Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 15, pp. 1985-1988, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00345-4

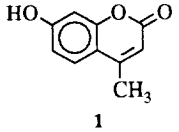
NOVEL 7-HYDROXYCOUMARIN BASED FLUORESCENT LABELS

Maciej Adamczyk,* Michael Cornwell, Jeff Huff, Sushil Rege, and Tata Venkata S. Rao

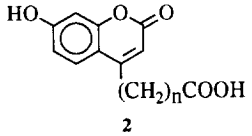
Abbott Laboratories, Diagnostics Division, D9NM/AP20, 100 Abbott Park Rd., Abbott Park, IL 60064

Abstract. Five new 7-hydroxy-8-(N-alkylaminomethyl)coumarin labels (**6a–e**) were prepared from 4-(4-carboxybutyl)-7-hydroxycoumarin (**4**) in 28–48% yield. Compared to 4-methylumbelliferone (4-MU, **1**), the new fluorescent labels exhibited higher emission intensity over a broader pH range. Extinction coefficients, quantum yields and pKa values are also reported for these labels. © 1997 Elsevier Science Ltd.

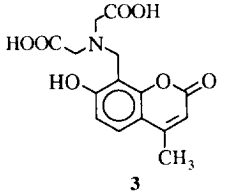
Fluorescent molecules are critically important for use as molecular probes and labels in biological research[1] and in commercial assays.[2] The interaction of fluorophore–ligand conjugates with their respective binding proteins can provide important information about the active site of the biomolecule.[3] While a variety of fluorescent probes[4] have been reported, coumarin derivatives are frequently used due to their intense fluorescence upon excitation above 330 nm.[5] The fluorescent properties of such probes depend upon the pH of the medium, the substituents on the coumarin label and the local microenvironment of the probe.[6,7] 4-Methyl umbelliferone (4-MU, **1**) and its derivatives have been widely used as molecular probes.[8] However, these probes lack functionality for covalent conjugation and require relatively high pH medium (pH ~9) to achieve maximum fluorescence intensity.[9] Our earlier study of umbelliferone based coumarin probes modified with a linker for conjugation[10] (**2**) revealed that an increase in the number of carbon atoms in the linker attached at the 4-position resulted in a decreased solubility in aqueous buffers. We found that a chain length of five or less carbons was optimal for bioconjugation. For 7-hydroxycoumarin, fluorescence is maximal at pH's above the pKa of the phenolic proton.[11] Lowering of the pKa of the coumarin phenolic proton should thus shift the useful fluorescence range towards neutral pH. Substitution at the 8-position of the coumarin nucleus with a functionality such as an amino methyl group (see **3**)[12] that can form a strong hydrogen bond with the phenolic hydroxy group should lower the pKa of the phenolic group. Indeed, Calcein Blue (**3**) and its derivatives display a lower pH optimum than 7-hydroxycoumarin deprived of an ortho substituted amino methyl group.[13,14] The strong chelation properties of Calcein Blue (**3**) to metal ions, its quenching of fluorescence by Cu^{++} and the homo-bifunctional nature limit its use for conjugation.[15]



1



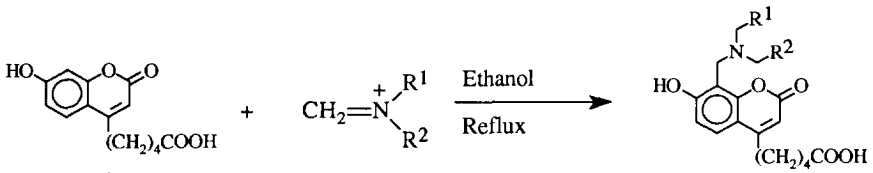
2



3

There is a need for new coumarin labels which display increased emission intensities relative to either **1** or **4** in the physiological pH range. Such labels must also display comparable quantum yields and incorporate functionality appropriate for covalent attachment to biomolecules. Thus, we present herein five new fluorescent 7-hydroxycoumarin labels (**6a–e**) and their physical and fluorescence properties.

Typically, 4-(4-carboxybutyl)-7-hydroxycoumarin (**4**)[10] in absolute ethanol was treated with 1 equiv. of freshly prepared Mannich reagent[16] (**5a–e**) and was refluxed for 3–4 h. The product (**6a–e,** Table)[17] precipitated after cooling to 0 °C and was purified by crystallization.



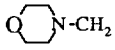
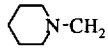
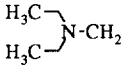
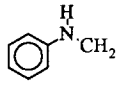
4 **5a–e** **6 a–e**

(**a**) R^1, R^2 = $-(CH_2)_2O(CH_2)_2-$, (**b**) R^1, R^2 = $-(CH_2)_5-$, (**c**) R^1, R^2 = $-(CH_2)_4-$
(**d**) R^1, R^2 = Et, (**e**) R^1= benzyl, R^2 = H;

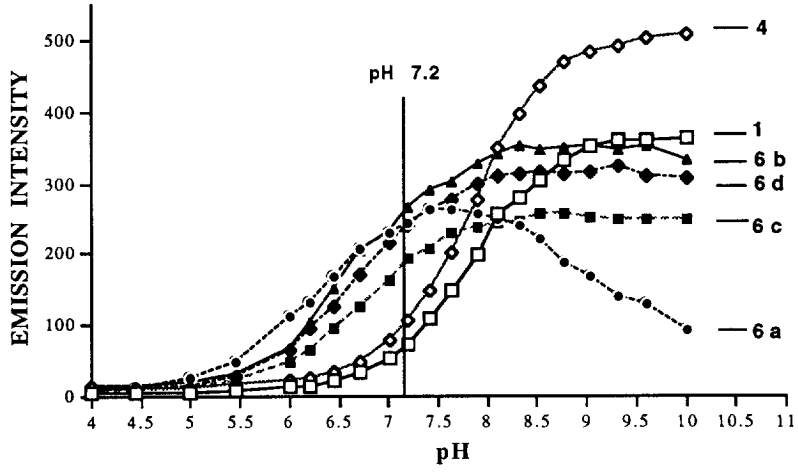
The physical properties, i.e., extinction coefficients, relative quantum yields, and pK_a values of each of the 7-hydroxy-8-dialkylaminocoumarins (**6a–d**) were determined. Molar extinction coefficients of compounds **6a–d** were determined at final concentrations ranging from 1×10^{-5} to 8×10^{-5} M in buffer at pH 10.[18] Fluorescence measurements were obtained at a final concentration of 1×10^{-7} M where self-quenching does not occur. The Figure shows the observed relationship between pH and emission intensity for these novel probes as compared to 4-MU (**1**) and 4-(4-carboxybutyl)-7-hydroxycoumarin (**4**). Analysis of the data shown in the Figure demonstrates the effect of substitution by an alkylaminomethyl group ortho to the phenolic hydroxyl in 7-hydroxycoumarins. The pH needed for high emission intensity was significantly lower for compounds **6a–d** than for either compound **1** or **4** (Figure). The 7-hydroxyl coumarins (**6b–d**) showed comparable quantum yields at pH 10. However, at the desired physiological pH 7.2, the Mannich derivatives **6a–d** all show a 4–5 fold higher fluorescence intensity than either compounds **1** or **4**. Thus, substituting the 8–position of 7-hydroxycoumarin by various aminomethyl derivatives resulted in lowering the pH required for attaining higher fluorescence.

In summary, we have synthesized several new 8-N-alkylaminomethyl-4-(4-carboxybutyl)coumarins (**6b–d**) that display higher relative quantum yield and lower pK_a values compared to 4-methylumbelliferone (**1**). As a consequence, higher fluroescence intensities are obtained at lower pH's with these new labels than for 4-methylumbelliferone (4-MU, **1**) and 7-hydroxy-4-(4-carboxybutyl)coumarin (**4**). The emission intensity at pH 7.2 is 4–5 times higher than either **1** or **4**. These properties should allow the labels to be utilized for binding studies of biomolecules near physiological pH.

Table. Physical properties of coumarin compounds **4** and **6a–e**

Comp	Substituent at 8-position	Extinction coefficient	Relative QY[a] (at pH 10)	pKa	Yield(%)[b]	m.p.[c] (°C)
1[d]	H	16408	0.7	7.8	-	-
4[e]	H	19030	0.82	7.8	86[e]	208–210
6a[f]		15565	d	d	42	173–175
6b		13155	0.80	6.6	45	115–116
6c		9137	0.82	6.7	48	154–155
6d		11225	0.83	6.6	28	164–165
6e[g]		g	g	g	48	158–160

a. **QY** = Quantum Yield. b. Isolated yield. c. Melting points were determined on product crystallized from ethanol. d. See ref 9. e. See ref 10. f. pKa and QY could not be determined due to a sharp decline in relative emission intensity at higher pH's. g. Data could not be collected due to poor solubility.



Figure

References and Notes

1. Rousseau, D. L. In*Optical Techniques in Biological Research* ; Academic: New York, 1984; Chapter 4.
2. (a) Mattingly, P. G. *Bioconjugate Chem.* **1992**, *3*, 430. (b) Adamczyk, M.; Fishpaugh, J.; Harrington, C.; Johnson, D.; Vanderbilt, A. *J. Immunol. Methods* **1993**, 187. (c) Adamczyk, M.; Fishpaugh, J.; Harrington, C.; Hartter, D.; Johnson, D.; Vanderbilt, A. *J. Immunol. Methods* **1993**, *162*, 47. (d) Fino, J. R.; Shipchandler, M.T.; Klein, L. D.; Kirkemo, C. L. *Anal. Biochem.* **1987**, *162*, 89.
3. (a) Thrieringe, R.; Shio, H.; Han, Y. S.; Cohen, G.; Lazarow, P. B. *Mol.Cell Biol.* **1991**, *11*, 510. (b) Lacowicz, J. R. *Principles of Fluorescence and Spectroscopy* ; Plenum: New York, 1983.
4. Haughlund, R. P. *Handbook of Fluorescent Probes* ; 1992.
5. (a) Forster, Y.; Haas, E. *Anal. Biochem.* **1993**, *209*, 9. (b) Corrie, J. E. T. *J. Chem. Soc. Perkin Trans. 1* , **1994**, 2975. (c) Kuzetsova, N. A.; Kaliya, O. L. *Russian Chem. Rev.* **1992**, *61*, 683; translated from *Uspehki Khimii* **1992**, *61*, 1243.
6. Haroutounian, S. A.; Katzenellenbogen, J. A. *Tetrahedron* **1995**, *51*, 1585.
7. Musgrove, E.; Rugg, C.; Hedley, D. *Cytometry* **1986**, *7*, 347.
8. Leonard, N. J.; Thomas, R. W. *Heterocycles* **1976**, *5*, 839.
9. Rel. Quantum Yield = 0.7,[19] Extinction Coefficient = 16408 $M^{-1}cm^{-1}$ at pH 10.0 and pKa = 7.8
10. Adamczyk, M.; Mattingly, P. G.; Pan, Y.; Rege, S. *Org. Prep. and Proc. Int.* **1996**, *28*, 627.
11. Haughlund, R.P. *Handbook of Fluorescent Probes* ; 1992, pp 82–85.
12. Mehta, R. H.; Vyas, R.; Shah, S. *J. Ind.Chem.Soc.* **1991**, *68*, 411 and references cited therein.
13. Murray, G. I.; Ewen, S. W. B. *J. Histochem. Cytochem.* **1992**, *40* , 1971.
14. Zao, S.; Aodeng, G.; Sai, Y. *Fenxi Shiyanshi* **1995**, *14*, 45.
15. Huitink, G. M.; Poe, D. P.; Diehl, H. *Talanta* **1974**, *21*, 1221.
16. A 25 mL round bottom flask with a magnetic stir bar was flame dried. Paraformaldehyde (1.2g, 13.3 mmol) (equiv to 40 mmol of formaldehyde) in absolute ethanol (10 mL) and KOH (100 mg) were added. The solids were dissolved by warming the flask (40–45 °C). The appropriate amine (40 mmol) was added dropwise to the reaction while cooling with an ice bath. The reaction was stirred for 1 h and was diluted with absolute ethanol (30 mL) to make a 1 mM stock solution of the Mannich reagent (**5a–e**).
17. The coumarin acid (**4**) (1 mmol) was dissolved in absolute ethanol (4 mL) in a 25 mL round bottom flask. The freshly prepared Mannich reagent (1 mL, 1 mmol) was added. The reaction was heated to reflux under N_2 for 3 h. After cooling to 0 °C, the precipitated solid was filtered off and washed with cold ethanol (5 × 10 mL). The product was recrystallized from boiling ethanol. All compounds gave satisfactory elemental analysis, NMR and MS.
18. The compounds had to be dissolved in either THF, DMSO or in both before diluting to the appropriate concentration. **4** in THF (150 μL), **6a** in DMSO:THF (80:80 μL), **6b** in THF (250 μL), **6c** in DMSO:THF (80:80 μL), **6d** in DMSO:THF (105 μL:100 μL). The quantum yield of the fluorophores relative to 4-methylumbelliferone (**1**)[19] was determined using the expression shown below.[20] $\mathbf{Q_F}$(sample)/$\mathbf{Q_F}$(standard) = **I**(sample)/**I**(standard) × ε(standard)/ε(sample) where $\mathbf{Q_F}$=Quantum yield, **I**(sample) and **I**(standard) refer to integrated emission intensities over all wavelengths of the sample and standard, and ε is the extinction coefficient, respectively. pKa determination: Fluorescence vs pH data were fit to sigmoidal 4-parameter logistic curve (4-PLC) and the inflection point was determined by standard calculations.[21]
19. Chen, R. F. *Anal. Lett.* **1968**, *1*, 423.
20. Voss Jr., E. W. In *Fluorescein Hapten: An Immunological Probe* ; CRC, 1984.
21. Myers, R. H. In *Classical and Modern Regression with Applications* ; 2nd ed; DWS-Kent: Boston, 1990.

(*Received in USA* 12 *June* 1997; *accepted* 26 *June* 1997)