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3-Carboxy-6-chloro-7-hydroxycoumarin: A highly fluorescent, water-soluble violet-excitable dye for cell analysis

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ABSTRACT

In our search for new violet-excitable dyes with improved photophysical and photochemical properties, we examined several halogen-substituted hydroxycoumarins and found that chlorinated derivatives are at least as bright as their fluorinated analogs. A monochlorinated hydroxycoumarin was found to have a high quantum yield (~0.98), and human leucocyte-specific monoclonal antibodies (CD3, CD4, and CD45) conjugated with this dye exhibited reliable performance in flow cytometry assays. Additional studies were performed, with BD Horizon V450–antibody conjugates being included in eight-color cocktails aimed at subsetting lymphocytes and myeloid cells. Such cocktails can frequently be unstable due to the tendency of one or more components to lose structural integrity, photobleach, or develop unwanted affinities for another component. However, the cocktails employed in this study enabled several different applications to be run and established that multicolor reagent mixtures containing V450–antibody conjugates are functional and stable.

Coumarin is a naturally occurring benzopyran found in plants. Many of its derivatives are highly fluorescent. The 7-amino-4-methyl-coumarin derivatives were widely studied during the 1970s and 1980s and were found to be useful for labeling biological molecules. For example, the three-acetic acid derivative known as AMCA (7-amino-4-methylcoumarin-3-acetic acid)[1] (Fig. 1), which has a carboxyl group available for activation and an excitation maximum at 350 nm, became a popular ultraviolet (UV)-excitable fluorescent probe for labeling proteins [1].

AMCA and its more recent descendant, Alexa Fluor 350, exhibit an intense blue fluorescence with a narrow emission peak in the range of 440–460 nm and have exceptional photostability; compared with fluorescein, AMCA is able to tolerate illumination more than three times longer. Moreover, the fluorescence intensity of AMCA is not affected by changes in pH over the range of 3–10. This is in marked contrast to other organic dyes, such as fluorescein isothiocyanate (FITC), that display considerable variability in their fluorescence intensities with pH [2–4].

Although the emission peak of AMCA does not overlap with the emission peaks of other major fluorescent probes and its quantum yield (QY) is not unusually low, its excitation maximum falls in an area of high cellular autofluorescence, sometimes making it appear to be dim relative to the background. This is due to the nature of cellular autofluorescence, which is largely the result of excitation of naturally occurring components such as flavins and nicotinamide adenine dinucleotide (reduced form) (NADH). These compounds are excited most efficiently in the UV region, so the laser used to excite AMCA also produces significant fluorescence from unstained cells. The emission characteristics of this autofluorescence are broad throughout the visible spectrum (e.g., blue, green, yellow, red) such that virtually none of the common dichroic mirror and bandpass filter combinations used in flow cytometry detection is able to eliminate it. The impact of this broad autofluorescence on flow cytometry becomes apparent with a densely expressed marker such as CD8 on mouse T-cells, when the signal-to-noise ratio of CD8 AMCA is significantly lower than that of CD8 FITC, because the UV laser used to excite AMCA produces much higher autofluorescence than the blue laser used to excite FITC [5].

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[1] *Abbreviations used:* AMCA, 7-amino-4-methylcoumarin-3-acetic acid; UV, ultraviolet; FITC, fluorescein isothiocyanate; QY, quantum yield; NADH, nicotinamide adenine dinucleotide (reduced form); DMF, dimethylformamide; mAb, monoclonal antibody; MWCO, molecular weight cutoff; NHS, *N*-hydroxysuccinimide; D/P, dye-to-protein ratio; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FSC, forward light scatter; SSC, 90° orthogonal light scatter; PMT, photomultiplier tube; HPLC, high-performance liquid chromatography; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin.

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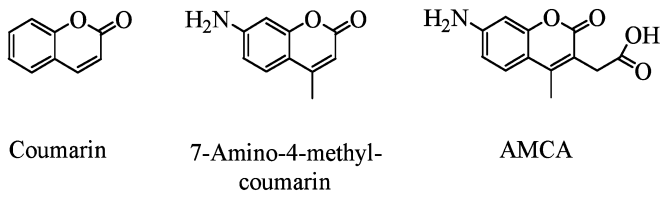

Fig. 1. Structures of coumarins and AMCA.

During the 1990s, when the 405-nm violet laser became readily available, new violet-excitable derivatives of 7-hydroxycoumarin were developed and hydroxy-substituted coumarins were widely used in the preparation of fluorescent protein conjugates and enzyme substrates [6,7]. Various derivatives of 7-hydroxycoumarin conjugated to enzyme substrates were used for assays of phosphatases, β-galactosidases, and β-lactamases [8]. The parent compound (3-carboxy-7-hydroxycoumarin) has an excitation maximum at 386 nm, and the pK_a of its phenolic hydroxyl group is approximately 7.5. In the physiological pH range of 6–8, the dye molecules are not fully deprotonated and, therefore, do not exhibit their maximum fluorescence intensity. Sun, Haugland, and Gee examined the effect of fluorination of 7-hydroxycoumarins on their photochemical properties and demonstrated that fluorinated versions such as 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Fig. 2) have higher QY values [9,10]. Moreover, fluorination of the phenolic ring elicits a bathochromic shift in excitation wavelength. Excitation maxima of halogenated alternatives are shifted to around 400 nm, allowing them to be excited in a region where cellular autofluorescence is lower. As a result, these new violet-excitable coumarins, when conjugated to antibodies, gave much better signal-to-noise ratios than UV-excitable aminocoumarins such as AMCA and Alexa 350 [11].

In our search for new violet-excitable dyes with improved photophysical and photochemical properties, we examined several halogen-substituted hydroxycoumarins and found that chlorinated derivatives are at least as bright as their fluorinated analogs. In particular, monochlorinated hydroxycoumarin (BD Horizon V450) has a QY equal to 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue), which was considered to be the brightest hydroxycoumarin for labeling proteins. We tested five V450–antibody conjugates in multicolor flow cytometry assays designed to identify subsets of lymphocytes and myeloid cells.

Materials and methods

Synthesis of monochlorinated hydroxycoumarin (V450)

6-Chloro-4-formylresorcinol (7 g), dimethylmalonate (6.5 g), 0.5 ml of piperidine, and 0.3 ml of acetic acid were heated under reflux for 3 h in 100 ml of methanol. After cooling to room temperature, the mixture was filtered and the filtrate was concentrated. The concentrated filtrate was poured into water, and resulting precipitate was filtered off with suction to collect the solid, which was air-dried. The crude product was further purified with silica gel chromatography to yield the desired 6-chloro-7-hydroxy-2-methoxycarbonylcoumarin.

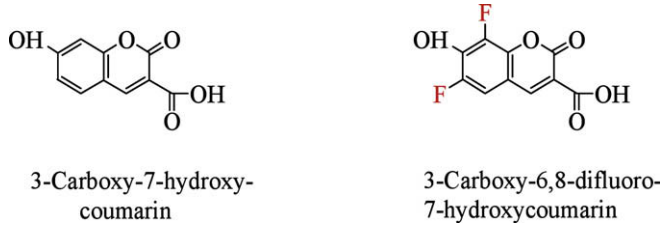

Fig. 2. Structures of 3-carboxy-7-hydroxycoumarin and its difluoro-substituted alternative.

6-Chloro-7-hydroxy-2-methoxycarbonylcoumarin (5 g) was dissolved in methanol (50 ml). To the methanol solution was added 6 N HCl (10 ml). The resulting solution was heated at 60–65 °C until the starting material was completely consumed. After cooling to room temperature, the mixture was filtered and the filtrate was concentrated. The concentrated filtrate was poured into water, and the resulting precipitate was filtered off with suction to collect the solid, which was washed with water and air-dried. The crude product was further purified by recrystallization of methanol–water to yield the desired 6-chloro-7-hydroxy-2-carboxycoumarin.

6-Chloro-7-hydroxy-2-carboxycoumarin (1 g) and *N*,*N*′-disuccinimidyl carbonate (1.6 g) were dissolved in dimethylformamide (DMF, 10 ml). To the DMF solution was added triethylamine (1.2 ml) and 4-dimethylaminopyridine (10 mg). The resulting solution was stirred at room temperature until the starting material was completely consumed. The mixture was filtered and the filtrate was concentrated. The concentrated filtrate was poured into water, and the resulting precipitate was filtered off with suction to collect the solid, which was washed with water and air-dried to yield the desired V450 protein labeling compound.

Absorption and emission spectra

Succinimidyl esters of 3-carboxy-6,8-difluoro-7-hydroxycoumarin (Pacific Blue) and V450 were reacted with 1 M glycine at pH 9.6 to stabilize their absorption maxima.

Fluorescence quantum yield

Fluorescence QY of glycinated V450 at different pH values was determined using quinine sulfate (Sigma–Aldrich) as a reference standard and literature values (0.51 in 50 mM sulfuric acid) for quantum efficiency [12]. All readings were taken at ambient temperature. V450–glycine or V450–antibody conjugates were serially diluted in 5-mM sodium phosphate buffers with $A_{375} \leqslant 0.05$, and quinine sulfate was serially diluted (with $A_{375} \leqslant 0.1$) in 50 mM sulfuric acid. Absorbance was measured in 1-cm cuvettes on an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies) at 375 nm, and corrected emission spectra over the ranges of 385–650 nm for quinine sulfate and 400–600 nm for V450 were recorded on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon) in 1-cm cuvettes using excitation at 375 nm. A set of emission correction factors, determined by the manufacturer of the Fluorolog-3 and valid over the range of 290–850 nm, was used by FluorEssence software to produce the spectra used in the QY determination. No attempt was made to control for polarization bias in the monochromators. QY was determined from data plotted as integrated fluorescence intensities (calculated by Microcal Origin software) versus absorbance for both quinine sulfate and V450. The resulting V450 QY is the ratio between the slopes of the resulting reference and sample curves multiplied by the known QY of quinine sulfate.

Titration scale labeling of antibody over a range of dye molar excesses

Monoclonal antibody (mAb) was buffer exchanged with labeling buffer on spin columns (Bio-Spin 30, Bio-Rad Laboratories) and concentrated to 2–10 mg/ml on Vivaspin 50-kDa molecular weight cutoff (MWCO) or equivalent centrifugal concentrators if necessary. The *N*-hydroxysuccinimide (NHS) ester of V450 or Pacific Blue dye was dissolved in dimethyl sulfoxide, and its concentration was determined spectrophotometrically. The dyes were