Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2229–2232

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

7-AMINO-4-METHYL-6-SULFOCOUMARIN-3-ACETIC ACID: A NOVEL BLUE FLUORESCENT DYE FOR PROTEIN LABELING

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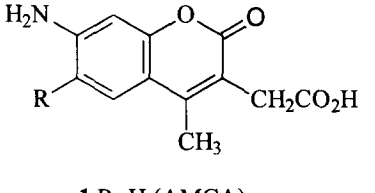
Received 10 May 1999; accepted 25 June 1999

Abstract: 7-Amino-4-methyl-6-sulfocoumarin-3-acetic acid (AMCA-S, also called Alexa™ 350) **2** was synthesized as a new water-soluble blue fluorescent dye for protein labeling. Compared with its nonsulfonated counterpart (AMCA) **1** the new dye gave significantly higher fluorescence quantum yields on proteins.

Coumarin derivatives have been widely used as fluorescent labels[1] and to prepare fluorogenic substrates for the study of enzyme activities.[2] The bright blue fluorescence emission of coumarin dyes provides a contrasting color that is easily distinguished from the green fluorescein and red rhodamine derivatives, thus allowing multicolor fluorescence applications, which includes immunohistochemistry, *in situ* hybridization and cell tracing. Among the coumarin compounds, 7-amino-4-methylcoumarin-3-acetic acid (AMCA) **1** has been used extensively for preparing blue fluorescent conjugates of proteins and nucleic acids.[3] The dye can be optimally excited at 350 nm and emits near 435 nm. However, like most of the other commonly used fluorescent dyes,[4] AMCA has significant fluorescence quenching when conjugated to proteins. Furthermore the relatively low water solubility of its succinimidyl ester makes AMCA inconvenient to use for preparing bioconjugates in aqueous media.

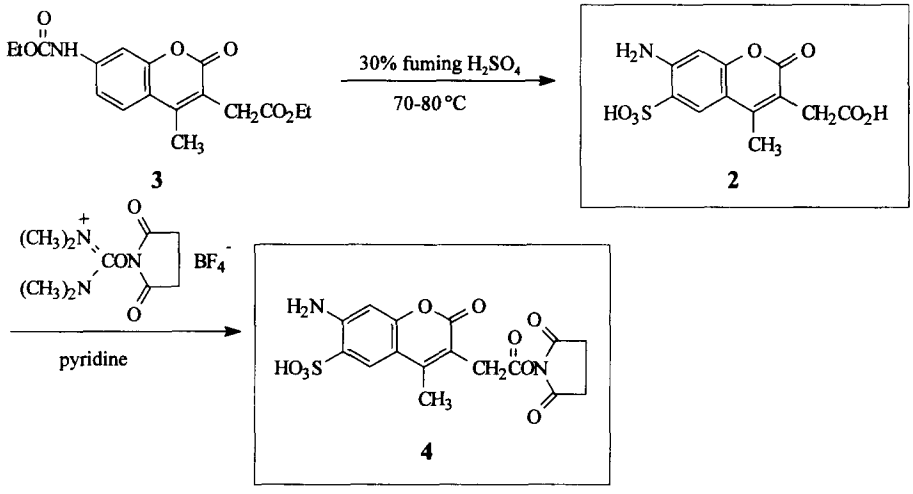


1 R=H (AMCA)
2 R=SO_3H (AMCA-S)

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00364-9

In this paper, we report the synthesis of 7-amino-4-methyl-6-sulfocoumarin-3-acetic acid (AMCA-S) **2**, the sulfonated derivative of **1**. The presence of the sulfonate group allows protein labeling in water or buffer solution without the addition of an organic cosolvent. Furthermore, the protein conjugates from **2** have significantly enhanced fluorescence quantum yields compared to those made from **1**.

The synthesis of AMCA-S and its succinimidyl ester **4** is outlined in Scheme 1. Sulfonation of the ethyl ester of 7-carboethoxyamido-4-methylcoumarin-3-acetic acid **3**[3b] with 30% fuming sulfuric acid at 70–80 °C gave **2**[5] in 20–30% yield after aqueous workup and preparative HPLC purification.[6] The succinimidyl ester **4**[5] was prepared from **2** and N,N,N',N'-tetramethylsuccinimidouronium tetrafluoroborate in pyridine, which acts both as a base and a solvent to give a yield of greater than 90%.[7]



Scheme 1

The fluorescence spectra of **1** and **2** are shown in Figure 1. While the absorption wavelength of AMCA-S is only slightly shifted to longer wavelengths by 5 nm compared with that of AMCA [Table 1], the fluorescent emission maxima of **1** and **2** are nearly identical. In addition **2** has a significantly higher extinction coefficient than **1** and this makes **2** a brighter dye due to increased efficiency of excitation.

Table 1. Spectral Data of the Coumarin Dyes

Dye	λ_{abs} (nm)[a]	λ_{em} (nm)[a]	ε (L/mol/cm)
1	345	435	15,500
2	350	434	20,100

[a]Measured in methanol

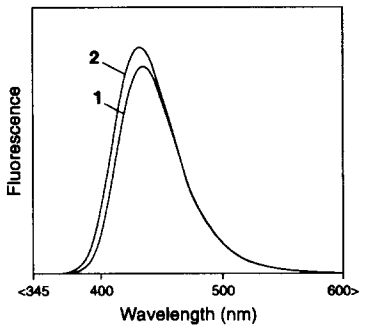


Figure 1. A comparison of the fluorescence spectra of **1** and **2** in methanol, recorded at equal dye concentrations.

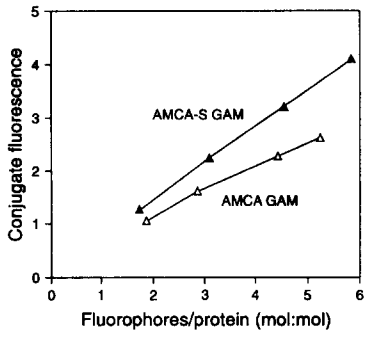


Figure 2. A comparison of total fluorescence of goat anti-mouse (GAM) conjugates of AMCA-S and AMCA. Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein.

Although the free dye **2** is only slightly more fluorescent than **1** (Figure 1), the conjugate fluorescence of the AMCA-S conjugate of goat anti-mouse (GAM) antibody is about 45% higher than the fluorescence of AMCA conjugate at 5 fluorophores/GAM, an optimal degree of substitution in protein labeling (Figure 2). The decreased quenching of fluorescence of the sulfonated AMCA-S dye relative to the nonsulfonated AMCA dye is similar to that observed in the sulfonated carbocyanine compounds.[8] Furthermore, even there is a bathochromic shift in the emission of **1** and **2** upon binding to the proteins, the AMCA-S conjugates (442 nm) have slightly shorter wavelength emission maxima than AMCA conjugates (448 nm). Thus the fluorescence of AMCA-S conjugates is much better separated from the other commonly used green fluorophores in the multicolor fluorescence applications.

In conclusion, we have synthesized a novel water-soluble and much brighter blue fluorescent dye **2** that should be useful for labeling proteins, nucleic acids and other biomolecules.

Acknowledgment: We thank Brian Hoyland for the preparative HPLC purification of **2**.

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5. **2**:^{1}H NMR (400 MHz, DMSO-d_6 with D_2O exchange): δ 7.84 (s, 1H), 6.49 (s, 1H), 3.4 (s, 2H), 2.34 (s, 3H). Anal calcd for $C_{12}H_{11}NO_7S{\cdot}0.25H_2O$: C, 45.35; H, 3.62; N, 4.41. Found: C, 45.29; H, 3.71; N, 4.37.
4:^{1}H NMR (400 MHz, DMSO-d_6 with D_2O exchange): δ 7.83 (s, 1H), 6.52 (s, 1H), 3.97 (s, 2H), 2.78 (s, 4H), 2.32 (s, 3H).

6. Preparative HPLC was performed on a Waters Prep LC2000 instrument equipped with a C18 reverse phase column 250 x 50 mm.

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