Syntheses of Caged AMPA Receptor Ligands for 2-Photon Excitation Studies

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Background

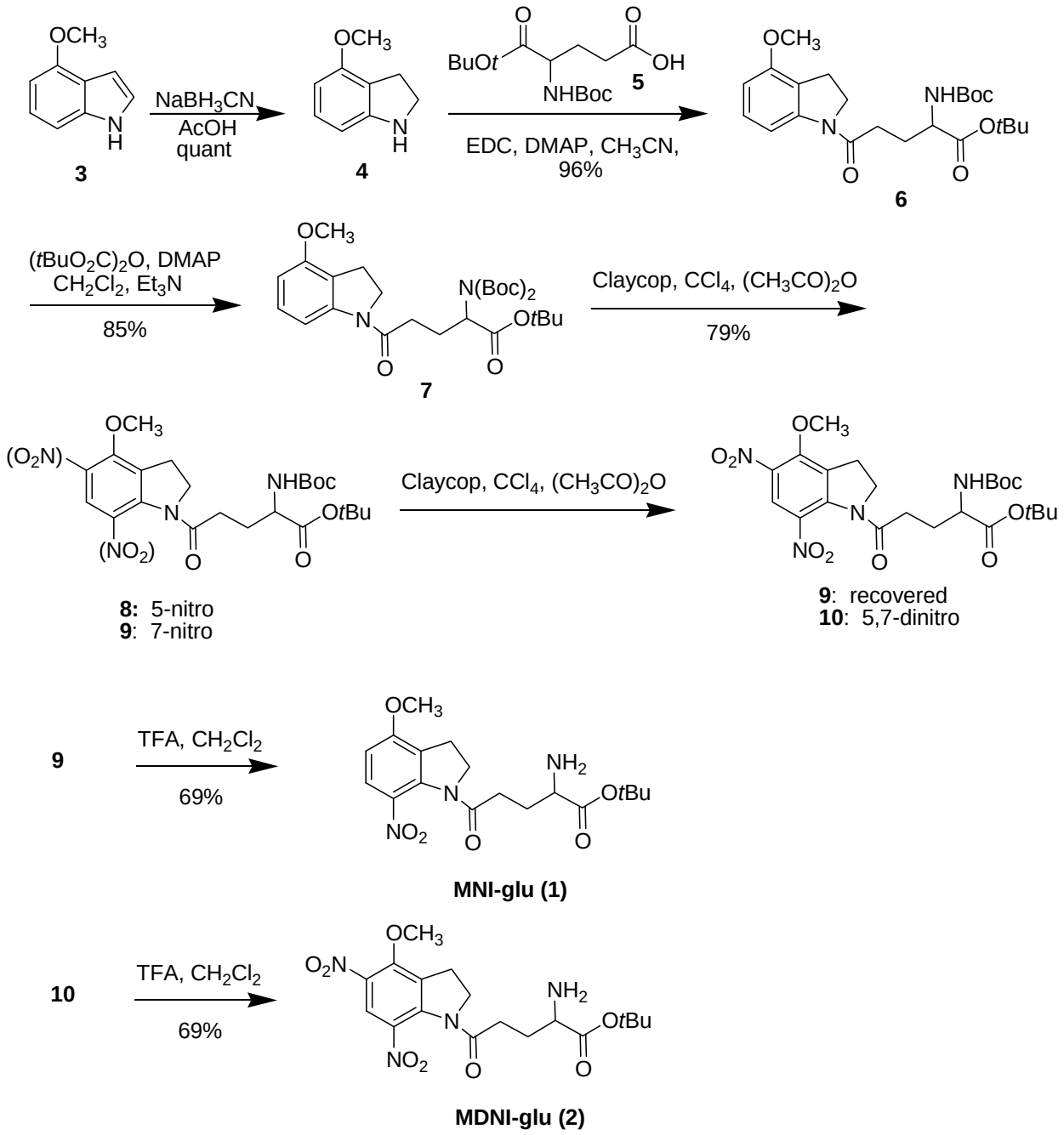
Caged neurotransmitters are biological signaling molecules that have been modified by the addition of a light-sensitive caging group that renders the molecule unable to activate receptors until a flash of light is used to remove the caging group.[1-5] Photolytic release from caged precursors is a powerful tool for studying kinetics of neurotransmitter-mediated processes.[2] Caged neurotransmitters must satisfy several stringent chemical and biological design criteria to be useful for experimental neuroscience: (1) They must be biologically inert (neither an antagonist or agonist); (2) They should be rapidly released upon photolysis (release half-time < 20 μs); (3) Uncaging should make efficient use of incident light (exhibit a large extinction coefficient and quantum yield); (4) Byproducts should not exhibit activity or be harmful to cells; (5) The caged precursor must not undergo spontaneous hydrolysis during storage or in physiological buffer. Current efforts in designing improved agents focus on faster release of probes and on production of new caged probes, especially for *in vivo* two-photon (2P) excitation.[4, 6, 7]

Among recently described caged glutamate probes, 4-methoxy-7-nitroindolinyl-glutamate (MNI-glu)[7, 8] (**1**) and 4-methoxy-5,7-dinitroindolinyl-glutamate (MDNI-glu)[9, 10] (**2**) are the most useful 2P excitation reagents known. These two probes have desirable features such as high 2P cross-sections, good solubility, hydrolytic resistance, and inactivity at neuronal glutamate receptors until photolyzed. A serious limitation of their wider use in neurophysiological studies is the availability of both caged precursors since only MNI-glu (**1**) is commercially available and is relatively expensive.

Chemistry

The synthetic route to MDNI-glu[9, 10] (**2**) was based upon a previously reported synthesis of MNI-glu2 which involved a difficult nitration of 4-methoxyindoline glutamate that proceeded in poor yield (19%). The synthetic pathway to MDNI-glu (**2**) is shown in Scheme 1. 4-Methoxyindole (**3**) was reduced in near quantitative yield to 4-methoxyindoline (**4**) using sodium cyanoborohydride in acetic acid. 4-Methoxyindoline (**4**) was then coupled to N-*tert*-Boc-L-glutamic acid α-*tert*-butyl ester (**5**) in the presence of EDC and DMAP yielding conjugate (**6**). The crucial step of the synthesis was nitration of 4-methoxyindoline glutamate, and we improved the overall yield of MDNI-glu by substituting the clay supported copper (II) nitrate (Claycop) reagent[11] in the key nitration step, based on an approach described in a previous report.[12] Treatment of (**7**) with Claycop in the presence of acetic anhydride gave a mixture of the 5- and 7-mononitro compounds, (**8**) and (**9**), in 1:3.5 ratio, respectively, that was difficult to resolve. A second nitration of that mixture ((**8**) and (**9**)) with excess Claycop afforded the desired 5,7-dinitro product (**10**) in a modest yield together with unreacted 7-nitro isomer (**9**). The 5,7-dinitro derivative (**10**) was readily separated by flash chromatography from compound (**9**). We tried to obtain the 5,7-dinitro derivative (**10**) directly in a double nitration of **7** using excess Claycop but obtained a complex mixture from which the (**10**) was difficult to isolate, so sequential nitration was preferred. Finally, deprotection of the amine and carboxyl groups of (**9**) and (**10**) with TFA furnished MNI-glu (**1**) and MDNI-glu (**2**), respectively.

Scheme 1. Syntheses of MNI-glu **(1)** and MDNI-glu **(2)**.



Experimental

General. ^{1}H and ^{13}C NMR spectra of all synthesized compounds (4-9) were in good agreement with literature data[7, 8] and NMR spectra of (1) and (2) were consistent with the structures of

MNI-glu[7, 8] and MDNI-glu,[9, 10] as were the MS spectra. The purities of (1) and (2) were ≥ 94 and 98%, respectively, by analytical HPLC. Thin-layer chromatography (TLC) analyses were carried out on Analtech (Newark, DE) silica gel GHLF 0.25 mm plates using UV and iodine detection. ESI and APCI-MS mass spectrometry was performed on an Agilent 1200 Series Mass Spectrometer and MALDI-MS was performed on a Shimadzu Biotech Axima. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian spectrometer at 400 MHz and 100 MHz respectively. Chemical shifts are reported in ppm using tetramethylsilane (TMS) as internal standard. Flash chromatography was carried out on a Teledyne Isco Combi*Flash*® Companion® instrument with UV detection at 254 nm. Analytical RP-HPLC analyses were performed on a Beckman instrument equipped with UV System Gold 168 detector using an Agilent SB-C18 column, 4.6 x 75 mm. Preparative RP-HPLC was performed on an Agilent 1200 instrument using an Agilent Prep-C18 column, 30 x 100 mm. The following purification methods were used in the method II: 20% B to 50% B over 15 min., and then 2 min. at 95%B. For both methods the solvents were: Solvent A: 0.05% TFA in water; Solvent B: 0.05% TFA in acetonitrile. All starting materials and reagents are commercially available from Sigma-Aldrich and were used without further purification.

4-Methoxyindoline (4).[7, 8] To a solution of 4-methoxyinoline **(3)** (11 g, 74.7 mmol) in acetic acid (50 mL) was added portion-wise $NaBH_3CN$ (4.7 g, 74.7 mmol) at 18-19°C, with stirring for a further 2 hr at room temperature. The pH of the reaction mixture was cautiously brought to 13 with 20% NaOH and the product was extracted with CH_2Cl_2 (3 x 50 mL). The organic layer was washed with brine, dried, and the solvent evaporated under reduced pressure to give a colorless oil (11 g, quantitative yield) which was used in the next step without further purification.

1-v-4-methoxyindoline (6).[7, 8] To a solution of crude **(4)** (11 g, 74 mmol) in dry acetonitrile (110 mL) was added 4-dimethylaminopyridine (DMAP) (27 g, 221 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (19.5 mL, 110.5 mmol). To this reaction mixture was added *N*-Boc-L-glutamic acid *α–t*-butyl ester **(5)** (26.8 g, 88.4 mmol) in dry acetonitrile (110 mL) under an argon atmosphere at 0°C. The mixture was stirred at room temperature overnight, and the solvent was evaporated. The residue was dissolved in CH_2Cl_2, washed with saturated $NaHCO_3$ and brine, dried, and evaporated to give a viscous oil. The crude

product was purified by flash chromatography with hexane and EtOAc (4:1) affording **(6)** (30 g, 94%) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.84 (d, J = 8.07 Hz, 1H), 7.17 (t, J = 8.07 Hz, 1H), 6.58 (d, J = 8.07 Hz, 1H), 5.21 (d, J = 7.72 Hz, 1H), 4.18-4.28 and 4.09-4.18 (2 x m, 1H, rotamers), 4.04 (t, J = 8.45 Hz, 2H), 3.84 (s, 3H), 3.11 and 2.97 (2 x t, J = 8.45 Hz, 2H), 2.43-2.59 (m, 2H), 2.23-2.31 (m, 1H), 1.99-2.06 (m, 1H), 1.47 (s, 9H), 1.42 (s, 9H).

1-{4*S*-[(4-*tert*-Butoxycarbonyl)-4-(di-*tert*-butoxycarbonylamino)]butanoyl}-4-methoxyindoline (7).[12] To a stirred solution of **(6)** (13.9 g, 32 mmol) in dry CH_2Cl_2 (60 mL) and triethylamine (192 mL) was added a solution of di-*tert*-butyl dicarbonate (18.4 mL, 80 mmol) followed by DMAP (0.47 g, 3.8 mmol) under an argon atmosphere. The reaction mixture was heated under reflux for 6 hr and the solvents removed under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with 1M $KHSO_4$, saturated aqueous $NaHCO_3$ and brine. The solution was dried and evaporated. The crude product was purified by flash chromatography with hexane and EtOAc (4:1) affording **(7)** (16 g, 93%) as a pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.86 (d, J = 8.07 Hz, 1H), 7.15 (t, J = 8.07 Hz, 1H), 6.56 (d, J = 8.07 Hz, 1H), 4.86 (dd, J = 9.24 and 8.22 Hz, 1H), 4.03-4.07 (m, 2H), 3.83 (s, 3H), 3.09 (t, J = 8.36 Hz, 2H), 2.40-2.61 (m, 3H), 2.18-2.28 (m, 1H), 1.48 (s, 18H), 1.46 (s, 9H) ; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.41, 169.63, 155.87, 152.57, 144.58, 129.11, 118.42, 110.33, 106.04, 83.15, 81.54, 81.24, 58.61, 55.51, 48.60, 32.81, 28.19, 28.09, 25.26, 24.73. MALDI-MS: 525.9 $(M+H)^+$.

1-[4*S*-(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)]butanoyl-4-methoxy-5-nitro-indoline (8) and 1-[4*S*-(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)] butanoyl-4-methoxy-7-nitroindoline (9).[13] A solution of **(7)** (2.4 g, 4.48 mmol) in CCl_4 (15 mL) and acetic anhydride (7.5 mL) was stirred with a suspension of Claycop (2.8 g) at room temperature for 1.5 hr after which the solid was filtrated off under vacuum and washed with CH_2Cl_2 (2 x 7 mL). The combined filtrates were washed with saturated $NaHCO_3$, brine, and dried and the solvents were evaporated off under reduced pressure. Purification by flash chromatography with hexane and EtOAc (4:1) gave a mixture of 5- and 7-nitro isomers (1.7 g, 79%) (1:3.5), **(8)** and **(9)**, respectively, as a yellow oil. The 5-nitro isomer **(8)** was obtained in a pure form by crystallization of the mixture of 5- and 7-nitro isomers (1:3) from EtOAc. **(8)** ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.03 (bs, 1H), 7.87 (d, J = 8.25 Hz, 1H), 5.19 (bs, 1H), 4.11-4.23 (m, 3H), 3.93

(s, 3H), 3.27 (t, 2H), 2.46-2.64 (m, 2H), 2.26-2.36 (m, 1H), 1.96-2.06 (m, 1H), 1.48 (s, 9H), 1.42 (s, 9H), MALDI-MS: 480.2 $(M+H)^+$. The pure 7-nitro isomer **(9)** was recovered from the mixture of 5- and 7-nitro isomers during the purification procedure of compound **(10)**. **(9)** ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.72 (d, J = 8.77 Hz, 1H), 6.62 (d, J = 8.77 Hz, 1H), 5.10-5.15 (2 x d, J = 7.02 Hz, 1H), 4.08-4.21 (m, 3H), 3.91 (s, 3H), 3.06 (t, J = 6.50 Hz, 2H), 2.50-2.64 (m, 2H), 2.05-2.25 (m, 2H), 1.50 (s, 9H), 1.42 (s, 9H). MALDI-MS: 480.8 $(M+H)^+$.

1-[4*S*-(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)]butanoyl-4-methoxy-5,7-dinitroindoline (10). A mixture of 5- and 7-nitro isomers **(8)** and **(9)** (1.7 g, 3.54 mmol) was stirred with a suspension of Claycop (3.4 g) in carbon tetrachloride (13 mL) and acetic anhydride (6.5 mL) at room temperature for 16 hrs followed by the work-up procedure described above for compounds **(8)** and **(9)**. Purification by flash chromatography with hexane and EtOAc (2:1) gave the pure 5,7-dinitro isomer **(10)** (0.8 g, 43%), the recovered 7-nitro isomer **(9)** (0.25 g) as well as mixture of mono- and di-nitro compounds (0.18 g). **(10)** ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.34 (s, 1H), 5.11 (dd, J = 5.58 and 4.99 Hz, 1H), 4.33 (t, J = 8.21 Hz, 2H), 4.01 (s, 3H), 3.34 (t, J = 8.21 Hz, 2H), 2.60-2.71 (m, 3H), 2.18-2.26 (m, 1H), 1.55 (s, 9H), 1.45 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.50, 167.17, 153.26, 149.57, 140.29, 138.14, 135.03, 130.18, 122.84, 87.11, 83.54, 61.80, 60.95, 50.34, 32.03, 28.07, 27.98, 26.95, 23.82.

1-[*S*- (4-amino-4-carboxybutanoyl)]-4-methoxy-7-nitroindoline (MNI-glu, 1).[7, 8] To a solution of **(9)** (100 mg, 0.2 mmol) in CH_2Cl_2 (2 mL) was added dropwise TFA (1 mL) at 0°C. The reaction mixture was stirred at room temperature for 2 hrs and then concentrated under reduced pressure. The residue was dissolved in water (5 mL) and purified by preparative HPLC (Method II) affording (45 mg, 69%) of product **(1)**. MNI-glu **(1)** was stored at -80°C. The purity of **(1)** was found to be ≥94% by analytical HPLC (Method I), retention time: 4.00 minutes. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.73 (d, 1H, J = 4.50 Hz), 6.84 (d, 1H, J = 4.50 Hz), 4.20-4.32 (m, 2H), 3.98 (t, 1H, J = 6.39 Hz), 3.92 (s, 3H), 3.09 (t, 2H, J = 8.02 Hz), 2.78 (dt, 2H, J = 6.94 and 2.25 Hz), 2.15-2.26 (m, 2H); ESI MS: m/z: 324.4 $(M+H)^+$.

1-[*S*- (4-amino-4-carboxybutanoyl)]-4-methoxy-5,7-dinitroindoline (MDNI-glu, 2).[9, 10] To a solution of **(10)** (0.8 g, 1.5 mmol) in CH_2Cl_2 (9 mL) was added dropwise TFA (9 mL) at 0°C.

The reaction mixture was stirred at room temperature for 1 hr and then worked-up and purified using the procedure as for compound **(1)**. MDNI-glu **(2)** was obtained in 54% yield (0.31 g) as a TFA salt and was stored at -80°C. The purity of **(2)** was found to be ≥98% by analytical HPLC (Method I), retention time: 7.28 minutes. ^{1}H-NMR (400 MHz, CD_3OD): :δ 8.28 (s, 1H), 4.59 (dd, 1H, J = 5.58 Hz), 4.37 (t, 2H, J = 8.07 Hz), 4.03 (s, 3H), 3.38 (t, 2H, J = 8.07 Hz), 2.76 (t, 2H, J = 7.28 Hz), 2.24-2.33 (m, 1H), 2.02-2.11 (m, 1H); ^{13}C-NMR (100 MHz, CD_3OD): δ 173.09, 172.89, 152.93, 140.28, 138.20, 134.89, 130.78, 121.69, 60.79, 50.47, 32.49, 26.79, 26.66.

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