Fr6** to normal Vilsmeier–Haack reaction conditions failed to provide a formylation product. However, a recently described method[16] for formylation of 2,4-difluorophenol using hexamethylenetetramine (HMTA) in boiling trifluoroacetic acid served our purpose well. Thus 3,5-difluoro-2,4-dihydroxybenzaldehyde (**14**) was isolated in 60% yield.[17] Subsequent condensation with malonic acid in CH_3SO_3H yielded 3-carboxy-6,8-difluoro-7-hydroxycoumarin **12** in 68% yield.

Conclusion:

In summary, 6,8-difluoro-7-hydroxy-4-methylcoumarin **6** (Marina Blue™) and 3-carboxy-6,8-difluoro-7-hydroxycoumarin **12** (Pacific Blue™) dyes have the most desirable photophysical properties. Their lower pK_as, increased resistance to photobleaching, and higher quantum yields make them superior fluorescent dyes for use as reporter molecules in biological systems. The results of bioconjugation of these dyes to proteins and other molecules and the synthesis and use of fluorinated coumarins as enzyme substrates for phosphatases and glycosidases will be reported in due course.

References and Notes:

1 Address correspondence to this author at EPIX Medicals, Inc. 71 Rogers Street, Cambridge, MA 02142. Fax: (617) 499-1415. E-mail: wsun@epixmed.com.

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13. The following β-ketoesters were used: ethyl acetoacetate, ethyl 4,4,4-trifluoroacetoacetate, ethyl pentafluorobenzoylacetate, diethyl acetylsuccinate.

14. ^{1}H-NMR (400 MHz) and ^{19}F-NMR (282 MHz) were taken in DMSO-d_6. Chemical shifts were reported downfield from TMS (δ) and upfield from $CFCl_3$ (ϕ) for ^{1}H and ^{19}F, respectively. **3**: δ 10.96 (s, 1H), 7.41 (dd, J = 7.4, 1.8, 1H), 6.95 (t, J = 8.6, 1H), 6.22 (s, 1H), 2.37 (s, 3H). ϕ 155.04 (d, J = 7.1). **4**: δ 7.57 (d, J = 11.6, 1H), 6.89 (d, J = 7.2, 1H), 6.20 (s, 1H), 2.35 (s, 3H). ϕ 135.06 (dd, J = 11.3, 7.1). **5**: δ 10.95 (s, 1H), 6.58 (d, J = 13.4, 1H), 6.11 (s, 1H), 2.43 (d, J = 6.0, 3H). ϕ 108.84 (m). **6**: δ 7.48 (d, J = 9.9, 1H), 6.31 (s, 1H), 2.36 (d, J = 1.0, 3H). ϕ 131.66 (t, J = 10.4, 1F), 149.31 (d, J = 9.0, 1F). **7**: δ 6.18 (s, 1H), 2.44 (d, J = 6.0, 3H). ϕ 140.04 (m, 1F), 155.24 (s, 1F), 158.74 (m, 1F). **8**: δ 7.48 (d, J = 12.1, 1H), 3.56 (s, 2H), 2.32 (s, 3H). ϕ 128.03 (dd, J = 12.3, 10.0, 1F), 147.46 (br, 1F). **10**: δ 7.42 (d, J = 11.3, 1H), 7.02 (d, J = 7.4, 1H), 6.86 (s, 1H). ϕ 59.45 (s, 3F), 133.15 (dd, J = 11.2, 7.3, 1F). **11**: δ 7.33 (d, J = 11.1, 1H), 6.99 (s, 1H). ϕ 59.38 (s, 3F), 129.93 (t, J = 9.9, 1F), 147.78 (d, J = 9.3, 1F). **12**: δ 8.53 (d, J = 1.2, 1H), 7.45 (d, J = 10.5, 1H). ϕ 131.67 (t, J = 12.0, 1F), 153.45 (br, 1F). **13** δ 8.53 (d, J = 1.2, 1H), 7.45 (d, J = 10.5, 1H), 4.23 (q, J = 7.1, 2H), 1.28 (t, J = 7.2, 3H). ϕ 131.67 (t, J = 11.9, 1F), 153.46 (br, 1F).

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17. **14**: δ 9.81 (s, 1H), 7.22 (dd, J = 10.3, 2.1, 1H). ϕ 143.78 (dd, J = 9.0, 7.0, 1F), 158.31 (d, J = 6.4, 1F). Anal Calcd for $C_7H_4F_2O_3$: C, 48.29; H, 2.32. Found: C, 47.87; H, 2.62.