

Synthesis and Photochemical Properties of a New Water-Soluble Coumarin, Designed as a Chromophore for Highly Water-Soluble and Photolabile Protecting Group

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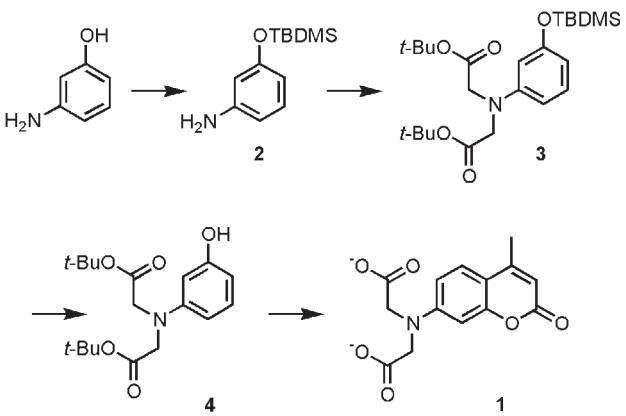
The absorption spectra and fluorescence spectra of a coumarin derivative (**1**), designed to be a water-soluble photolabile protecting group for caged compounds, were successfully observed in aqueous buffer solutions at pH 2.0–12.5 for the first time. Coumarin **1** is highly soluble (>5 mM) in aqueous buffer at pH 7.2 and is stable in the dark at room temperature for up to a week. The pK_a value for the protonation of anilino nitrogen in coumarin **1** was determined to be 4.5. Coumarin **1** has a fluorescence lifetime of 2.0 ns and undergoes intersystem crossing to the triplet state. Therefore, compound **1** can be used for the light absorbing part of the new water-soluble caged compounds, such as caged amino acids or caged nucleotides, which can release free amino acids or nucleotides, respectively, by excitation in the visible region.

Photochemical properties of conjugated molecules have been extensively studied in organic solvents. Solvent properties, such as polarity and hydrogen-bonding ability, sometimes affect the properties of molecules both in the ground state and excited state. However, photochemistry in water has hardly been studied because many organic molecules do not dissolve in water.

In the course of our study on the photochemistry in water,[1–5] we have come to study caged compounds soluble in water. Although a lot of studies concerning caged compound have been done, quite a few have been reported on a water-soluble photolabile protecting group. In this respect, we are synthesizing a new chromophore for caged compounds that is capable of uncaging carboxylic acid and phosphoric acid in water.

The points for the development of a new chromophore for photolabile protecting group in caged compound are: 1) sufficient solubility and stability in water at neutral pH and 2) high quantum yield for photolysis by excitation in the visible wavelength region. The latter is closely related to the lifetime for the singlet excited state. It is also advantageous if the photolysis proceeds in the triplet excited state, because the radical recombination is not efficient to result in enhancing the quantum yield for photolysis.

Coumarin derivatives have been successfully employed as a photolabile protecting group in caged compounds because of their favorable properties such as rapid photolytic release with high efficiency, stability under physiological conditions, and large extinction coefficients at longer wavelength.[6–11] Among coumarin-based caged compounds, 7-diethylamino-4-methylcoumarin (DECM) caging group showed significant properties such as high efficiency of photolysis in the visible wavelength region.[12–14] However, DECM itself is not soluble in water. Insolubility in water is an obvious problem for caging group, especially when the biologically active molecule does not have strong hydrophilic groups either. In this respect, we designed a new coumarin derivative **1** (Scheme 1), being designed as a water-soluble version of DECM, which has a dialkylamino group at the 7-position and two carboxyl groups to make it water soluble. When our studies have almost finished, we come to realize the report of the use of coumarinylmethyl esters for the caging of phosphate and other groups.[15] However, there is no report on the photochemical properties of coumarin derivative in pure water. Therefore, it is worthwhile to report the photophysical features of coumarin derivatives in pure water. We now wish to report the synthesis of coumarin **1** and the studies of photochemical and photophysical properties in water.



Scheme 1.

Experimental

Materials. Coumarin **1** was synthesized by the four steps as shown in Scheme 1.

Compound **2**: A mixture of 3-aminophenol (6.98 g, 64 mmol), imidazole (10.9 g, 160 mmol) and *tert*-butyldimethylchlorosilane (TBDMS) (11.6 g, 77 mmol) in DMF (56 mL) was stirred for 2 h at 0 °C under nitrogen. A saturated aqueous solution of $NaHCO_3$ (100 mL) was then added and the solution was extracted three times with ether. The organic layer was collected, washed with

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brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by flash chromatography (SiO_2, hexane–AcOEt 5:1) to give 8.4 g of **2** in 66% yield. ^{1}H NMR ($CDCl_3$) δ 6.98 (t, $J = 8.0$ Hz, 1H), 6.30–6.18 (m, 3H), 3.58 (s, 2H), 0.97 (s, 9H), 0.18 (s, 6H).

Compound **3**: A mixture of **2** (4.13 g, 19 mmol), ethyldiisopropylamine (9.56 mL, 56 mmol), *tert*-butyl bromoacetate (6.14 mL, 42 mmol), and sodium iodide (8.32 g, 56 mmol) in acetonitrile was refluxed for 18 h under nitrogen. The solution was poured into water and was extracted with dichloromethane. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was evaporated. The residue was purified by flash chromatography (SiO_2, hexane–AcOEt 7:1) to give 6.82 g of **3** in 80% yield; ^{1}H NMR ($CDCl_3$) δ 7.03 (t, $J = 8.0$ Hz, 1H), 6.25–6.07 (m, 3H), 3.97 (s, 4H), 1.46 (s, 18H), 0.97 (s, 9H), 0.17 (s, 6H).

Compound **4**: To a solution of **3** (5.35 g, 12 mmol) in THF–ethanol (10:1, 110 mL) was added 1.0 M THF solution of tetrabutylammonium fluoride (17.7 mL). After stirring for 2 h under nitrogen, water was added, and the solution was extracted with dichloromethane. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was evaporated, followed by purification by flash chromatography (SiO_2, hexane–AcOEt 5:1) to give 3.45 g of **4** in 87% yield; ^{1}H NMR ($CDCl_3$) δ 7.00 (t, $J = 8.0$ Hz, 1H), 6.20–6.06 (m, 3H), 3.97 (s, 4H), 1.45 (s, 18H).

Coumarin **1**: To a mixture of **4** (0.74 g, 2.2 mmol) and ethyl 3-oxobutanoate (0.29 g, 2.2 mmol) was added *p*-toluenesulfonic acid monohydrate (20.3 mg, 0.11 mmol). The mixture was stirred at 60 °C for 24 h under nitrogen. After cooling to room temperature, AcOEt (25 mL) and hexane (10 mL) were added to the mixture to precipitate of the crude product, and the supernatant was removed. The residue was purified by recrystallization from mixed solvent of ethanol and hexane (5:1) to give **1** (0.25 g) in 39% yield. ^{1}H NMR (CD_3OD) δ 7.64 (d, $J = 4.2$ Hz, 1H), 6.73–6.70 (m, 1H), 6.53 (d, $J = 2.4$ Hz, 1H), 6.07 (s, 1H), 4.31 (s, 4H), 2.44 (s, 3H); ^{13}C NMR (CD_3OD) δ 175.0, 165.1, 157.4, 156.9, 153.7, 128.1, 113.0, 111.5, 111.1, 100.4, 55.1, 19.4. Anal. Calcd for $C_{14}H_{13}NO_6$: C, 57.73; H, 4.50; N, 4.81%. Found: C, 57.86; H, 4.77; N, 4.61%.

Measurements. The ^{1}H and ^{13}C NMR spectra were measured with a Bruker ARX-400 (400 MHz for ^{1}H NMR) and Bruker AVANCE 500 (125 MHz for ^{13}C NMR) spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard. The UV absorption and fluorescence spectra were recorded on a Shimadzu UV-1600 UV–visible spectrophotometer and on a Hitachi F-4500 fluorescence spectrometer, respectively. Fluorescence lifetimes were determined with Horiba NAES-1100 time-resolved spectrofluorometer. Laser flash photolysis was performed by using an excimer laser (Lambda Physik LPX-100, 308 nm, 20 ns fwhm) as the excitation light source and a pulsed xenon arc (Ushio UXL-159) as a monitoring light source. A photomultiplier (Hamamatsu R-928) and a storage oscilloscope (LeCroy LT264) were used for the detection.

Results

Synthesis. Direct *N*,*N*-dialkylation of 7-amino-4-methylcoumarin was not successful probably because of the low reactivity of anilino nitrogen due to the electron-withdrawing effect of carbonyl group on coumarin ring. Instead of 7-amino-4-methylcoumarin, 3-aminophenol was used as a starting material. The four step synthesis, including the protection of the hydroxy group,[16] *N*,*N*-dialkylation, deprotection,[17] and final coumarin synthesis,[18] proceeded smoothly to give coumarin **1** in 18% overall yield, as described in Scheme 1.

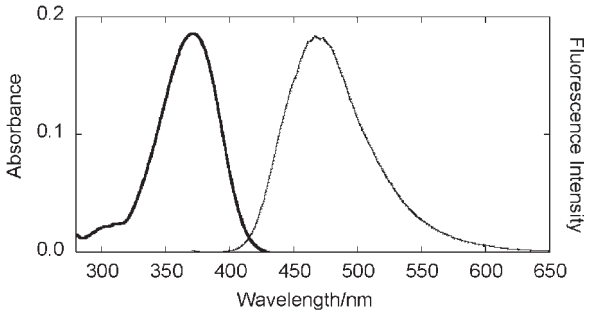

Fig. 1. Absorption and fluorescence spectra of coumarin **1** (1.02×10^{-5} M) in HEPES buffer at pH 7.2.

Steady State Absorption and Fluorescence Spectra. For the absorption and the fluorescence measurements, a McIlvaine buffer solution[19] was used in the range of pH 2.0 to 6.4, a HEPES buffer solution (HEPES 40 mM, KCl 100 mM) was used for pH at 7.2, and a KCl buffer solution (KCl 100 mM) was used in the range of pH 6.4 to 12.5. Coumarin **1** is soluble (>5 mM) in HEPES buffer at pH 7.2. Figure 1 shows the UV absorption and fluorescence spectra of coumarin **1** in HEPES buffer solution at pH 7.2. The absorption maximum of coumarin **1** was at 371 nm with the extinction coefficient of 17600 M^{-1} cm^{-1}. The fluorescence spectrum was observed in the same buffer with the peak at 472 nm. The value of the Stokes shift of coumarin **1** was 5800 cm^{-1}. The fluorescence band did not depend on the excitation wavelength at pH 7.2. The stability of coumarin **1** in the neutral buffer was tested by monitoring the absorption spectra. The HEPES buffer solution of coumarin **1** (0.5×10^{-6} M) at pH 7.2 was kept in the dark at room temperature under air. After a week, the solution gave the identical absorption spectra to that of a freshly prepared solution, indicating that coumarin **1** is stable in aqueous neutral buffer in the dark at room temperature for a week.

The pK_a value for the protonation of the anilino nitrogen in coumarin **1** was determined by monitoring the absorption spectra (Fig. 2) in a McIlvaine buffer solution[19] at various pH (pH 2.0–6.4). The UV absorption spectra of coumarin **1** largely changed between pH at 6.0 and 3.0, where the intensity of the absorption band at 353 nm decreased and that at 370 nm increased with increasing pH. The pK_a value was calculated to be 4.5. The absorption spectra slightly changed in the acidic solution between pH 3.0 and 2.0, probably because the protonation of the two carboxylic acid moieties in coumarin **1** affects its absorption spectra. However, the exact pK_a values for the two carboxylic acids could not be determined. Coumarin **1** shows stability and solubility in acidic conditions even at pH 2.0. The absorption spectra of coumarin **1** did not change in the range of pH 6.4 to 12.5 in KCl buffer, indicating that coumarin **1** takes a single form in the ground state in aqueous buffer between pH 6.4 and 12.5.

The fluorescence spectra of coumarin **1** were also pH dependent. The emission band with the maximum at 440 nm at pH 2 was ascribed to the protonated form (Fig. 3a). At pH 5, the fluorescence bands from both the protonated and non-protonated form were observed, depending on the excitation wavelength (Fig. 3b). The fluorescence spectrum of cou-

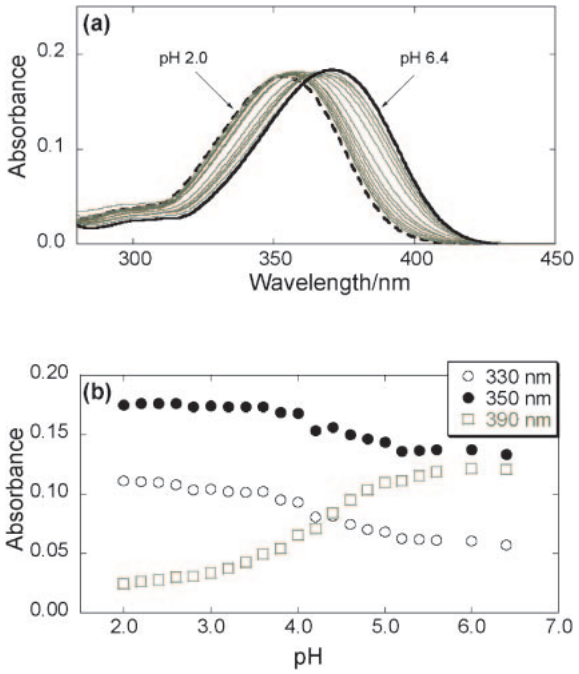

Fig. 2. (a) Absorption spectra of **1** in McIlavine buffer at various pH (dotted line: pH 2.0, solid line: pH 6.4, and thin lines: pH 2.4–6.0) at room temperature. (b) Plots of the absorbance for **1** at 330 nm (○), 350 nm (●), and 390 nm (□) as a function of pH.

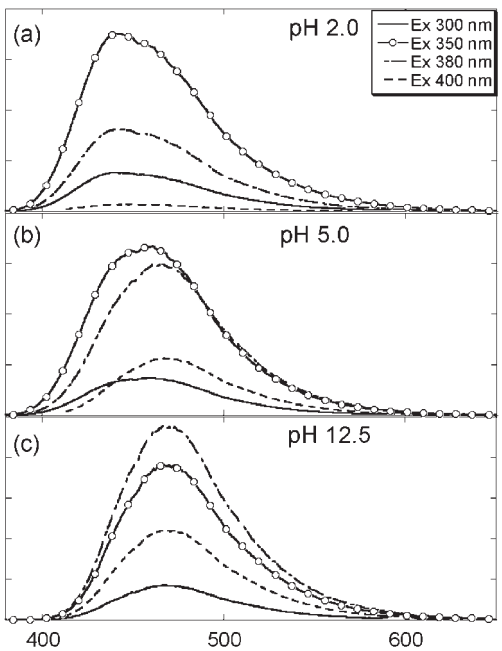

Fig. 3. Fluorescence spectra of coumarin **1** in HEPES buffer (1.02×10^{-5} M) at pH 2.0 (a), at pH 5.0 (b), and at pH 12.5 (c).

marin **1** at pH 12.5 was almost identical to that at pH 7.2. The values of the fluorescence quantum yield in buffer at pH 7.2 were determined to be 0.32. The fluorescence lifetime of coumarin **1** was 2.0 ns.

Transient Absorption Spectra. The transient absorption spectra of **1** were measured in HEPES buffer at pH 7.2. On irradiation at 308 nm light under argon atmosphere, a weak band was observed in the range of 500–700 nm (Fig. 4). The lifetimes of the transient species were 15 μs under argon and 2.1 μs under air. The quenching rate constant by oxygen was calculated to be 1.4×10^9 M^{-1} s^{-1}. These results suggest that the transient band is ascribed to the triplet excited state.

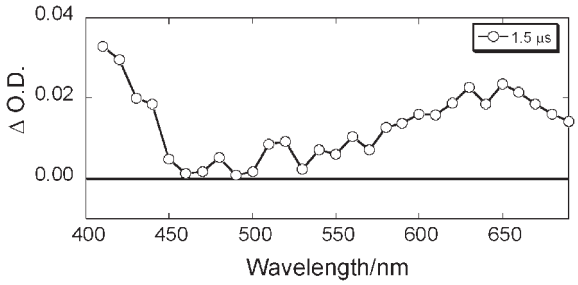

Fig. 4. Transient absorption spectra of coumarin **1** in HEPES buffer solution under argon at pH 7.2.

Discussion

A water-soluble coumarin **1** was synthesized in 18% overall yield from readily available starting materials. Some of the characteristics of coumarin **1**, as a chromophore for caged compounds, are mentioned below.

(1) Solubility in aqueous buffer solution: In general, caged compounds consist of a biological active molecule and a chromophore, or a photolabile protecting group. Coumarin derivatives have already been used as chromophores for cage nucleotides, such as ATP, ADP, and amino acid, such as L-glutamic acid, mainly because of their sufficient quantum yield for photolysis and large extinction coefficient at longer wavelength region (300–400 nm). In many cases, however, the coumarin moiety in a caged compound is hydrophobic or weakly hydrophilic. For example, although DECM-caged compounds exhibited rapid and efficient photolysis, DECM itself did not dissolved in aqueous buffer at pH 7.2. This result indicates that the water-solubility of caged compounds is essentially determined by the hydrophilicity of the biologically active compound part, solubility may be a problem when the biologically active compounds are not strongly hydrophilic. Coumarin **1** has a solubility of more than 5 mM in aqueous buffer at pH 7.2, which good for use with caged compounds because (1) the caged compounds can be used at higher concentrations, and (2) it can inhibit hydrophobic interactions with proteins, lipids in membranes, or even self assembly, in aqueous solution. Furthermore, coumarin **1** did not precipitate even under acidic conditions (pH 2.0), probably because of the protonation of the anilino nitrogen below pH 4.5. Coumarin **1** also is stable in the dark at room temperature in buffer at pH 7.2. From these results, coumarin **1** is a good candidate for use as a caging group not only for hydrophilic biological active compounds but also hydrophobic compounds without solubility problems.

(2) Photochemical properties: Coumarin **1** was designed as a water-soluble version of DECM. Since DECM-caged compounds have large extinction coefficients around 370–380 nm and undergo efficient photolysis, coumarin **1** must have similar photochemical properties, i.e., the caged compounds with coumarin **1** must be photolizable with high efficiency. In a steady-

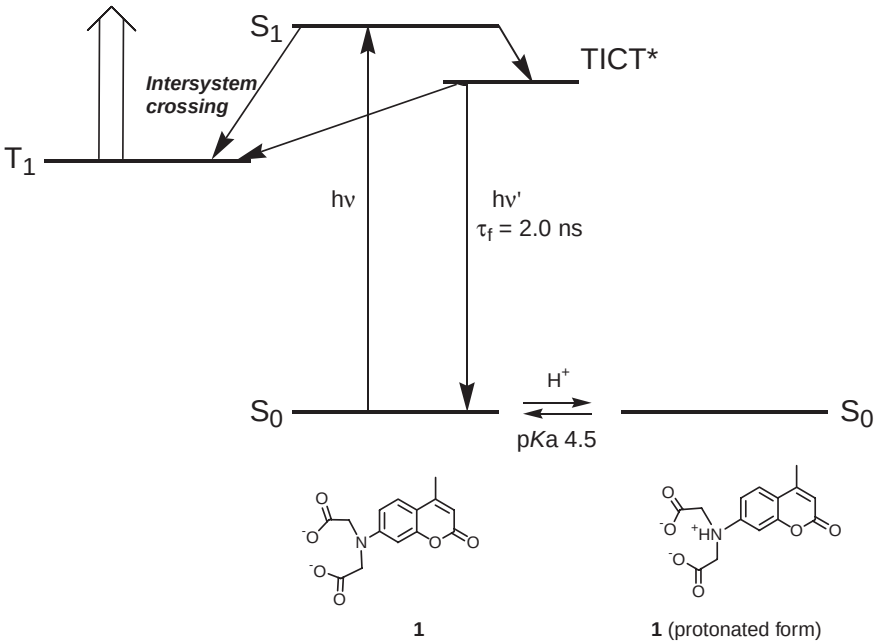

Fig. 5. Energy diagram for **1**.

state absorption study, coumarin **1** had an absorption maximum at 371 nm with the extinction coefficient of 17600 M^{-1} cm^{-1} in HEPES buffer. In the fluorescence spectrum, coumarin **1** had a maximum at 472 nm in HEPES buffer. These values are similar to those of DECM derivatives in MeOH–HEPES 1:4 mixture. MeOH was used for the measurements of the absorption and the fluorescence spectra of DECM derivatives due to their insolubility in water. The value of the Stokes shift of **1** was 5800 cm^{-1}, suggesting that fluorescence emission of **1** in aqueous solution is mainly from a twisted intramolecular charge-transfer (TICT) state.[20–23] The fluorescence lifetime was 2.0 ns, indicating that **1** may have enough lifetime to induce photolysis, thus, releasing the biologically active molecules.

In the transient absorption spectra, the transient band ascribed to the triplet excited state was observed in the range of 500–700 nm. The lifetimes of the transient species were 15 μs under argon and 2.1 μs under air. The quenching rate constant by oxygen was calculated to be 1.4×10^9 M^{-1} s^{-1}. These results suggest that photolysis of the caged compounds with coumarin **1** may partially proceed from the triplet excited state, in which the recombination reaction of the radical pair can be suppressed in comparison with the singlet excited state.

In conclusion, we have successfully prepared a new water-soluble coumarin derivative **1**. Energy diagram for coumarin **1** is shown in Fig. 5. This molecule shows absorption spectrum at visible region and yields TICT*, the lifetime of which is 2.0 ns, via the singlet excited state,[24] indicating that this chromophore can be used as the light-absorbing part of the water soluble caged compounds such as caged amino acids or caged nucleotides, which can give free amino acids or nucleotides, respectively, by excitation at visible region. Furthermore, the observation of the long-lived triplet state of coumarin **1** indicates that coumarin **1** can be used as a chromophore for the triplet state reactions because radical recombination reaction does not favored.

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