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Synthesis of carbamate-type caged derivatives of a novel glutamate transporter blocker

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Abstract—L-*threo*-β-Benzyloxyaspartate (L-TBOA) and (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (L-TFB-TBOA) are potent nontransportable blockers for glutamate transporters. We synthesized a carbamate-type coumarin derivative of L-TBOA **3a** as a caged blocker and compared **3a** with the corresponding ester-type analogs **1**. The carbamate **3a** was less sensitive to photolysis than the ester **1** but was more stable in the aqueous solution. The [6,7-bis(carboxymethoxy)-coumarin-4-yl]methylcarbonyl (BCMCMC) group exhibited good results both in photoreactivity and stability. Therefore, we examined photolysis of *N*-BCMCMC-TBOA **3b** and *N*-BCMCMC-TFB-TBOA **4**, which immediately released blockers to show glutamate uptake inhibition.

1. Introduction

Caged compounds whose activities are masked by a photocleavable group are useful tools to elucidate complex intracellular processes.[1–3] The concentration jumps of an active compound can be generated at the desired time and position by a photochemical reaction using caged compounds with UV-irradiation and the technique has attracted much attention toward overcoming the limitations in time resolution.

L-Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate receptors are believed to be involved in learning, memory, and pathological phenomena such as ischemia-related neuronal death. On the other hand, glutamate transporters, five subtypes of which were cloned as excitatory amino acid transporters (EAAT1-5), maintain extracellular glutamate concentrations at low levels to limit the receptor activation and to protect neurons from the excitotoxicity.[4] Signal transmission can occur on a millisecond order, thus, methods with a good kinetic resolution are required. Excellent caged glutamate derivatives were reported for the investigation of the function of glutamate.[5] However, these derivatives activate both glutamate receptors and EAATs. Since EAATs together with glutamate receptors regulate the fast signal transmission at excitatory synapses, caged blocker of glutamate transporters would be promising for methods for the selective inactivation of EAATs. In order to inhibit EAATs efficiently, we have developed L-TBOA (L-*threo*-β-benzyloxyaspartate), which is a potent nontransportable blocker for all subtypes of EAATs.[6] As L-TBOA did not affect receptors, including ionotropic and metabotropic glutamate receptors, its caged compound, which can block the glutamate uptake at the desired time and position by UV-irradiation, has potential as a useful tool for investigation of EAATs. In the previous paper, we reported the synthesis of its ester-type caged derivatives **1**–**2** and showed that they rapidly provided L-TBOA by UV-irradiation.[7] However, they were gradually hydrolyzed at room temperature in the aqueous or the DMSO solution. As excess amounts of caged compounds are generally applied to cells or tissue culture, stability in the aqueous solution before UV-irradiation is crucial for practical use. Here, we describe the synthesis of caged TBOA analogs, possessing a stable caging group even in the aqueous solution.

Keywords: Excitatory amino acid; Glutamate transporter blocker; Caged compound; Photolysis; Carbamate.
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Moreover, we synthesized a caged derivative of a novel TBOA analog (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (L-TFB-TBOA), which was about 50- to 1500-fold more potent than L-TBOA in the uptake assay.[8]

2. Results and discussion

As two carboxylic acids and one amino group of L-TBOA are necessary for the inhibitory activity on EAATs, its activity could be masked by introduction of a photocleavable group to the amino group (Fig. 1). A carbamate group is generally more stable against hydrolysis than an ester group.[9] We first planned to synthesize the carbamate-type caged L-TBOA having the [7-(carboxymethoxy)coumarin-4-yl]methoxycarbonyl (CMCMC) group, because the [7-(carboxymethoxy)coumarin-4-yl]methyl (CMCM) ester[2c] of L-TBOA **1a** showed superior sensitivity for photolysis to 2-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) esters **2**[10] and good solubility in the aqueous solution (PBS(+), pH 7.4).[7] The protected CMCM-OH **6a** was obtained from the corresponding aldehyde **5a**[2c] with $NaBH_4$ reduction and converted to **7a** with 1,1-carbonyldiimidazole (Scheme 1).[9] Introduction of the CMCMC group using **7a** to the amino group of the protected glutamate **8** was achieved in high yield. However, the corresponding carbamate derivative of L-TBOA **12a** was not obtained at all from the amine **11** and **7a** under the same conditions. The reactivity of the amino group of **11** was very low probably due to the steric hindrance of two *tert*-butyl esters and the benzyloxy group. In contrast, treatment of **11** with chloroformate **7b** prepared from trichloromethylchloroformate (phosgene dimer) and **6a** provided **12a** in 51% yield.[11] Deprotection of **12a** with TFA followed by HPLC purification gave *N*-(7-carboxymethoxycoumarin-4-yl)methoxycarbonyl-TBOA (*N*-CMCMC-TBOA: **3a**: λ_{max}: 322 nm). We examined the chemical stability of **3a** both in the aqueous solution and in the DMSO solution. In the aqueous buffer as well as the DMSO solution, **3a** was stable at room temperature and no decomposed product was observed by HPLC at least for three months (Fig. 2).

Next, we compared the photosensitivity of **3a** with those of the ester-type compounds **1**. All compounds were decomposed by the irradiation of a UV-handy lamp (365 nm, max power at 10 cm from the lamp: 2.3 mW/cm^2). Kinetic analyses revealed the half-life of **1a**, **1b**, and **3a** was 3.7, 3.9, and 19.1 min, respectively (Fig. 3). Compared to the ester-type compounds, the carbamate-type caging group was less sensitive for the photolysis by UV-handy lamp. Therefore, in order to increase the photosensitivity, an additional carbomethoxy group was introduced on the coumarin ring of **3a**, expecting both bathochromic and hyperchromic effects.[2] *N*-[6,7-bis(carboxymethoxy)coumarin-4-yl]methoxycarbonyl-

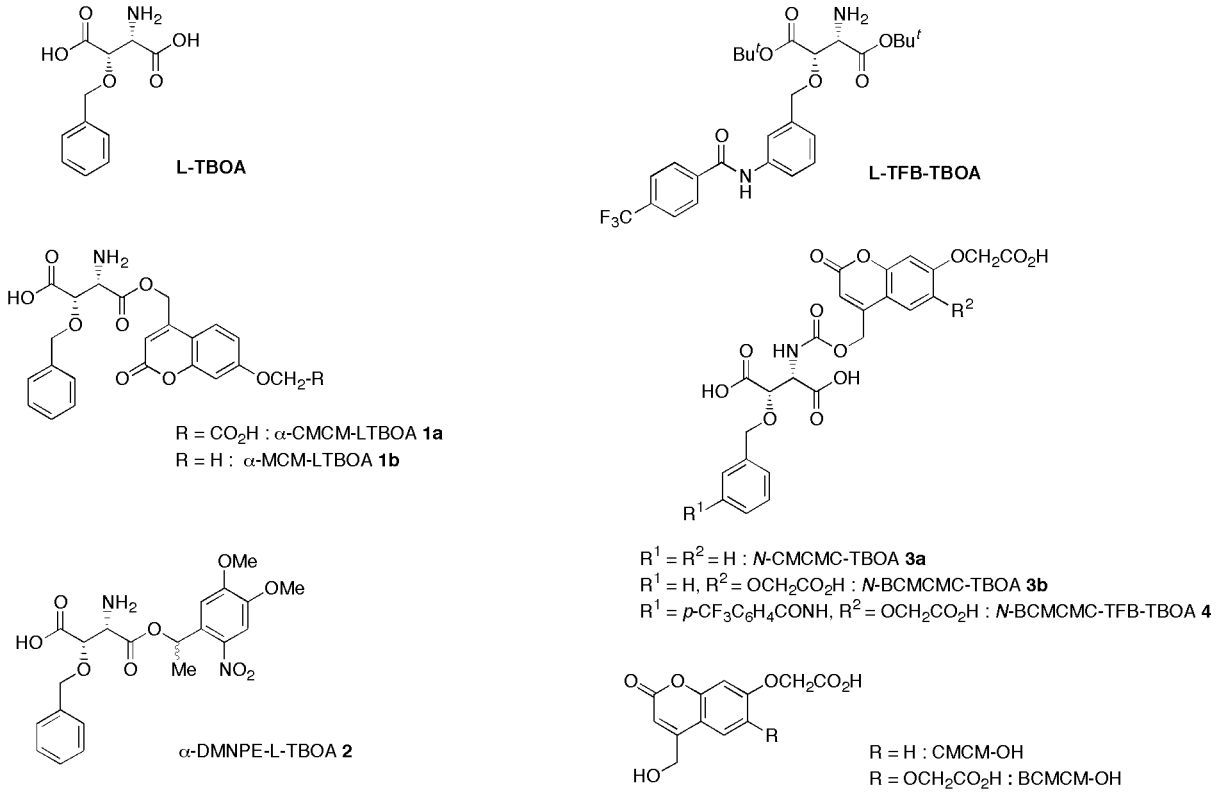
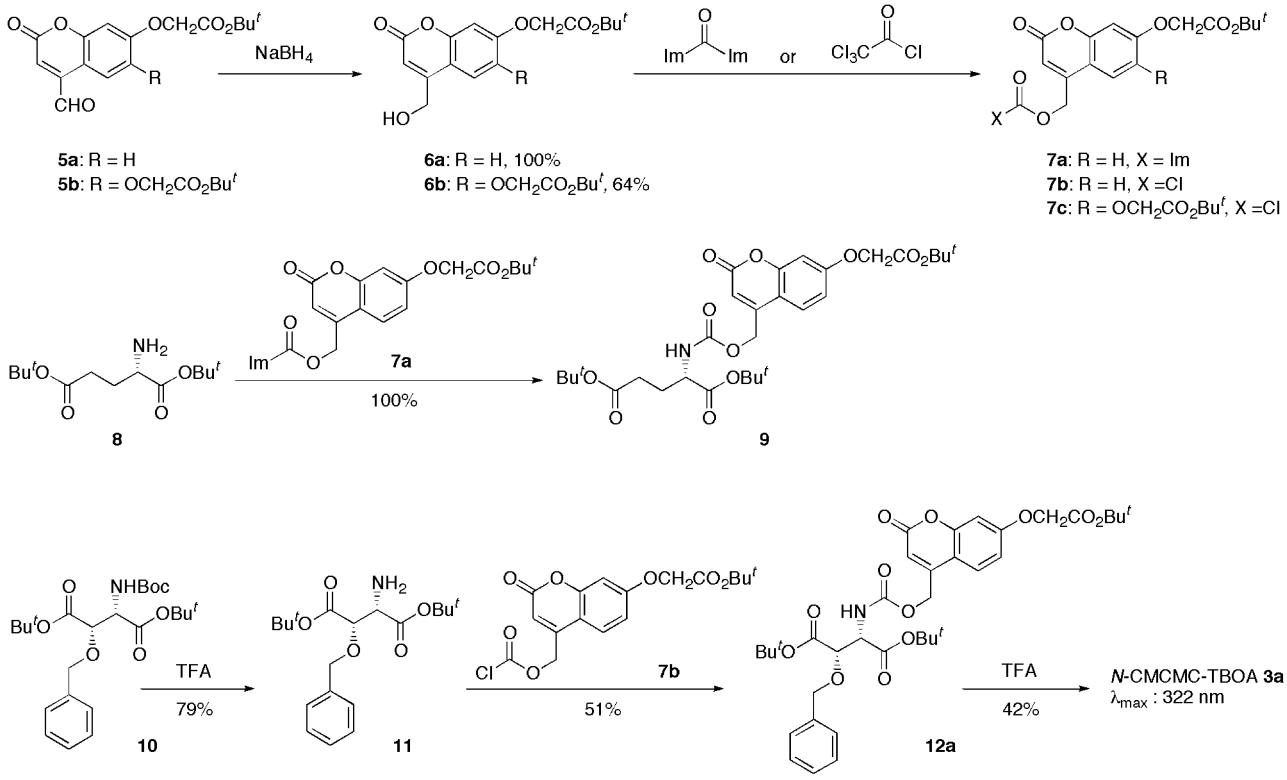


Figure 1. Glutamate transporter blockers and their caged derivatives. CMCM: [7-(carboxymethoxy)coumarin-4-yl]methyl, MCM: (7-methoxycoumarin-4-yl)methyl, DMNPE: 2-(4,5-dimethoxy-2-nitrophenyl)ethyl, BCMCM: [6,7-bis(carboxymethoxy)coumarin-4-yl]methyl, CMCMC: [7-(carboxymethoxy)coumarin-4-yl]methoxycarbonyl, BCMCMC: [6,7-bis(carboxymethoxy)coumarin-4-yl]methoxycarbonyl.



Scheme 1.

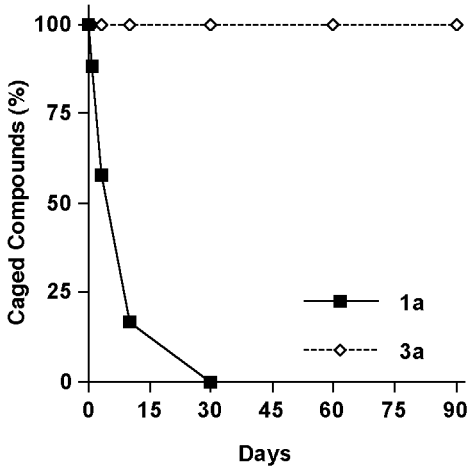


Figure 2. Stability of caged compounds (**1a**, **3a**) in PBS(+) (pH 7.4). The concentration of the caged compounds were quantified by HPLC and values are presented as the average of two determinations.

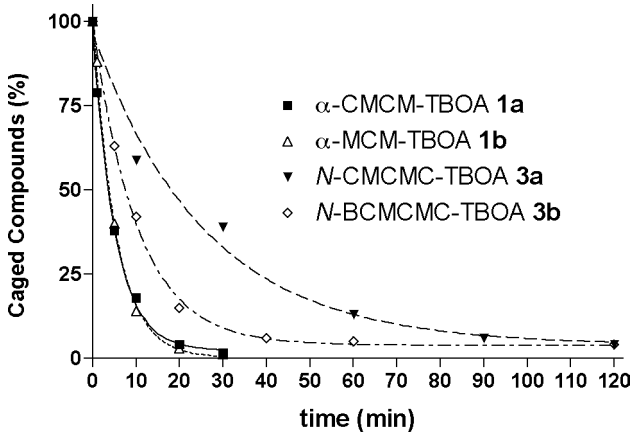
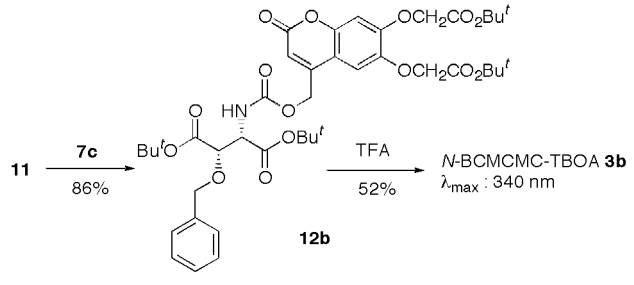


Figure 3. Decomposition of caged compounds (**1a**, **1b**, **3a**, and **3b**) with UV-handy lamp irradiation. The concentrations of the remaining caged compounds during irradiation were measured by HPLC. Values are presented as the average of three determinations.

TBOA (*N*-BCMCMC-TBOA: **3b**) was synthesized in the same manner as **3a** (Scheme 2). The λ_{max} (340 nm) of **3b** was shifted to longer wavelength to be closer to the irradiation wavelength (365 nm) and the extinction coefficient (ε) significantly increased at λ_{max} as well as at 365 nm. As expected, **3b** was more rapidly decomposed by UV-irradiation than **3a** and the half-life of **3b** was improved to 7.8 min (Fig. 3), which was almost comparable to that of **1a**. No decomposition product from **3b** was observed in the aqueous solution for three months (data not shown), indicating the superior



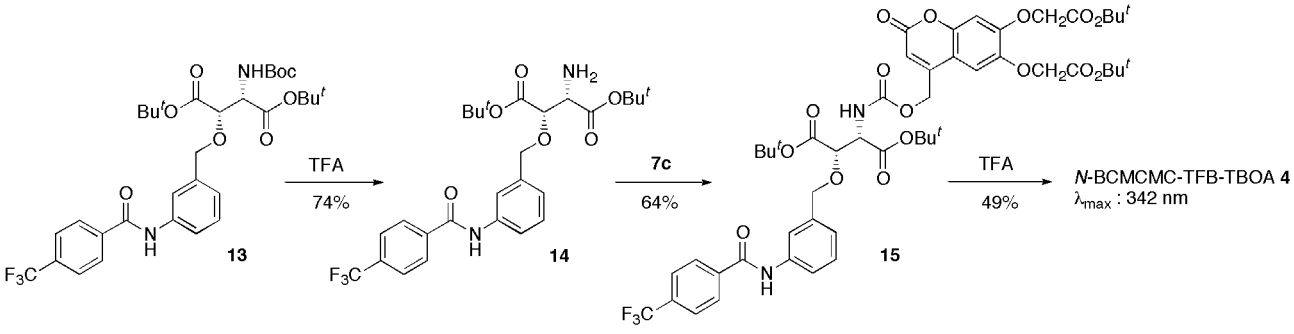
Scheme 2.

stability of carbamate-type caging groups to that of the ester-type (Fig. 2).

L-TFB-TBOA is a novel blocker, which blocks EAATs about 50- to 1500-fold more potently than L-TBOA with longer duration.[8] An excess amount of a caged compound sometimes interferes in other intracellular processes. The application dose of caged L-TFB-TBOA would be expected to decrease and, thus, unexpected effects could be prevented. Therefore, based on the results above, we designed *N*-BCMCMC-TFB-TBOA **4** (Fig. 1). The Boc group of **13** was removed by TFA and the resulting amino group of **14** was reacted with **7c** to give a protected caged-derivative **15**. Removal of *tert*-butyl esters by TFA provided the desired **4** as shown in Scheme 3.

The stability and the photoreactivity of **4** were then assessed. It was stable in the aqueous solution for three months (data not shown). In the case of UV-handy lamp irradiation experiments, the concentration of both the decomposed **4** and the produced L-TFB-TBOA can be directly qualified by HPLC since L-TFB-TBOA has strong UV absorption unlike L-TBOA. HPLC analysis revealed that **4** was smoothly decomposed while a new peak, which showed the same retention time as that of L-TFB-TBOA (R_t: 12.8 min), increased with UV-irradiation. The half-life of the decomposition of **4** and that of the production of L-TFB-TBOA were 5.5 and 5.8 min, respectively, and both can be fitted to single exponential equations. The production of L-TFB-TBOA was also confirmed by Q-TOF-MS as the molecular ion peak [m/z $(M+H)^+$ 427] was dominantly observed in the irradiated samples. However, although the peak of **4** monitored by HPLC almost completely disappeared within 30 min, L-TFB-TBOA reached a plateau at ca. 40% of the expected amount.[12] Despite attempts to find other products, no remarkable peak except for L-TFB-TBOA or BCMCM-OH could be detected either in HPLC or in Q-TOF-MS. These observations were consistent with our previous results for the esters **1**[7] and the recent report that photolysis of 6-bromo-4-(1,2-dihydroxyethyl)-7-hydroxycoumarin (Bhc) derivatives of acetal gave only a half of the expected yield.[13] Although the causes of the differences remain unclear, we confirmed that the blocker activity of the resulting L-TFB-TBOA in the uptake assay was not impeded by photolysis byproducts.[14]

We then examined photolysis of these compounds by using YAG Laser (SureLite II, Continuum, Santa Clara, CA, 355 nm, 8 ns pulse, 40 mJ).[15] Using a laser pulse, which has higher energy and much smaller focus than a UV-handy lamp, the active compound can be generated at the desired time and position in a moment. Therefore, the laser pulse in a sample specimen would be more useful for physiological applications. The chemical yields of disappearance (Y_{dis}), quantum yields of disappearance (Φ_{dis}) and $\varepsilon_{355}\Phi_{dis}$ values (indication of the efficiency of photolysis: high value reflects high efficiency) with laser pulse irradiation are summarized in Table 1.[7,16] Indication values of the efficiency of photolysis ($\varepsilon_{355}\Phi_{dis}$) of **3b** and **4** were larger than that of **1a**, which was not consisted with the results of half-life with UV-handy lamp irradiation (Fig. 3) probably because the disparity of the features of light sauces. Laser beam



Scheme 3.

Table 1. Spectroscopic and photolytic characteristics of the caged compounds

Compound	UV absorption λ_{max} (nm)[a] (ε: $M^{-1}\,cm^{-1}$)	Laser photolysis 1 shot (40 mJ) 355 nm			
		Y_{dis}[b]	Φ_{dis}[b]	ε_{355}[c]	$\varepsilon_{355}\Phi_{dis}$[d]
α-CMCM-TBOA **1a**	325 (11,381)[e]	8 ± 2.3	0.02	2455	49
N-CMCMC-TBOA **3a**	322 (5796)	1 ± 0.8	0.006	738	4.4
N-BCMCMC-TBOA **3b**	340 (15,457)	24 ± 3.6	0.03	12,120	363
N-BCMCMC-TFB-TBOA **4**	287 (5372) 342 (5047)	10 ± 1.2	0.02	4052	81

[a] ε: Wavelength of absorbance maximum (λ_{max}) and extinction coefficient in 100 μM solution in PBS(+) solution.
[b] Y_{dis}: Chemical yield of disappearance (%). Φ_{dis}: Quantum yield of disappearance (%).
[c] ε_{355}: extinction coefficient at 355 nm ($M^{-1}\,cm^{-1}$).
[d] $\varepsilon_{355}\Phi_{dis}$: Product of the quantum yield of disappearance and extinction coefficient (indication of the efficiency of photolysis).
[e] ε_{max}, ε_{355}, Φ_{dis}, and $\varepsilon_{355}\Phi_{dis}$ values of **1** reported in Ref. 7 are corrected to these values.

is polarized and monochromic while UV-handy lamp has a spectrum band (330–400 nm). Moreover, the longer irradiation time of the UV-handy lamp could in principle offer advantages over laser pulses if the lifetimes of multiple excited states of **1** are in a relatively longer range rather than those of **3b** and **4**,[17] although remarkable difference of the lifetimes between the tested compounds was not suggested at this stage. The present result showed the excellent photosensitivity of BCMCMC derivatives for flash photolysis using YAG laser. Together with the photosensitivity and the stability in the aqueous solution, the ability of the BCMCMC group as a caging group was evaluated.

We next measured the caging effects on biological activity by the glutamate uptake inhibition assay using EAAT2 stably expressed on MDCK cells.[7] The activities of **3a** ($IC_{50} > 100\,\mu M$) and **3b** ($IC_{50} > 100\,\mu M$) before the irradiation were less potent than that of L-TBOA ($IC_{50} = 1.3 \pm 0.12\,\mu M$) (Fig. 4). The effects of the carbamate-type caging groups were almost the same as that of the ester (**1a**: $IC_{50} = 124\,\mu M$).[7] The activity of L-TFB-TBOA ($IC_{50} = 4.1 \pm 0.6\,nM$)[18] was effectively masked (**4**: $IC_{50} > 1000\,nM$) by the BCMCMC group (Fig. 5). Moreover, **4** did not show any inhibition at under 100 nM. Therefore, compared with the effective concentration of L-TFB-TBOA, much higher concentration of **4** could be applied into biological preparations. On the other hand, blocker activity was obviously restored by the laser irradiation. The concentration of L-TFB-TBOA produced from **4** with 1 shot of the laser pulse was estimated to be 20 nM from the uptake assay as well as HPLC. Since it is an averaged value in the whole sample (100 μL), the localized concentration around the target point would be higher in the moment of the irradiation and would be enough to inhibit uptake. These results indicate that L-TFB-TBOA can be quickly and effectively generated at the desired time and position by using the combination of **4** with the laser pulse and that **4** would be useful for the analysis of the inactivation of EAATs.

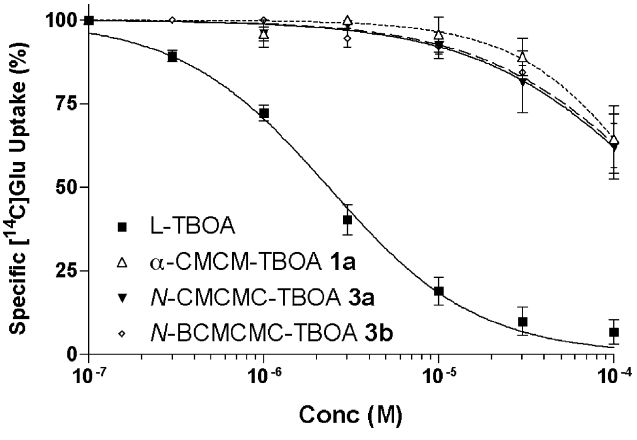


Figure 4. Inhibition of [^{14}C]Glu uptake in MDCK cells expressing EAAT2 by nonirradiation sample of caged compounds (**1a**, **3a**, and **3b**) and L-TBOA.

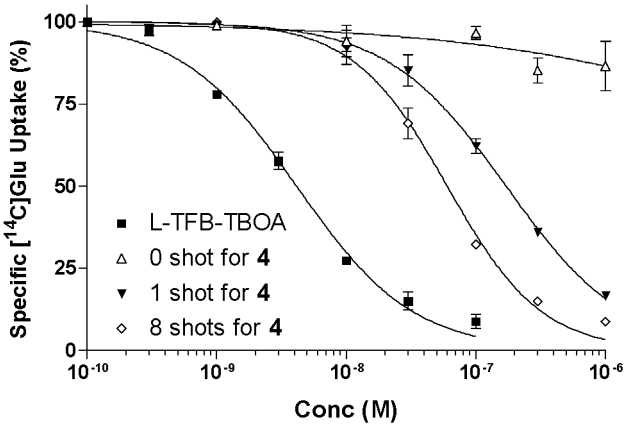


Figure 5. Inhibition of [^{14}C]Glu uptake in MDCK cells expressing EAAT2 by *N*-BCMCMC-TFB-TBOA (**4**) before and after YAG laser irradiation (355 nm). Nonirradiated **4** (△) was much less potent than L-TFB-TBOA (□). Irradiated samples of **4** restored the activity according to the frequency of laser shots (1, 8 shots). Values are presented as the mean ± SEM of at least three determinations.

3. Conclusion

In summary, we have synthesized carbamate-type caged blockers for glutamate transporters, *N*-BCMCMC-TBOA **3b** and *N*-BCMCMC-TFB-TBOA **4**. (1) They were more stable than the ester-type analogs in the aqueous solution and could be stocked at least for 3 months. (2) The blocker activity of **4** was well masked before UV-irradiation. (3) They were highly reactive for the photolysis using a UV-handy lamp or YAG laser to restore biological activity. (4) As the effective dose of **4** at inhibiting EAATs was lower than that of **3b**, the side effects against other intracellular processes could be decreased. Therefore, these compounds, especially **4**, would be useful tools for the analysis of signal transduction and for the elucidation of the physiological roles of transporters.

4. Experimental

^{1}H NMR (400 MHz) was measured on JEOL EX-400 spectrometer. Chemical shifts are reported as δ values in ppm relative to $CHCl_3$ (7.26) in $CDCl_3$ or DMSO (2.50) in DMSO-d_6. IR spectra were recorded on Nicolet FT/IR spectrometer AVATAR 360. Mass spectra were obtained on JEOL JMS-HX110 spectrometer for high-resolution FAB or Micromass Q-Tof mass spectrometer for Q-TOF-MS and Micromass MSI mass spectrometer for ESI-MS. HPLC was carried out with a JASCO UV-975 (detector), PU-980 (pump) high-pressure liquid chromatography. For the purification of the caged compounds, a column of SP-120-5-ODS-AP (Daiso Co. Ltd. Japan), 250 mm × 20 mm ID was used and peaks were monitored at only 254 nm to avoid decomposition. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. Analytical and preparative TLC were performed on Merck Silica gel 60 F_{254} TLC plates Art. 5715 (layer thickness, 0.25 mm) and 5744 (0.5 mm), respectively, and monitored by UV light (254 and

365 nm) or ninhydrin solution. Column chromatography was carried out on silica gel (Silica Gel 60, partial size 63–210 μM, Kanto Chemical).

4.1. 7-(*tert*-Butoxycarbonylmethoxy)-4-(hydroxymethoxy)coumarin (6a)

To a solution of 7-(*tert*-butoxy-carbonylmethoxy)coumarin-4-carbaldehyde[2c] (**5a**: 1.78 g, 5.87 mmol) in MeOH (60 mL), $NaBH_4$ (346.5 mg, 9.16 mmol) was added at 0 °C and the mixture was stirred for 3 h at room temperature. After being treated with 1 N HCl solution, the mixture was extracted with EtOAc. The extract was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to obtain 1.47 g of **6a** (100% yield). IR ν_{max}^{KBr} cm^{-1}: 3500, 1755, 1697, 1610, 1394, 1154, 1090. ^1H NMR ($CDCl_3$) δ 1.48 (s, 9H), 4.55 (s, 2H), 4.84 (s, 2H), 6.46 (s, 1H), 6.75 (d, 1H, $J = 2.4$ Hz), 6.86 (dd, 1H, $J = 2.4$, 11.2 Hz), 7.41 (d, 1H, $J = 8.8$ Hz). MS (ESI) m/z calcd for $C_{16}H_{18}O_6(M+H)^+$ 307, found 307.

4.2. Di-*tert*-butyl *N*-{[7-(*tert*-butoxycarbonylmethoxy)-coumarin-4-yl-methoxy]carbonyl}glutamate (9)

To a solution of **6a** (78.3 mg, 0.31 mmol) in CH_2Cl_2 (5 mL), 1,1-carbonyldiimidazole (45.6 mg, 0.28 mmol) was stirred for 3 h at room temperature, and then and the resulting solution, di-*tert*-butyl glutamate hydrochloride (51.5 mg, 0.17 mmol) and Et_3N (28 μL, 0.2 mmol) were stirred at reflux overnight. The solvent was distilled off and the residue was purified on a silica gel-plate (hexane/EtOAc = 2/1) to obtain 103 mg of compound **9** (100% yield). ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H), 1.48 (s, 9H), 1.50 (s, 9H), 1.94–2.38 (m, 4H), 4.26–4.27 (m, 1H), 4.58 (s, 2H), 5.26 (s, 2H), 5.59 (d, 1H, $J = 7.6$ Hz), 6.35 (s, 1H), 6.79 (d, 1H, $J = 2.4$ Hz), 6.90 (dd, 1H, $J = 2.4$, 8.8 Hz), 7.43 (d, 1H, $J = 8.8$ Hz).

4.3. Di-*tert*-butyl (2*S*,3*S*)-3-benzyloxyaspartate (11)

To a solution of di-*tert*-butyl (2*S*,3*S*)-3-benzyloxy-*N*-*tert*-butoxycarbonylaspartate[6a,b] (**10**: 149 mg, 0.33 mmol) in CH_2Cl_2 (2 mL), TFA (1 mL) was added at 0 °C and the mixture was stirred for 1 h at 0 °C. Saturated aqueous $NaHCO_3$ solution was added and the mixture was extracted with $CHCl_3$. The extract was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to obtain 92 mg of **11** (79% yield). ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.52 (s, 9H), 3.76 (d, 1H, $J = 2.8$ Hz), 4.31 (d, 1H, $J = 2.8$ Hz), 4.42 (d, 1H, $J = 11.2$ Hz), 4.80 (d, 1H, $J = 11.2$ Hz), 7.32–8.33 (m, 5H).

4.4. Di-*tert*-butyl (2*S*,3*S*)-3-benzyloxy-*N*-{[7-(*tert*-butoxycarbonylmethoxy)coumarin-4-yl-methoxy]carbonyl}-aspartate (12a)

To a solution of **6a** (176 mg, 0.7 mmol) in THF (5 mL), trichloromethylchloroformate (0.17 mL, 1.4 mmol) was added prior to reflux for 2 h, and then the solvent was distilled off. The residue was dissolved in CH_2Cl_2 (2 mL) and the resulting solution and *i*-Pr_2NEt (0.4 mL, 2.2 mmol) were added to a solution of compound **11** (85 mg, 0.24 mmol) in CH_2Cl_2 (2 mL), after which the mixture was stirred for 10 min. The solvent was distilled off and the residue was purified on a silica gel-plate (hexane/EtOAc = 2/1) to obtain 84 mg of compound **12a** (51% yield). ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H), 1.47 (s, 9H), 1.50 (s, 9H), 4.45–4.41 (m, 2H), 4.57 (s, 2H), 4.75–4.82 (m, 2H), 5.24 (d, 2H, $J = 6.4$ Hz), 5.67 (d, 1H, $J = 9.6$ Hz), 6.33 (s, 1H), 6.78 (d, 1H, $J = 2.4$ Hz), 6.88 (dd, 1H, $J = 2.4$, 8.8 Hz), 7.32–7.34 (m, 5H), 7.42 (d, 1H, $J = 8.8$ Hz).

4.5. (2*S*,3*S*)-3-Benzyloxy-*N*-{[7-(carboxymethoxy)coumarin-4-yl-methoxy]carbonyl}aspartate (*N*-CMCMC-TBOA: 3a)

Compound **12a** (97 mg, 0.12 mmol) was dissolved in CH_2Cl_2 (2 mL) and to the solution cooled in an ice bath TFA (0.5 mL) was added. The mixture was stirred for 1 h at 0 °C and then at room temperature for 4 h. The solvent was distilled off and the residue was purified by HPLC (10–30% CH_3CN/H_2O/0.1%TFA) to obtain 25 mg of **3a** (42% yield). ^1H NMR (DMSO-d_6) δ 4.45 (d, 1H, $J = 12.0$ Hz), 4.50 (d, 1H, $J = 3.6$ Hz), 4.62 (dd, 1H, $J = 3.6$, 10.0 Hz), 4.76 (d, 1H, $J = 12.0$ Hz), 5.25 (d, 1H, $J = 16.4$ Hz), 5.33 (d, 1H, $J = 16.4$ Hz), 6.4 (s, 1H), 6.95 (dd, 1H, $J = 2.4$, 9.2 Hz), 6.99 (d, 1H, $J = 2.4$ Hz), 7.28–7.60 (m, 5H), 7.61 (d, 1H, $J = 9.2$ Hz), 7.89 (d, 1H, $J = 9.2$ Hz). HRMS (FAB) m/z calcd for $C_{24}H_{22}NO_{12}(M+H)^+$ 516.1142, found 516.1142. Fluorescence $\lambda_{ex}/\lambda_{em}$ (1 μM): 320 nm/405 nm.

4.6. 6,7-Bis(*tert*-butoxycarbonylmethoxy)-4-(hydroxymethoxy)coumarin (6b)

Compound **6b** (1.70 g, 64%) was prepared by the same procedure as **6a**, from 6,7-bis(*tert*-butoxycarbonylmethoxy)coumarin-4-carbaldehyde[2c] (**5b**: 2.64 g, 6.07 mmol) and $NaBH_4$ (321.5 mg, 8.50 mmol). IR ν_{max}^{KBr} cm^{-1}: 3544, 1739, 1641, 1616, 1370, 1152, 1102. ^1H NMR ($CDCl_3$) δ 1.49 (s, 9H), 1.49 (s, 9H), 4.61 (s, 2H), 4.64 (s, 2H), 4.79 (d, 2H, $J = 9.6$ Hz), 6.46 (s, 1H), 6.71 (s, 1H), 7.03 (s, 1H). MS (ESI) m/z calcd for $C_{22}H_{28}O_9(M+H)^+$ 437, found 437.

4.7. Di-*tert*-butyl (2*S*,3*S*)-3-benzyloxy-*N*-{[6,7-bis(*tert*-butoxycarbonylmethoxy)coumarin-4-yl-methoxy]carbonyl}-aspartate (12b)

Compound **12b** (110.6 mg, 75%) was prepared by the same procedure as **12a**, from **6b** (342.7 mg, 0.79 mmol), trichloromethylchloroformate (0.18 mL, 1.48 mmol), **11** (63 mg, 0.18 mmol), and *i*-Pr_2NEt (0.31 mL, 1.78 mmol). ^1H NMR ($CDCl_3$) δ 1.36 (s, 9H), 1.37 (s, 9H), 1.40 (s, 9H), 1.42 (s, 9H), 4.34–4.37 (m, 2H), 4.55 (s, 2H), 4.57 (s, 2H), 4.69–4.75 (m, 2H), 5.13 (s, 2H), 5.61 (d, 1H, $J = 10.0$ Hz), 6.28 (s, 1H), 6.67 (s, 1H), 6.89 (s, 1H), 7.19–7.26 (m, 5H).

4.8. (2*S*,3*S*)-3-Benzyloxy-*N*-{[6,7-bis(carboxymethoxy)coumarin-4-yl-methoxy]carbonyl}aspartate (*N*-BCMCMC-L-TBOA: 3b)

Compound **3b** (61.6 mg, 52%) was obtained by the same procedure as **3a**, from compound **12b** (164 mg, 0.20 mmol) and TFA (2 mL). ^{1}H NMR (DMSO-d_6) δ 4.44 (d, 1H, $J = 12.0$ Hz), 4.49 (d, 1H, $J = 3.2$ Hz), 4.61 (dd, 1H, $J = 3.2, 9.6$ Hz), 4.75 (d, 1H, $J = 12.0$ Hz), 4.78 (s, 2H) 4.86 (s, 2H), 5.21 (d, 1H, $J = 16.8$ Hz), 5.31 (d, 1H, $J = 16.8$ Hz), 6.41 (s, 1H), 7.00 (s, 1H), 7.10 (s, 1H), 7.28–7.32 (m, 5H) 7.90 (d, 1H, $J = 9.6$ Hz). HRMS (FAB) m/z calcd for $C_{26}H_{24}NO_{15}(M+H)^+$ 590.1146, found 590.1127. Fluorescence $\lambda_{ex}/\lambda_{em}$ (1 μM): 340 nm/444 nm. $[\alpha]_D^{26}$ −9.01 (*c* 0.32, DMSO).

4.9. Di-*tert*-butyl (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoyl]-aminobenzyloxy}aspartate (14)

To a solution of compound di-*tert*-butyl (2*S*,3*S*)-*N*-*tert*-butoxycarbonyl-3-3-[4-(trifluoromethyl)benzoyl]aminobenzyloxyaspartate[8] (**13**: 149 mg, 0.23 mmol) in CH_2Cl_2 (2 mL), TFA (1 mL) was added at 0 °C and the mixture was stirred for 1 h at 0 °C. Saturated aqueous $NaHCO_3$ solution was added and the mixture was extracted with $CHCl_3$. The extract was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to obtain 91 mg of compound **14** (74% yield). ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.52 (s, 9H), 3.80 (d,1H, $J = 2.8$ Hz), 4.33 (d, 1H, $J = 2.8$ Hz), 4.43 (d, 1H, $J = 11.6$ Hz), 4.82 (d, 1H, $J = 11.6$ Hz), 7.13 (d, 1H, $J = 7.6$ Hz), 7.35(t, 1H, $J = 7.6$ Hz), 7.50 (s, 1H), 7.77 (d, 2H, $J = 8.4$ Hz), 7.88 (s, 1H), 8.00 (d, 2H, $J = 8.4$ Hz).

4.10. Di-*tert*-butyl (2*S*,3*S*)-*N*-[6,7-bis(*tert*-butoxycarbonylmethoxy)coumarin-4-yl-methoxy]carbonyl-3-{3-[4-(trifluoromethyl)benzoyl]aminobenzyloxy}aspartate (15)

To a solution of **6b** (301 mg, 0.69 mmol) in THF (4 mL), trichloromethylchloroformate (0.17 mL, 1.4 mmol) was added prior to reflux for 2 h, and then the solvent was distilled off. The residue was dissolved in CH_2Cl_2 (2 mL) and the resulting solution and *i*-Pr_2NEt (0.3 mL, 1.7 mmol) were added to a solution of compound **14** (91 mg, 0.17 mmol) in CH_2Cl_2 (2 mL), after which the mixture was stirred for 10 min. The solvent was distilled off and the residue was purified on a silica gel-plate (hexane/EtOAc = 2/1) to obtain 111 mg of compound **15** (64% yield). ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H), 1.46 (s, 9H), 1.49 (s, 9H), 1.50 (s, 9H), 4.42–4.47 (m, 2H), 4.61 (s, 2H), 4.63 (s, 2H), 4.78–4.81 (m, 2H), 5.20 (d, 2H, $J = 6.0$ Hz), 5.71 (d, 1H, $J = 10.4$ Hz), 7.10 (d, 1H, $J = 7.6$ Hz), 7.36 (t, 1H, $J = 7.6$ Hz), 7.54 (s, 1H), 7.76 (d, 2H, $J = 8.4$ Hz), 7.80 (d, 1H, $J = 7.6$ Hz), 8.02 (d, 2H, $J = 8.4$ Hz).

4.11. (2*S*,3*S*)-*N*-[6,7-Bis(carboxymethoxy)coumarin-4-yl-methoxy]carbonyl-3-{3-[4-(trifluoromethyl)-benzoyl)amino-benzyloxy}aspartate (*N*-BCMCMC-L-TFB-TBOA: 4)

Compound **15** (111 mg, 0.11 mmol) was dissolved in CH_2Cl_2 (4 mL) and to the solution cooled in an ice bath TFA (0.5 mL) was added. The mixture was stirred for 1 h at 0 °C and then at room temperature for 4 h. The solvent was distilled off and the residue was purified by HPLC (10–50% CH_3CN/H_2O/0.1%TFA) to obtain 42 mg of **4** (49% yield). ^{1}H NMR (DMSO-d_6) δ 1.6 (d, 3H, $J = 6.4$ Hz), 3.8 (s, 3H), 3.9 (s, 3H), 4.2 (d, 1H, $J = 11.2$ Hz), 4.4 (d, 1H, $J = 3.2$ Hz), 4.5 (d, 1H, $J = 4.6$ Hz), 4.7 (d, 1H, $J = 11.2$ Hz), 6.4 (q, 1H, $J = 6.4$ Hz), 7.04–7.06 (m, 2H), 7.20–7.22 (m, 4H), 7.51 (s, 1H). Q-TOF-MS m/z 777 $(M+H)^+$, 427. HRMS (FAB) m/z calcd for $C_{34}H_{28}F_3N_2O_{16}(M+H)^+$ 777.1391, found 777.1395. $[\alpha]_D^{25}$ +92.7 (*c* 0.65, DMSO).

4.12. Photolysis of caged compounds with UV-handy lamp

A solution (300 μL) of 100 μM of the caged compound in PBS(+) buffer (phosphate buffered saline including 137 mM NaCl, 2.7 mM KCl, 8.1 mg Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) was placed in quartz cuvettes with a path length of 1 mm in a dark box and irradiated at room temperature for 5–120 min. Irradiation condition was as follows: Spectroline ENF-260C/J (Spectronics Co., USA), 365 nm, max power at 10 cm from the lamp: 2.3 mW/cm^2. Irradiated solution was analyzed by HPLC [Column; SP-120-5-ODS-AP (Daiso Co. Ltd, Japan), 150 mm × 6 mm ID: Eluent: 30% CH_3CN/H_2O/0.1%TFA for **1a**, **1b**, **3a**, and **3b** or 30–50% CH_3CN/H_2O/0.1%TFA for **4**; Flow rate; 1 mL/min.; Detection; 320 and 254 nm.] As an internal standard for the quantitative analyses, 10 μM of 3′,4′-dimethoxyacetophenone (R_t: 11.1 min) for **1a** (R_t: 5.3 min), **1b** (R_t: 10.7 min), **3b** (R_t: 9.0 min), and **4** (R_t: 25.0 min) or 40 μM of 2-(3,4-dimethoxyphenyl)ethanol (R_t: 6.7 min) for **3a** (R_t: 12.5 min) was included in the sample solution.

4.13. Photolysis of caged compounds with YAG-laser

A solution (100 μL) of 100 μM of the caged compound in PBS(+) was placed in quartz cuvettes with a path length of 1 mm and irradiated at room temperature. For the uptake assay 1 μM solution of **4** was used. Irradiation condition was as follows: SureLite II (Continuum, Santa Clara, CA, USA), 355 nm, 8 ns pulse, 40 mJ. Irradiated solution was analyzed by HPLC as above.

4.14. [^{14}C]Glutamate uptake assay

MDCK cells stably expressing EAAT2 were seeded onto 96-well plates and cultured for 2 days before the uptake assay. The subconfluent cells were washed two times with 300 μL of modified PBS(+) that contained 10 mM D-glucose, pH 7.4 and preincubated in 300 μL of the same buffer at 37 °C for 12 min. After aspiration of the buffer, cells were incubated with 1 μM L-[^{14}C]glutamate in 80 μL of modified PBS(+) in the absence or presence of test compounds at various concentrations at 37 °C for 12 min. To determine the uptake, cells were washed three times with ice-cold buffer and radioactivity was measured by scintillation counting in 100 μL of

Microscinti 40 (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA). Nonspecific incorporation was determined in sodium-free solution (140 mM choline chloride, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM D-glucose, pH 7.4). Specific uptake of [^{14}C]glutamate is given relative to the control. All values displayed are mean ± SEM of at least three determinations. Dose–response curves were generated by using computer software GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

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11. The same reaction using **7b** prepared from triphosgen and **6a** yielded **12a** in only 8%.
12. Even if L-TFB-TBOA was irradiated for 60 min in the presence or in the absence of BCMCM-OH, the amount monitored by HPLC did not change, indicating that L-TFB-TBOA does not decompose by UV-irradiation.
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14. The concentration of L-TFB-TBOA in the photolysis samples estimated from the uptake assay was well consistent with that determined from the HPLC analysis. We also confirmed that neither BCMCM-OH nor CMCM-OH affected the glutamate uptake.
15. The transient absorption during the laser irradiation was measured using a photomultiplier tube and monochromatic light from a 150-W xenon lamp. The photocurrent was fed into an oscilloscope (TDS340AP, Sony-Tektronics, Tokyo, Japan). The accurate rate of the photolysis of coumarin-carbamate **3b**, **4** could not be obtained in the same case as the esters **1**,[7] because these coumarin-derivatives showed very strong fluorescence, of which the decay curves were very complex. Nevertheless, the change in the transient spectroscopy was very fast and was completed within 1.5 μs. It was reported that the photolytic uncaging and release of the carbamate-type derivatives occur in two steps, consisting of an initial light-induced cleavage and a subsequent light-independent, pH-dependent decarboxylation step that proceeds on the millisecond time scale.[9] The latter step would be rate-limiting for release of free amino acid (L-TBOA or L-TFB-TBOA) if the photocleavage process of the coumarin-moiety is fast. To estimate the kinetics of **3b** and **4**, electrophysiological measurements would be needed.
16. The chemical yields of the photolysis by repeated irradiation (8 shots) were 5.5 ± 1.5% (**3a**), 43 ± 1.0% (**3b**), and 42 ± 1.9% (**4**), respectively.
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18. The activity of L-TFB-TBOA was slightly less potent than the previous result ($IC_{50} = 1.9 \pm 0.1$ nM).[8] The value of IC_{50} would vary according to the condition of using cells.