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Synthesis and Photoreactivity of Caged Blockers for Glutamate Transporters

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Abstract—L-TBOA (L-*threo*-β-benzyloxyaspartate) is, so far, the most potent non-transportable blocker for glutamate transporters. We synthesized α-CMCM-L-TBOA (**1a**) possessing [7-(carboxymethoxy)coumarin-4-yl]methyl ester as a caging group. α-CMCM-L-TBOA (**1a**) is biologically inactive until UV irradiation and the photolysis of **1a** immediately released L-TBOA to show glutamate uptake inhibition. The photoreactivity of the coumarin-type caging group was superior to that of the *o*-nitrobenzyl-type caging group.

L-Glutamate plays major roles in the excitatory neurotransmission in mammalian central nervous systems (CNS). Glutamate transporters (excitatory amino acid transporters: EAATs) maintain the extracellular glutamate concentrations at low level to limit the receptor activation and to protect neurons from the excitotoxicity.[1] Recent studies have revealed that EAATs modulate synaptic transmission.[2] Signal transmission can occur on a milisecond order and, thus, methods for the activation and/or inactivation of EAATs with good time resolution are strongly required.

Caged compounds whose activities are masked by a photocleavable group are a useful tool to overcome the limitations in time resolution.[3] An active compound can be generated at the desired time and position by a photochemical reaction using pulsed UV-light irradiation. In order to elucidate the regulation of EAATs at excitatory synapses, we synthesized caged derivatives of L-TBOA (L-*threo*-β-benzyloxyaspartate), which is, so far, the most potent non-transportable blocker for all subtypes of glutamate transporters (EAAT1-5).[4]

Although *o*-nitorobenzyl-type groups have been most widely used as photocleavable protective groups, recently coumarin derivatives were reported as more sensitive caged compounds.[5,6] Therefore, we synthesized the [7-(carboxymethoxy)coumarin-4-yl]methyl (CMCM) ester of L-TBOA (**1a**: α-CMCM-L-TBOA) and the (7-methoxycoumarin-4-yl)methyl (MCM) ester (**1b**: α-MCM-L-TBOA). We also synthesized the 2-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) esters (**3a**, **b**) to compare the photosensitivity.[7] By photolysis, coumarin-esters (**1a**, **b**) generate L-TBOA and the corresponding alcohols (**2a**, **b**) (eq 1) whereas DMNPE-esters (**3a**, **b**) provide L-TBOA and 4′,5′-dimethoxy-2-nitrosoacetophenone (**4**) (eq 2).

The preparation of esters from carboxylic acid (**5**) and alkyl halide or alcohol under basic conditions[8] resulted in significant epimerization of the amino group or the benzyloxy group and low yield. Therefore, we used diazoalkane under neutral conditions.[5b,9a] Treatment of **5** with the corresponding diazoalkane **6a**[9a] in benzene provided the protected ester (**7a**) in 69% yield (Scheme 1). No epimerization was detected by 400 MHz ^{1}H NMR. Deprotection of **7a** with TFA followed by HPLC purification gave α-CMCM-L-TBOA (**1a**).[10] MCM analogue **1b** was synthesized in the same manner.[10]

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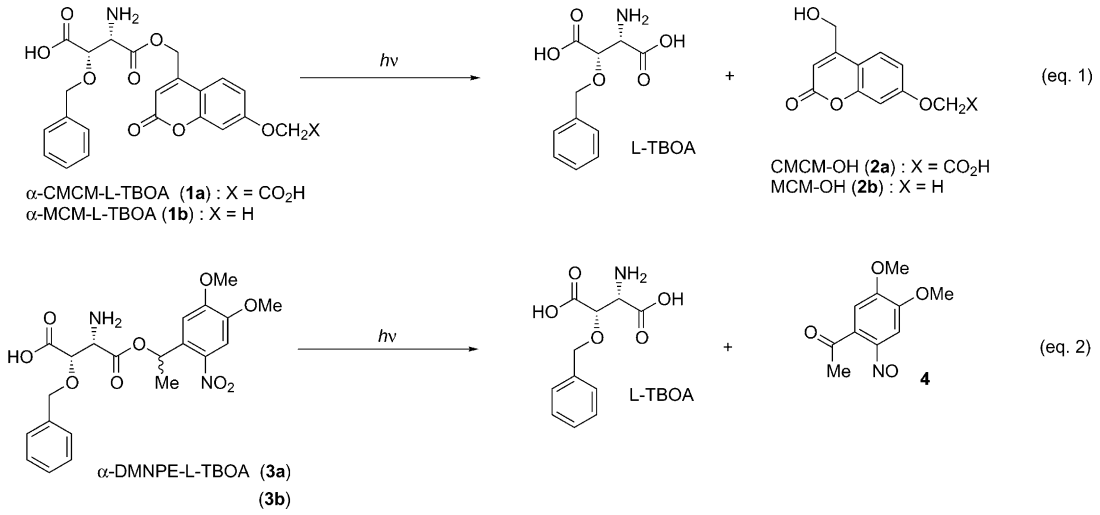
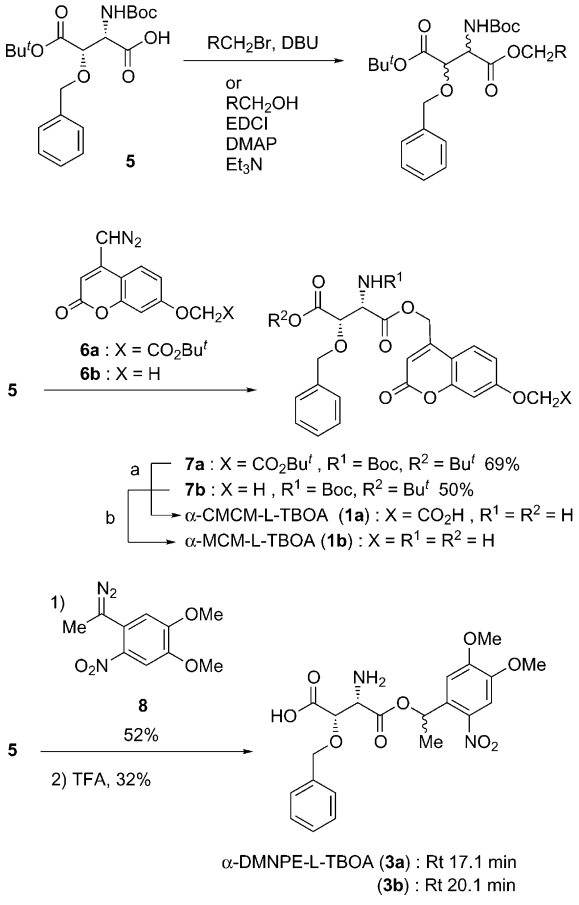


Similarly, α-DMNPE-L-TBOA (**3a**, **b**) were synthesized from **5** and diazoalkane **8**.[9b–d] Although the diastereomers (**3a** and **3b**) were separated by HPLC (Rt: 17.1 and 20.1 min), the configuration of the DMNPE group could not be determined (Scheme 1).[11]

Prior to the physiological application, we checked the photosensitivity of the caged compounds. A solution of caged TBOAs during steady-state irradiation with UV lamp (max power at 10 cm from the lamp: 2.3 mW/cm^2) was analyzed by HPLC.[12,13] HPLC analyses indicated that the peak of **1a** (Rt: 5.3 min) decreased during UV-irradiation while that of **2a** (Rt: 4.5 min) increased (Fig. 1a–d). CMCM-ester (**1a**) completely disappeared within 20 min. We found that a part of CMCM-OH (**2a**) was further converted to the decarboxylated alcohol MCM-OH (**2b**: Rt: 8.0 min)[14] but decarboxylation from **1a** to **1b** (Rt: 10.7 min) was not observed. The rate of disappearance of α-MCM-L-TBOA (**1b**) was almost the same as that of **1a** (data not shown). On the other hand, DMNPE-ester (**3a**) hardly decomposed after 30 min (Fig. 2a and b). The peaks of **3a** (Rt: 17.1 min) and **3b** (Rt: 20.1 min) were still detected after 180 min-irradiation (Fig. 2c and d). Although the photolysis products were not identified, **3a** and **3b** provided the same peaks (Rt: 15.0, 18.9 and 23.2 min). Quantitative analyses revealed the half-life of **1a** and **1b** was 3.7 and 3.9 min, respectively, while that of **3a** and **3b** was 57.2 and 45.2 min, respectively. Therefore, it is found that coumarin-esters are much more photosensitive than nitrobenzyl-esters.[5,6] Although the irradiated caged TBOA solution contained byproducts, the disappearance of the starting materials can be fitted to single exponential decay, suggesting the photolysis proceeds without any interference of the photolysis products.



Scheme 1. (a) TFA, 50%; (b) TFA, 22%.

Restoring of L-TBOA from **1a** by photolysis was directly confirmed by the glutamate uptake inhibition assay on EAAT2 stably expressed on MDCK cells,[15] since it was difficult to quantitate L-TBOA by HPLC. The activity of the caged compound should be masked until the photoirradiation. Before UV irradiation, the inhibitory activity of **1a** (IC_{50}: 124 μM) was 100-fold less potent than that of L-TBOA (IC_{50}: 1.3 μM) and **1a** was practically inactive at the effective concentration range of L-TBOA. Its activity was restored time-dependently as shown in Figure 3, as we expected. The IC_{50} values of **1a** with UV irradiation (365 nm) were as follows: 24 μM (1 min), 8.6 μM (5 min), 7.0 μM (10 min), 5.6 μM (20 min). However, the activity was not completely restored after 20-min irradiation, in which the peak of **1a** disappeared in HPLC, and even if **1a** was irradiated for more than 20 min, the inhibitory activity was not

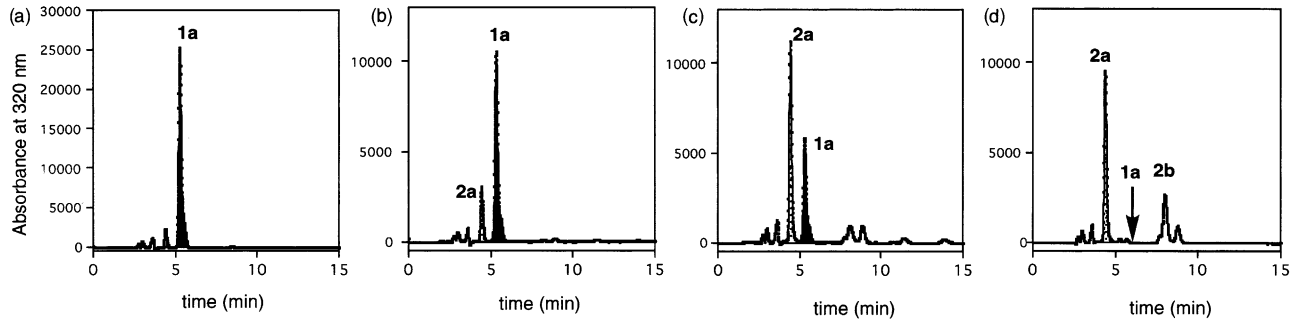


Figure 1. HPLC analysis of α-CMCM-L-TBOA [**1a**: 100 μM solution in PBS(+) buffer] and the products with UV irradiation (365 nm) monitored at 320 nm. Condition of HPLC: 32% CH_3CN/H_2O/0.1% TFA. Irradiation time of sample: (a) 0 min, (b) 1 min, (c) 5 min, (d) 20 min.

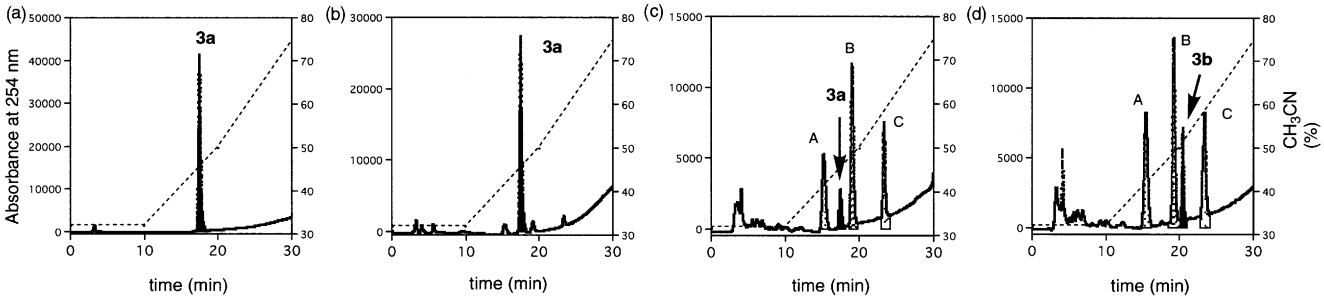


Figure 2. HPLC analyses of α-DMNPE-L-TBOA [**3a** and **3b**: 100 μM solution in PBS(+) buffer] and the products with UV irradiation (365 nm) monitored at 254 nm. Condition of HPLC: Dashed lines indicate the acetonitrile gradient (32–77% CH_3CN/H_2O/0.1% TFA). Irradiation time of sample: (a) 0 min, (b) 30 min, (c) 180 min for α-DMNPE-L-TBOA (**3a**), (d) 180 min for α-DMNPE-L-TBOA (**3b**). Photolysis of **3a** and **3b** gave the peaks of the same retention time (A: 15.0, B: 18.9 and C: 23.1 min).

improved. The activity of L-TBOA was not disturbed after 120-min-irradiation and CMCM-OH (**2a**) or MCM–OH (**2b**) produced by photolysis did not affect the inhibitory activity (data not shown). These results indicate that an unknown byproduct(s) was produced by the photoreaction. The byproduct(s) could not be specified by HPLC in both 320 and 254 nm detection, suggesting the byproduct is L-TBOA possessing a decomposed coumarin ring.[16] There was no obvious difference in IC_{50} values among the other caged TBOAs (**1b**: 124 μM, **3a**: 103 μM and **3b**: 111 μM), showing that type of ester group is not important for the caging effect.

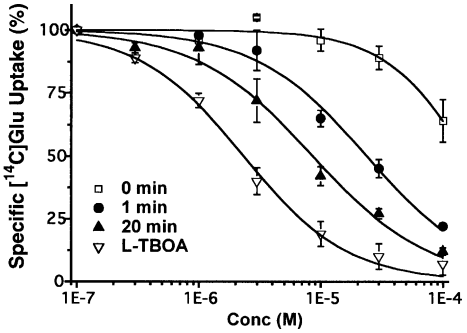


Figure 3. Release of L-TBOA from α-CMCM-L-TBOA (**1a**) with UV lamp irradiation (365 nm). Inhibition of [^{14}C]Glu uptake in MDCK cells expressing EAAT2 was measured as previously reported.[15] Non-irradiated α-CMCM-L-TBOA (**1a**) (□) was much less potent than L-TBOA (▽). Irradiated samples of **1a** restored the activity according to the irradiation time (1–20 min). Values are presented as mean ± SEM of at least three determinations.

The photosensitivity of the caged TBOAs and the generation of L-TBOA were confirmed by the UV lamp irradiation. Using laser-pulse, which is higher energy than UV light, in a sample specimen would be more useful for physiological applications because the active compound can be generated at the desired time and position in a moment. Therefore we next examined photolysis of these compounds by using YAG Laser (SureLite II, Continuum, Santa Clara, CA, USA, 355 nm, 8 ns pulse, 40 mJ).[17] The photochemical quantum yields (ϕ_q) were determined from the disappeared amount of the caged compounds and the absorbed light intensity (Table 1). The concentrations of the remaining caged compounds after one shot irradiation were measured from HPLC and the chemical reaction yields (ϕ_c)

Table 1. Spectroscopic and photolytic characteristics of caged compounds

Compd	UV absorption λ_{max} (nm)[a] (ε: M^{-1} cm^{-1})	Laser photolysis 1 shot (40 mJ) 355 nm		
		ϕ_c[b]	ϕ_q[b]	$\varepsilon_{355}\phi_q$ (ε_{355}: M^{-1} cm^{-1})[c]
α-CMCM-L-TBOA (**1a**)	325 (4600)	8 ± 2.3	0.04	44 (1100)
α-MCM-L-TBOA (**1b**)	325 (4300)	13 ± 0.6	0.06	66 (1100)
α-DMNPE-L-TBOA (**3a**)	352 (4000)	3 ± 2.1	0.005	20 (3900)
α-DMNPE-L-TBOA (**3b**)	352 (3300)	n.d.[d]	n.d.[d]	n.d.[d]

[a]ε, wavelength of absorbance maximum (λ_{max}) and extinction coefficient in 100 μM solution in PBS(+) solution.
[b]ϕ_c, chemical yield; ϕ_q, quantum yield.
[c]$\varepsilon_{355}\phi_q$, product of the quantum yield and extinction coefficient (indication of the efficiency of photolysis).
[d]Not determined.

were obtained by the subtracted concentrations.[18] The light intensity was determined by using potassium ferrioxalate actinometry, and the absorption factor at 355 nm (ε_{355}) of each compound was obtained from the UV spectra. Chemical yields (ϕ_c), quantum yields (ϕ_q) and $\varepsilon_{355}\phi_q$ values (indication of the efficiency of photolysis: high value reflects high efficiency)[19] of coumarin-esters (**1a**, **b**) were better than those of DMNPE-ester (**3a**). As the ε_{355} values of **1a**, **b** were relatively small, laser irradiation with closer wavelength to λ_{max} (325 nm) would more efficiently provide L-TBOA. Alternatively, introduction of substituents on the coumarin ring to shift λ_{max} to longer wavelength would be useful.[5,10]

It is well-known that decomposition of nitrobenzyl compounds can be monitored by the transient absorption at 420 nm of its aci-nitro intermediate.[20] 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 half-life of aci-nitro intermediates for **3a** and **3b** was 445 and 507 ms, respectively. On the other hand, the accurate rate of the photolysis of coumarin-esters (**1a**, **b**) could not be obtained from the transient absorption spectra at intervals of 20 nm from 380 to 600 nm with or without O_2, because these coumarin-esters showed very strong fluorescence at 400 nm,[10] of which the decay curves were very complex (data not shown).[5d] In the transient absorption of α-CMCM-L-TBOA (**1a**) at 500 nm, a very sharp peak was detected at 0.15 μs. It was reported that the transient absorption at 480 nm in degassed acetonitrile is assigned to the triplet state of *N*,*N*,*N*-tributyl-*N*-(4-methylene-7-methoxycoumarin) ammonium borates.[5d] However, we could not conclude whether the observed peak of **1a** reflects its triplet state in aqueous solution, because such peak was also detected in the transient absorption of CMCM-OH (**2a**: 540 nm) but not in those of α-MCM-L-TBOA (**1b**) and MCM-OH (**2b**). Nevertheless, the change in the transient spectroscopy was completed within 1.5 μs and the reaction pathway of the photolytic cleavage of **1a** and **1b** is thought to be the same as that of the coumarin caged compounds reported previously.[5,6] Therefore, the liberation of L-TBOA from **1a** and **1b** is much faster than that from **3a** and **3b**, in agreement with the photosensitivity of these compounds using UV lamp, and the rapid generation of the blocker would enable the precise kinetic analysis of EAATs.

The HPLC analysis of the non-irradiated sample **1a** revealed a small contamination of **2a**, suggesting decomposition of the caged compound in a solution. Therefore, we examined the chemical stability of **1a** both in aqueous solution and in DMSO (Fig. 4) In the aqueous buffer [PBS(+), pH 7.4], **1a** was stable at −20 °C but gradually decomposed at 0 °C and it was rather unstable at room temperature. Hydrolysis of the coumarin-ester occurred even in the dark condition.[21] In contrast, DMSO solution was extremely stable and only a little decomposition was observed after more than 30 days (0 °C, dark). As far as a stock solution of **1a** is prepared in DMSO and is added to the buffer just prior to the physiological experiments, it would be stable enough during the experiments. Similarly, we also confirmed that **1b** is stable in DMSO for more than 30 days (data not shown). Both DMNPE-esters (**3a**, **b**) were more stable in the aqueous buffer [PBS(+), pH 7.4] than the coumarin-esters at 0 °C, but the benzyl-esters were also hydrolyzed. However, the chemical stability of diastereomers (**3a, b**) at room temperature was different. Only **3b** unexpectedly decreased by 50% for a month even in DMSO at room temperature. The reason for the difference in diastereomers (**3a** and **3b**) is unclear.

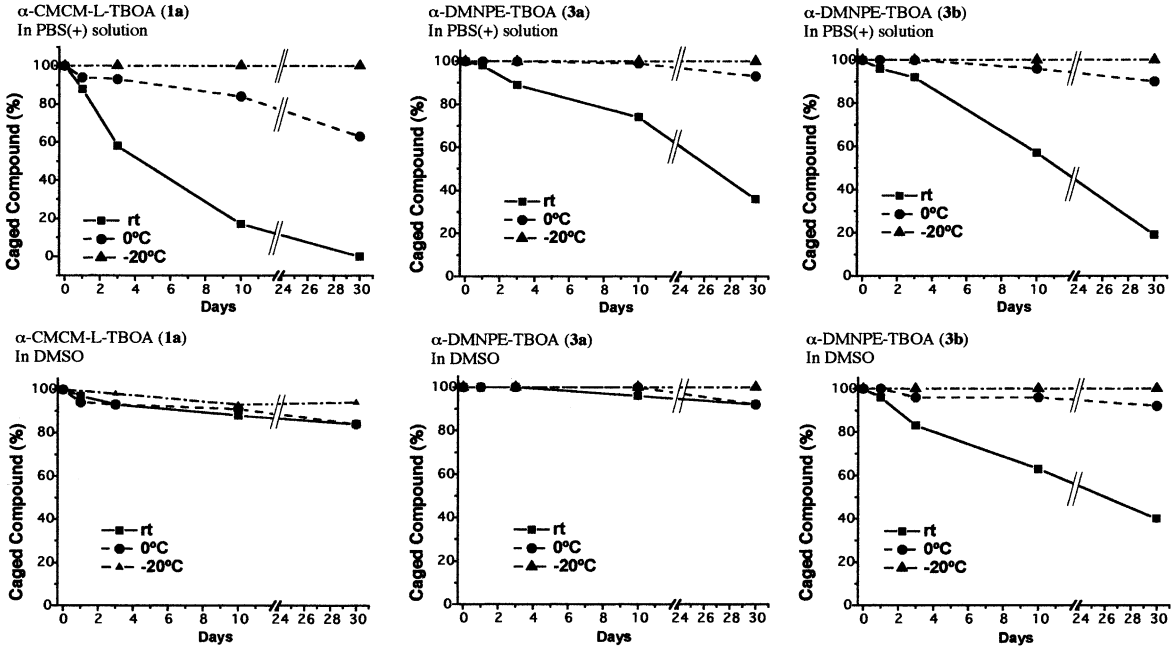


Figure 4. Stability of caged compounds (**1a**, **3a**, and **3b**) in PBS(+)(pH 7.4) or DMSO. The concentrations of the caged compounds were quantified by HPLC.

In summary, we have synthesized caged blockers for glutamate transporters, α-CMCM-L-TBOA (**1a**) and α-MCM-L-TBOA (**1b**), which are practically inactive around the effective concentration of L-TBOA while rapidly generating L-TBOA by UV lamp irradiation or laser-pulse irradiation. They are stable in DMSO solution and can be stocked for several weeks. Therefore, these compounds would be useful tools for elucidating the physiological roles of transporters. Applications of these samples to electrophysiological studies are now in progress.

Acknowledgements

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10. **α-CMCM-L-TBOA** (**1a**): ^{1}H NMR (DMSO-d_6) δ 4.4 (d, 1H, J = 11.6 Hz), 4.5 (d, 1H, J = 3.2 Hz), 4.7 (br, 1H), 4.7 (d, 1H, J = 11.6 Hz), 4.8 (s, 2H), 5.3 (d, 1H, J = 15.6 Hz), 5.5 (d, 1H, J = 15.6 Hz), 6.4 (s, 1H), 6.9 (dd, 1H, J = 2.4, 8.8 Hz), 7.0 (d, 1H, J = 2.4 Hz), 7.2–7.3 (m, 5H), 7.6 (d, 1H, J = 8.8 Hz). HRMS (FAB) m/z calcd for $C_{23}H_{22}NO_{10}$ $(M+H)^+$ 472.1244, Found 472.1244. $[\alpha]_D^{28}$ +225.2° (c 0.46, DMSO), mp 148–150 °C. Fluorescence $\lambda_{ex}/\lambda_{em}$ (1 μM): 320 nm/401 nm. **α-MCM-L-TBOA** (**1b**): ^{1}H NMR (DMSO-d_6) δ 3.9 (s, 3H), 4.5 (d, 1H, J = 12 Hz), 4.6 (d, 1H, J = 4.6 Hz), 4.7–4.8 (m, 2H), 5.3 (d, 1H, J = 15.6 Hz), 5.5 (d, 1H, J = 15.6 Hz), 6.4 (s, 1H), 6.9 (dd, 1H, J = 2.4, 8.8 Hz), 7.0 (d, 1H J = 2.4 Hz), 7.2–7.3 (m, 5H), 7.6 (d, 1H, J = 8.8 Hz). HRMS (FAB) m/z calcd for $C_{22}H_{22}NO_8$ $(M+H)^+$ 429.1345, Found 428.1361. $[\alpha]_D^{28}$ −13.1° (c 0.35, DMSO). Fluorescence $\lambda_{ex}/\lambda_{em}$ (1 μM): 320 nm/407 nm.
11. **α-DMNPE-L-TBOA** (**3a**): ^{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). HRMS (FAB) m/z calcd for $C_{21}H_{25}N_2O_9$ $(M+H)^+$ 449.1560, Found 449.1563. $[\alpha]_D^{25}$ −749.1° (c 0.50, DMSO). **α-DMNPE-L-TBOA** (**3b**): ^{1}H NMR (DMSO-d_6) δ 1.44 (d, 3H, J = 6 Hz), 3.84 (s, 3H), 3.90 (s, 3H), 4.4 (d, 1H, J = 2.8 Hz), 4.51–4.54 (m, 2H), 4.8 (d, 1H, J = 11.6 Hz), 6.1 (q, 1H, J = 6.4 Hz), 7.1 (s, 1H), 7.6–7.35 (m, 5H), 7.57 (s, 1H). HRMS (FAB) m/z calcd for $C_{21}H_{25}N_2O_9$ $(M+H)^+$ 449.1560, Found 449.1578. $[\alpha]_D^{26}$ −65.7° (c 0.52, DMSO).
12. Condition of analytical HPLC: Column; SP-120-5-ODS-AP (Daiso Co. Ltd. Japan), 150 mm × 6 mm I.D.: Eluent; CH_3CN/H_2O/TFA = 32:68:0.1. Flow rate; 1 mL/min; Detection; 320 nm and 254 nm. As an internal standard for the quantitative analyses, 10 μM of 3′,4′-dimethoxyacetophenone (Rt: 11.1 min) for **1a**, **3a** and **3b** or 40 μM of 2-(3,4-dimethoxyphenyl)ethanol (Rt: 6.7 min) for **2a** was included in the sample solution.
13. Irradiation condition: UV handy-lamp; Spectroline ENF-260C/J (Spectronics Co. USA), 365 nm: Sample solution; 100 μM in PBS(+) buffer (phosphate buffered saline including 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). A solution (300 μL) of the caged compounds was placed in quartz cuvettes with a path length of 1 mm in a dark box and irradiated at room temperature for 1–240 min.
14. We confirmed that CMCM-OH (**2a**) was gradually converted to MCM-OH (**2b**) with UV irradiation. The structure of the product was determined by ^{1}H NMR. **CMCM-OH** (**2a**): ^{1}H NMR (DMSO-d_6) δ 4.7 (s, 2H), 4.8 (s, 2H), 5.6 (br, 1H), 6.2 (s, 1H), 6.93–6.97 (m, 2H), 7.61 (d, 1H, J = 8.8 Hz). Mp >260 °C. Fluorescence $\lambda_{ex}/\lambda_{em}$ (1 μM): 320 nm/400 nm. **MCM-OH** (**2b**): ^{1}H NMR (DMSO-d_6) δ 3.8 (s, 3H), 4.7 (dd, 2H, J = 1.6, 5.6 Hz), 5.5 (t, 1H, J = 5.6 Hz), 6.2 (s, 1H), 6.9 (dd, 1H, J = 2.4, 9.2 Hz), 7.0 (d, 1H, J = 2.4 Hz), 7.6 (d, 1H, J = 9.2 Hz). Fluorescence $\lambda_{ex}/\lambda_{em}$ (1 μM): 320 nm/402 nm.
15. The uptake assay was performed as previously described[4a] except that MDCK cells stably expressing EAAT2 were used instead of the transiently transfected COS-1 cells. The relative specific uptake of [^{14}C]glutamate (1 μM) was determined from three different experiments. The Michaelis constant (K_m) and IC_{50} values of L-glutamate for EAAT2 were 63 ± 3.8 and 41 ± 1.9 μM, respectively.
16. MCM-OH (**2b**) was decomposed 20% for 30 min and 50% for 120 min with UV lamp irradiation (365 nm).
17. A sample [100 μM in PBS(+) solution, 100 μL] placed in a quartz cuvette (path length 1 mm) was irradiated with laser pulse.
18. The chemical yields of the photolysis by repeated irradi-

ation (8 shots) were 38±1.6% (**1a**), 34±5.6% (**1b**), and 21±2.9% (**3a**), respectively.
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21. For the purification of the caged compounds, column of SP-120-5-ODS-AP (Daiso Co. Ltd. Japan), 250 mm × 20 mm I.D. was used and peaks were monitored at only 254 nm to avoid decomposition. Because **1a** was hydrolyzed during the lyophilization after HPLC purification, we could not remove a small contamination of L-TBOA from **1a**. It may contribute to the activity at the higher concentrations.