Synthesis of a caged glutamate for efficient one- and two-photon photorelease on living cells

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We have synthesized a biologically inert, photosensitive derivative of the major excitatory amino acid, L-glutamate (which we call MDNI-glu) that makes more efficient use of incident light than all other caged glutamates. Laser flash photolysis of MDNI-glu in acutely isolated hippocampal brain slices evoked a rapid increase in intracellular Ca^{2+} concentration in astrocytes.

Photochemically initiated release ("uncaging") of signalling molecules in or on cells is now a standard technique by which physiologists, neuroscientists, biochemists, and cell biologists switch on the biological process they study. Molecules as diverse as cAMP (and many other nucleotides), calcium, IP_3, glutamate (and many other neurotransmitters), peptides, T4 lysozyme, antibodies, proteases, and kinases have all been caged.[1] Such compounds have become essential chemical tools for many biologists because the uncaging technique has many useful features and advantages compared to other methods for changing solute concentration. Namely, release can be (a) intracellular, (b) ultrafast, (c) timed, controlled and repeated at any point during an experiment, (d) physically non-perturbing and (e) highly localized (*i.e.* sub-cellular). It is this last property that we focus on in this report. We have synthesized a new caged glutamate (4-**m**ethoxy-5,7-**di**nitro**i**ndolinyl-glutamate, or MDNI-glu, **1**). The MDNI caging chromophore substantially improves the properties of previous caged glutamates (*e.g.* CNB-glu or MNI-glu), and will thus significantly reduce the power requirements for uncaging on living cells, giving access to new biological experiments because of extended cell viability.

The synthesis of MDNI-glu is outlined in Fig. 1 and is based upon our synthesis MNI-glu.[2] 4-Methoxyindole was reduced in essentially quantitative yield using $NaBH_3CN$ in acetic acid to the 4-methoxyindoline. This was coupled to N-*tert*-BOC-L-glutamic acid α-*tert*-butyl ester using standard DCCD coupling conditions to give methoxyindoline **2**. Dinitration of compound **2** was accomplished by sequential nitration with acetyl chloride and silver nitrate to give **3**, followed by treatment of **3** with fuming nitric acid

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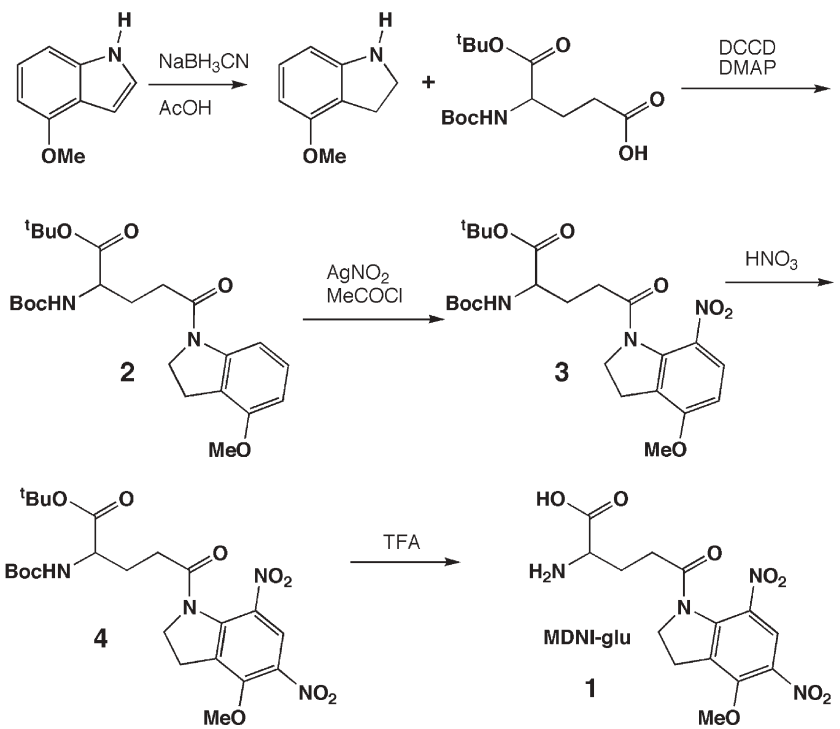

Fig. 1 Synthesis of MDNI-glu.

in acetic anhydride to give **4** (19% yield), which was deprotected by TFA treatment to give MDNI-glu.†

The quantum yield for uncaging of MNI-glu in water at pH 7 is 0.085.[3] HPLC analysis of a photolyzed solution of MNI-glu and MDNI-glu (mixed in a 2 : 1 ratio, ensuring equal absorptivities) revealed that the latter is about 5.5 times more photosensitive than the former, implying that the additional nitro group at the 5-position of MDNI-glu has a dramatic effect on the decay of the key intermediate **5** (Fig. 2, this reaction mechanism is well established).[4] The presence of an electron-withdrawing group at the 5-position promotes deprotonation at C–2, preventing reversion of **5** to **1**, enhancing the quantum yield of N–O bond scission to give the desired glutamate.[4]

We have previously determined that the 2-photon cross section for uncaging of MNI-glu in physiological buffer is 0.06 GM (GM is 10^{-50} cm^4 s photon^{-1}).[2] We have tested the effect of the 5-nitro group on this physico-chemical property. We utilized the direct output from a mode-locked Ti:sapphire laser (10 W Verdi-Mira, Coherent) to perform 2-photon uncaging. The beam from the Mira laser has a diameter of approximately 0.8 mm and an energy of 900 mW at 730 nm. Irradiation of an equimolar solution (pH 7.4 and 300 mOsm) of MNI-glu and MDNI-glu for 3–4 hours in a cuvette (total volume of 20 μL) revealed that the 2-photon cross section for uncaging of MDNI-glu is about 0.06 GM. We could detect no hydrolysis of either caged glutamate by HPLC during this period.

The absorption spectrum of MDNI-glu is shown in Fig. 3 (orange line). The caged compound undergoes smooth photolysis to the indole photoproduct (**6**), as shown by the clean isobestic point. The identity of **6** was confirmed by NMR and MS.

The properties of several caged glutamates are summarized in Table 1. It can be seen from these data that MDNI-glu makes more efficient use of incident light (*i.e.* the product $\phi.\varepsilon$) than all other caged glutamates.

When a solution of MDNI-glu (200 μM) was applied to acutely isolated brain slices from mouse hippocampus, no stimulation of glutamate receptors could be detected by confocal imaging of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Irradiation of this solution with a focused laser (continuous wave Ar, 30 mW at 351–364 nm, 3 μm diameter, for 30 ms) evoked rapid increase in $[Ca^{2+}]_i$ *via* activation of metabotropic glutamate receptors on the

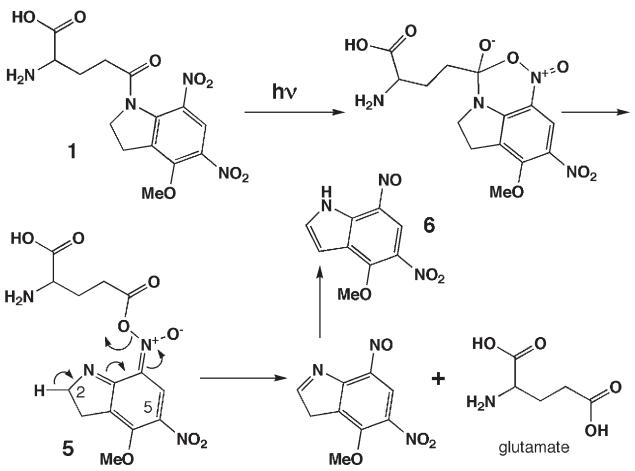

Fig. 2 Photorelease of glutamate from MDNI-glu.

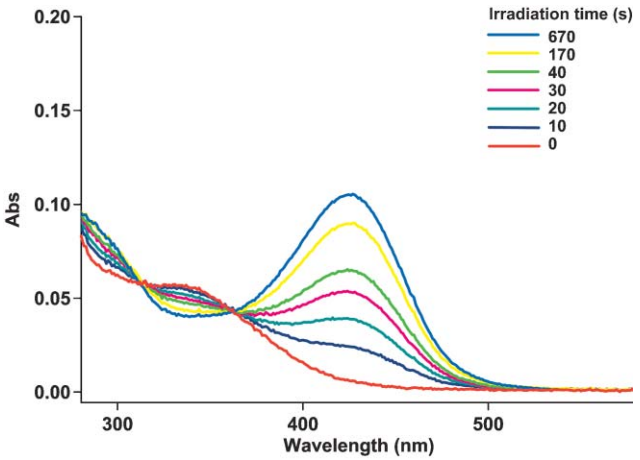

Fig. 3 Absorption spectrum of MDNI-glu (orange), and time-course of conversion to photoproduct **6**.

astrocyte cell body (see Fig. 4: cells were loaded with fluo-4 AM, and imaged using laser-scanning confocal microscopy. The period for each image frame was 1.4 s, and the frames were at 9 s intervals. Uncaging (at the red dot in frame 1) was immediately before the 2nd frame. The scale bar, lower right, is 30 μm and the images are 8 bit depth).

In this report we introduce a new photochemical protecting group for carboxylates (MDNI) and have used this moiety to cage L-glutamate. MDNI-glu (**1**) has a superior combination of physico-chemical properties when compared to all other previous synthesized caged glutamates (Table 1). Photorelease of glutamate from MDNI-glu evoked rapid increases in $[Ca^{2+}]_i$ in living brain slices, demonstrating the effectiveness of the caged compound for neurobiological experiments. We believe that photolysis of MDNI-caged neurotransmitters will permit much improved 4-dimensional mapping of excitatory neuroreceptors on living cells (both in brain slices[9] and *in vivo*) with 2-photon uncaging at energies much lower than were previously required.[2,6,9,10] MNI-glu requires 5–10 mW of 720 nm light to mimic quantal release.[2] MDNI-glu will require approximately 2.5-fold less energy in the image plane to produce similar quantities of neurotransmitter. Since 2P-induced photo-damage is known to be highly non-linear,[11] use of this new photosensitive neurotransmitter for 2-photon uncaging in brain slices and *in vivo* should provide access to a whole panoply of long-term experiments.

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Table 1 Summary of the properties of caged glutamates

Chromophore	Quantum yield (ϕ)	2-photon cross section (730 nm)/ GM	Extinction coefficient (ε)(350 nm)/ M^{-1} cm^{-1}	$\phi.\varepsilon$
MDNI	0.47	0.06	8600	4042
MNI	0.085	0.06	4300	366
NI[5]	0.043	No datum	2700	116
Bhc[6]	0.019	0.95	17300	329
CNB[7]	0.15	<0.001	500	75
pHP[8]	0.08	No datum	200	16

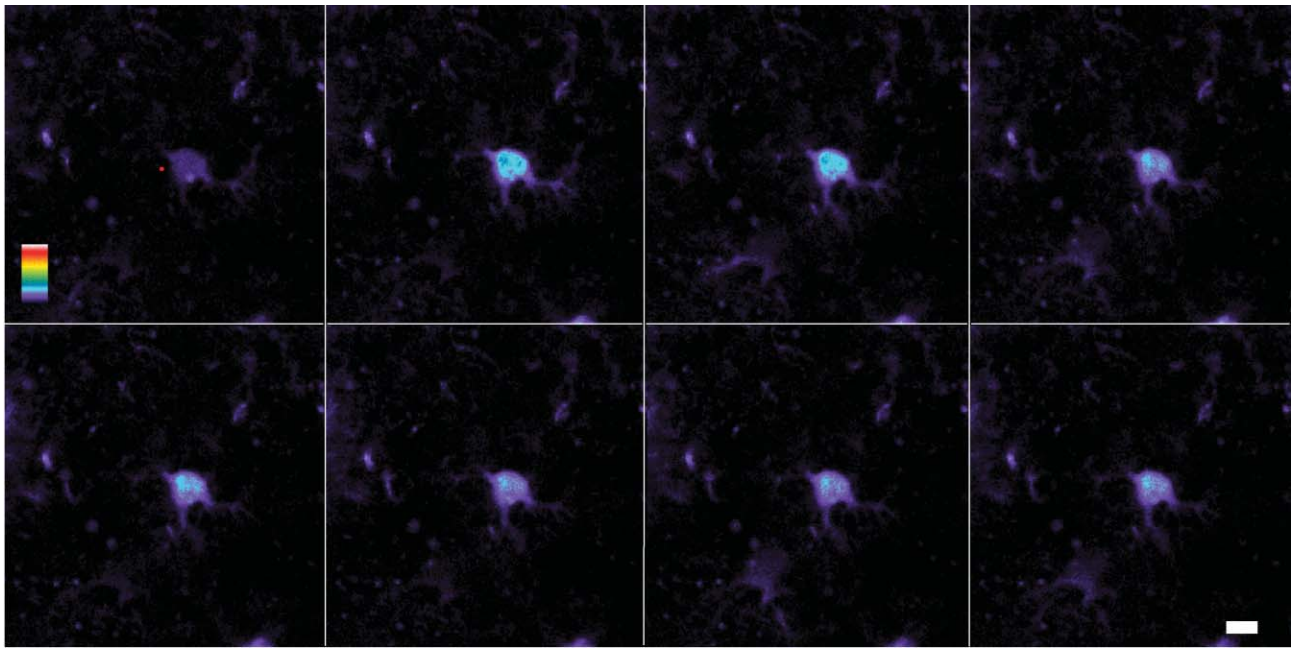

Fig. 4 Calcium imaging in astrocytes in mouse brain slices after extracellular uncaging of MDNI-glu.

Notes and references

† Abbreviations: CNB, α-carboxy-*o*-nitrobenzyl; MNI, 4-methoxy-7-nitroindolinyl; NI, 7-nitroindolinyl-5-acetate; Bhc, 6-bromo-7-hydroxycoumarin-4-ylmethyl; pHP, *p*-hydroxyphenacetyl. MDNI-glu **(1)**. ^{1}1H NMR (CD_3OD) δ 8.23 (s, 1H), 4.6 (m, 1H), 4.38 (t, J = 8.1, 2H), 4.04 (s, 3H), 3.39 (t, J = 8.1, 2H), 2.76 (t, J = 7.3, 2H), 2.26 (m, 1H), 2.05 (m, 1H); ^{13}C NMR (CD_3OD) δ 172.0, 171.8 153.6, 140.8, 139.1, 135.7, 131.5, 122.6, 61.8, 57.8, 51.3, 32.6, 27.8, 24.2. Analysis calculated for $C_{14}H_{16}N_4O_8$: C, 45.66; H, 4.38; N, 15.21%; found: C, 45.73; H, 4.43; N, 15.14%.

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