

Synthesis and Photocleavage of 7-[{Bis(carboxymethyl)amino}-coumarin-4-yl]methyl-Caged Neurotransmitters

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7-[{Bis(carboxymethyl)amino}coumarin-4-yl]methyl-caged neurotransmitters (glutamate and GABA) were synthesized. Both caged compounds showed sufficient stability in the dark, were water-soluble at pH 7.2 without using organic solvents, and exhibited relatively high quantum yield for photolysis upon irradiation with 390 nm light.

Photolabile precursors of bioactive effecter molecules (caged compounds) are powerful tools for understanding, controlling, and manipulating cell physiology.[1–6] In many caged compounds, covalent bond formation with a photoremovable protecting group masks important features for biological recognition, and photocleavage (un-cage) of the caged compound produces rapid jumps in the concentration of the bioactive molecules. For biological use, photolabile protecting groups must undergo rapid and efficient photolysis upon photo excitation at wavelengths that are not harmful to the cells. In addition, enhancement of the hydrophilicity of the photolabile protecting group makes the caged compound more soluble in aqueous buffers without using unnecessary organic solvents.

Functionalized coumarins have been recognized and widely used as suitable chromophores caging for chemical messenger molecules, such as cyclic nucleotide monophosphates (cNMPs),[7–12] cytidine diphosphate,[13] and glutamic acid.[14,15] Furuta et al. first reported that (7-methoxycoumarin-4-yl)-methyl-caging group (MCM-caging group, Scheme 1) has certain advantages, such as stability in the dark in physiological buffer, high extinction coefficient at longer wavelength region, and relatively high quantum yield of photorelease, over other photolabile protecting groups.[7] Hagen et al. have introduced both axial and equatorial diastereomers of caged-cNMPs using a (7-diethylaminocoumarin-4-yl)methyl-caging group (DEACM-caging group).[16,17] The DEACM-caged cNMPs photorelease free cNMPs by photoirradiating at longer wavelength more efficiently than the other coumarinylmethyl-caged cNMPs. DEACM-caging group, itself, is however hydrophobic, and therefore, an organic solvent must be used to dissolve DEACM-caged cNMPs. Thus, they have developed 7-[bis(carboxymethyl)amino]-4-methylcoumarin-caged cyclic nucleotides (BCMACM-caged cNMPs), in which the BCMACM-caging group maintains its positive photochemical properties and there is an enhancement in the aqueous solubility due to the carboxylate groups in its structure.[18]

As a parent chromophore for BCMACM-caging group, we have studied on the photochemical properties of 7-[bis(carboxymethyl)amino]-4-methylcoumarin (**1**).[19] This molecule is soluble in aqueous buffers at physiological pH without using organic solvents, absorbs in the visible region, and shows an excited singlet lifetime of 2.0 ns, which is considered to be sufficiently long to undergo photolysis efficiently. Thus, it should be possible to use **1** as the light-absorbing part of the water soluble caged compounds not only for cNMPs but also for other biologically active substances. Here, we describe the photocleavage of BCMACM-caged glutamate **6** and BCMACM-caged GABA **7**. Both compounds showed sufficient solubility in aqueous buffer at pH 7.2, were stable in the dark and had a reasonable quantum yield on photolysis. Because glutamate and GABA are the major excitatory and inhibitory neurotransmitters, respectively, in the central nervous system, **6** and **7** should facilitate research on the mechanism of the neurotransmitter receptor.

Results and Discussion

Caged compounds **6** and **7** were prepared as shown in Scheme 1 by condensing alcohol **2** with *N*-Boc-protected glutamic acid and *N*-Boc-protected GABA to yield **4** and **5**, respectively, followed removal of *tert*-butyl ester groups with trifluoroacetic acid (TFA). The TFA deprotection resulted in the pure caged glutamic acid **6** and caged GABA **7**, respectively, which was confirmed by using ^{1}H NMR, UV, and fluorescence spectroscopy, and HPLC analysis. 7-[Bis(carboxymethyl)amino]-4-(hydroxymethyl)coumarin (**3**) was prepared and used as an authentic photoproduct.

The thermal stability of the caged compounds **6** and **7** was measured in HEPES buffer solution at pH 7.2. Caged compounds **6** and **7** were pratically stable for 1 h in the dark at room temperature but were gradually hydrolyzed to give the half-lives of longer than 200 h for both **6** and **7**. Caged compounds **6** and **7** showed very little hydrolysis in the dark at room temperature during 2 h of measurement. Hydrolysis was not detected after 2 days, when the caged compounds were dissolved in HEPES buffer at pH 7.2 and were stored at $-4\,^\circ$C in the dark.

The photochemical properties of the **1**, **3**, **6**, and **7** in HEPES buffer (pH 7.2) are summarized in Table 1. The absorption and emission bands in both Figs. 1a and 1b originate from the coumarin chromophore. The band properties of the caged compounds **6** and **7** are very similar to each other, and appeared at longer wavelengths than those of model compounds **1** and **3**. The fluorescence quantum yields (Φ_f) of **6** and **7** are lower than those of model compounds **1** and **3** (Table 1) prob-

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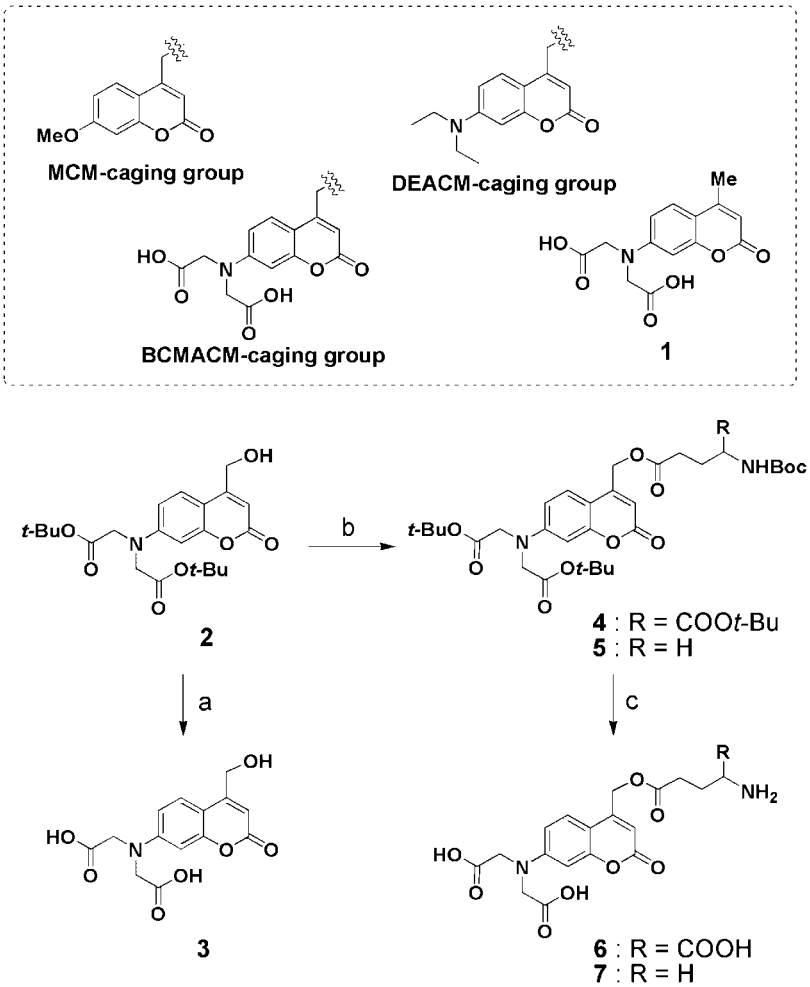


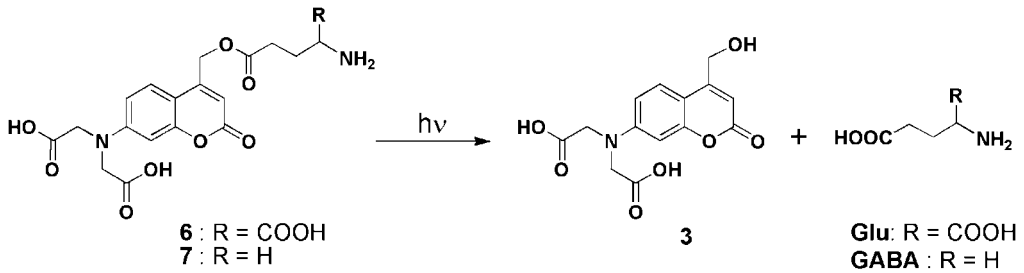
Scheme 1. (a) TFA, CH_2Cl_2, H_2O, rt, 20 min; (b) EDC, DMAP, CH_2Cl_2, *N*-Boc-Glu-O-*t*-Bu for **4**, or *N*-Boc-GABA for **5**, rt, 40 min.

Table 1. Absorption Maxima (λ_{abs}^{max}), Extinction Coefficients (ε^{max}), Fluorescence Maxima (λ_f^{max}), Fluorescence Quantum Yields (Φ_f), Stokes Shifts, Quantum Yields for Photolysis (Φ_p), and Solubility of Compounds **1**, **3**, **6**, and **7** in HEPES Buffer (pH 7.2) at Room Temperature

Compounds	λ_{abs}^{max}/nm (ε^{max}/M^{-1} cm^{-1})	λ_f^{max}/nm (Φ_f)	Stokes shifts/cm^{-1}	Φ_p	Solubility /mM
1	371 (17600)	472 (0.32)	5800	—	>5
3	375 (16900)	489 (0.22)	6200	—	>5
6	379 (13100)	498 (0.10)	6300	0.10	>1
7	381 (15000)	498 (0.10)	6200	0.20	>1

ably due to the existence of competitive decay pathway involving the photocleavage from the excited singlet state. The large values of Stokes shifts for all compounds are characteristic for 7-aminocoumarin derivatives, which undergo photoinduced intramolecular charge transfer in their excited singlet state.[19]

Efficient release of the neurotransmitters upon photoirradiation of caged compounds is needed for biological applications. Upon irradiation at 380 nm in aqueous buffer, the absorption spectra of caged glutamate **6** shifted to shorter wavelength region (Fig. 2a). The blue shift of the absorption spectrum is due to the production of alcohol **3** by photolysis, which was detected by using HPLC as a major photoproduct and confirmed by using an authentic sample of **3** (Fig. 2b). A similar spectral change during the irradiation was observed by photolysis of caged GABA **7**. As depicted in Scheme 2, formation of alcohol **3** during irradiation should indicate the production of "free" neurotransmitters. The quantum yield of photolysis (Φ_p) was determined by HPLC analysis to be 0.10 for **6** and 0.20 for **7**. The quantum yield of **6** is similar to that of previously reported DECM-caged glutamate (Φ_p 0.11),[15] whereas the efficiency for photolysis of caged GABA **7** is twice as high as those of caged glutamates (Table 1). The fluorescence lifetimes of **6** and **7** were 700 and 760 ps, respectively, and are shorter than **1** (2.0 ns). The rate constant of the cleavage reaction was estimated to be 1.4×10^8 and 2.6×10^8 s^{-1} for **6** and **7**, respectively, from the fluorescence lifetime and the quantum yield of photolysis. According to the reported mechanism for the photocleavage of MCM-caged acids,[9] an ion pair of



Scheme 2.

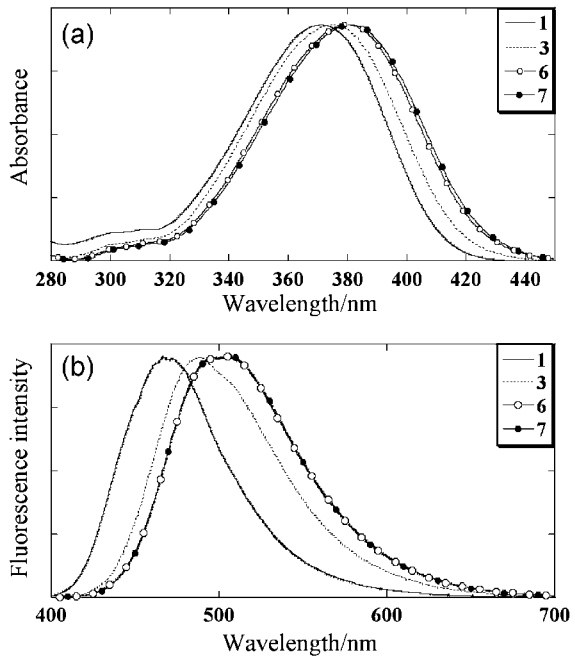


Fig. 1. (a) UV–vis absorption spectra for **1**, **3**, caged glutamate **6**, and caged GABA **7** in HEPES buffer at pH 7.2.

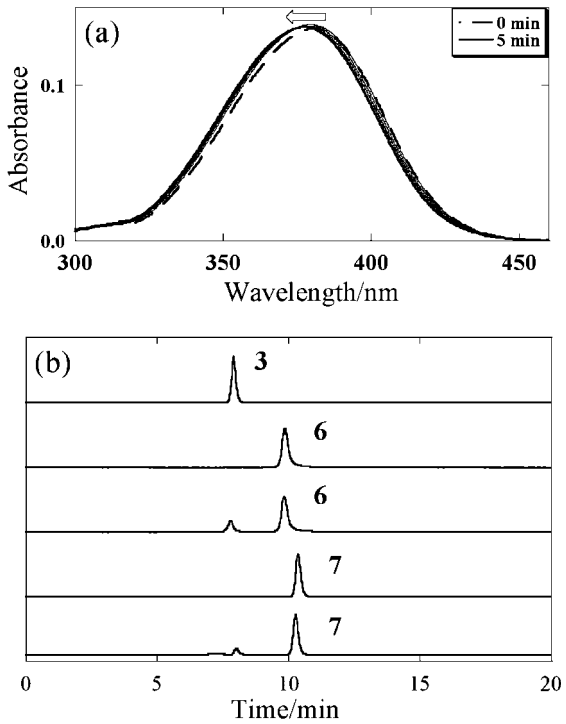


Fig. 2. (a) Change in the absorption spectra of **6** in HEPES buffer (1.0×10^{-5} M) at pH 7.2 on photoirradiation with 380 nm light. (b) HPLC chromatograms of **3** as an authentic sample, **6** before irradiation, **6** after irradiation, **7** before irradiation, and **7** after irradiation from the top to the bottom, respectively.

coumarinylmethyl cation and the anionic product are produced by photoexcitation, and the following escape process to produce the free acids might compete with the recombination reaction to give starting caged compound. In this mechanism, the quantum yield of photocleavage can be affected by the ability of the cleaved group to escape from the solvent cage, which should depend on the molecular structure. The quantum yields (Φ_p) and the extinction coefficients (ε) are high for caged glutamates, resulting in good photosensitivity at longer wavelength.

The coumarin chromophore and glutamate moiety in **6** or GABA moiety in **7** is linked by ester bond, which should be hydrolyzed by intracellular esterase. Such caged compounds, therefore, can be used extracellularly, where **6** and **7** were confirmed to be stable in the dark in physiological buffer. Thus, it is expected that **6** and **7** can activate the glutamate or GABA receptors, respectively, on the cell surface by flash photolysis.

In summary, BCMACM-caged glutamate **6** and BCMACM-caged GABA **7** were prepared from readily available starting materials, and their photochemistry was investigated. Both compounds **6** and **7** showed sufficient stability in the dark, were water-soluble at pH 7.2 without using organic solvents, and had relatively high quantum yield for photolysis on irradiation with 390 nm light. These favorable properties will make it possible to obtain high concentrations of free neurotransmitters by flash photolysis.

Experimental

Compound 3. Compound **2** (43 mg) was dissolved in dichloromethane (3 mL), and the reaction mixture was cooled to 0 °C. Trifluoroacetic acid (1 mL) and water (50 μL) was added, and the solution was stirred for 20 min at room temperature. The solvent was evaporated under reduce pressure, and the residue was washed three times with 1 mL of diethyl ether and twice with 1 mL of acetonitrile to give 26 mg (84% yield) of **3** as a pure product.

^{1}H NMR (CD_3OD) δ 7.51 (d, $J = 8.8$ Hz, 1H), 6.65 (dd, $J = 8.8$, 2.4 Hz, 1H), 6.51 (d, $J = 2.4$ Hz, 1H), 6.30 (s, 1H), 4.80 (s, 2H), 4.27 (s, 4H).

Compound 4. A mixture of Boc-Glu-*O*-*t*-Bu (146 mg, 0.48 mmol), DMAP (7.8 mg, 0.064 mmol), and EDC (91 mg, 0.48

mmol) in dichloromethane (20 mL) was stirred for 10 min at room temperature. Compound **2** (102 mg, 0.24 mmol) in dichloromethane was then added to the reaction mixture, and the reaction mixture was stirred in the dark for 40 min at room temperature. The reaction mixture was poured into water, and the separated organic layer was washed with $NaHCO_3$ aq and brine, dried over $MgSO_4$, and filtered. Then, the solvent was evaporated under reduce pressure. The residue was purified by flash chromatography (SiO_2, hexane–AcOEt 3:1) to give 0.151 g of **4** in 89% yield. ^{1}H NMR ($CDCl_3$) δ 7.34 (d, $J = 8.8$ Hz, 1H), 6.52 (dd, $J = 8.8$, 2.8 Hz, 1H), 6.47 (d, $J = 2.8$ Hz, 1H), 6.21 (s, 1H), 5.23 (s, 2H), 5.23–5.12 (m, 1H), 4.28–4.20 (m, 1H), 4.06 (s, 4H), 2.61–2.45 (m, 1H), 2.27–2.20 (m, 1H), 1.96–1.93 (m, 1H), 1.48 (s, 18H), 1.44 (s, 18H). ^{13}C NMR ($CDCl_3$) δ 172.1, 171.2, 168.8, 161.3, 155.7, 155.4, 151.3, 149.1, 128.3, 124.5, 109.2, 108.4, 108.2, 99.3, 82.5, 82.4, 79.9, 61.4, 54.3, 53.2, 30.1, 28.3, 28.1, 28.0. Found: C, 61.13; H, 7.51; N, 3.72%. Calcd for $C_{36}H_{52}N_2O_{12}$: C, 61.35; H, 7.44; N, 3.97%.

Compound 5. A mixture of Boc-GABA (98 mg, 0.48 mmol), DMAP (7.8 mg, 0.064 mmol), and EDC (91 mg, 0.48 mmol) in dichloromethane (20 mL) was stirred for 10 min at room temperature. Compound **2** (101 mg, 0.24 mmol) in dichloromethane (30 mL) was added to the reaction mixture, and the reaction mixture was stirred in the dark for 40 min at room temperature. The reaction mixture was poured into water, and the separated organic layer was washed with $NaHCO_3$ aq and brine, dried over $MgSO_4$ and filtered. The solvent was then evaporated under reduce pressure. The residue was purified by flash chromatography (SiO_2, hexane–AcOEt 3:1) to give 0.134 g of **5** in 92% yield. ^{1}H NMR ($CDCl_3$) δ 7.34 (d, $J = 8.8$ Hz, 1H), 6.52 (dd, $J = 8.8$, 2.8 Hz, 1H), 6.47 (d, $J = 2.8$ Hz, 1H), 6.21 (s, 1H), 5.23 (s, 1H), 4.62 (s, 1H), 4.06 (s, 4H), 3.22–3.178 (m, 2H), 2.49 (t, $J = 7.2$ Hz, 2H), 1.91–1.83 (m, 2H), 1.48 (s, 18H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$) 172.4, 168.8, 161.4, 156.0, 155.7, 151.3, 149.2, 143.6, 129.6, 129.1, 127.5, 124.5, 109.2, 108.4, 108.2, 99.3, 82.6, 79.4, 61.3, 54.3, 51.9, 39.8, 31.3, 28.4, 28.1, 25.3. MALDI-TOF-MS: Found: m/z 604.30. Calcd for $C_{31}H_{44}N_2O_{10}$: M, 604.30.

General Procedures for the Preparation of the Caged Glutamate 6 and 7. Compound **4** or **5** (40 mg) was dissolved in dichloromethane (3 mL) and the reaction mixture was cooled to 0 °C. Trifluoroacetic acid (1 mL) and water (50 μL) were added, and the solution was stirred in the dark for 20 min at room temperature. The solvent was evaporated under reduce pressure, and the residual oil was redissolved in acetonitrile (10 mL). Chloroform (10 mL) was then added to the solution, and the resulting solid was filtered and washed with methanol to give caged glutamate **6** in 73% yield and caged GABA **7** in 77% yield, respectively. The purity of caged compounds **6** and **7** was confirmed by HPLC analysis (see instrumentation section) using 0.2% TFA(aq)/acetonitrile (83:17) as an eluent [retention time (**6**) = 9.8 min; retention time (**7**) = 10.4 min].

Caged Glutamate 6: ^{1}H NMR (DMSO) δ 8.18 (s, 2H), 7.53 (d, $J = 9.0$ Hz, 1H), 6.53 (dd, $J = 9.0$, 2.0 Hz, 1H), 6.38 (d, $J = 2.0$ Hz, 1H), 6.10 (s, 1H), 5.30 (s, 2H), 4.16 (s, 4H), 3.88 (t, $J = 7.0$ Hz, 1H), 2.73–2.69 (m, 1H), 2.66–2.61 (m, 1H), 2.12–2.09 (m, 1H), 2.04–2.02 (m, 1H). ^{13}C NMR (DMSO) 172.1, 171.3, 170.5, 160.2, 155.0, 150.9, 150.3, 125.4, 108.8, 106.6, 106.5, 97.6, 61.3, 51.3, 40.0, 29.0, 25.1, ESI-MS observed m/z 437.11 ($[M + H]^+$).

Caged GABA 7: ^{1}H NMR (DMSO) δ 7.68 (s, 2H), 7.54 (d, $J = 9.0$ Hz, 1H), 6.53 (dd, $J = 9.0$, 2.0 Hz, 1H), 6.32 (d, $J = 2.0$ Hz, 1H), 6.10 (s, 1H), 5.30 (s, 2H), 4.11 (s, 4H), 2.83–2.82 (m, 2H), 2.61–2.58 (m, 2H), 1.85–1.80 (m, 2H). ^{13}C NMR (DMSO) 172.1, 171.6, 160.2, 155.1, 150.9, 150.3, 125.5, 108.8, 106.7, 106.7, 97.6, 61.3, 40.0, 38.1, 30.0, 22.3. ESI-MS observed m/z 393.12 ($[M + H]^+$).

Measurements. The ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX-400 (400 MHz for ^{1}H NMR) and Bruker AVANCE 600 (600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR) spectrometer. FAB mass spectra were recorded on a JEOL MS-600H mass spectrometer. ESI mass spectra were recorded on an Applied Biosystems Qstar/Pulsar *i* spectrometer. 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 on a Horiba NAES-1100 time resolved spectrofluorometer.

Solution of **6** and **7** were photolyzed with UV light (365 nm, slit 5 nm) from 150 W xenon lamp from a JASCO FP777 fluorescence spectrometer, and analytical HPLC (ODS100V) was performed to determine the decrease in the amount of **6** and **7** in the photolysis. Quantum efficiencies were calculated as $(I\varepsilon t_{10\%})^{-1}$, where I is the irradiation intensity in einsteins cm^{-2} s^{-1}, ε is the molar extinction coefficient in cm^2 (mol substrate)$^{-1}$ (10^3 times the conventional extinction coefficient in M^{-1} cm^{-1}), and $t_{10\%}$ is the irradiation time in seconds for 10% conversion to product. Total UV intensity I was measured by using chemical actinometry with potassium ferrioxalate in the same setup. The intensities were 2.8×10^{-8} einsteins cm^{-2} s^{-1}.

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