Electronic Supplementary Material (ESI) for Chemical Communications

Wavelength-orthogonal photolysis of neurotransmitters *in vitro*

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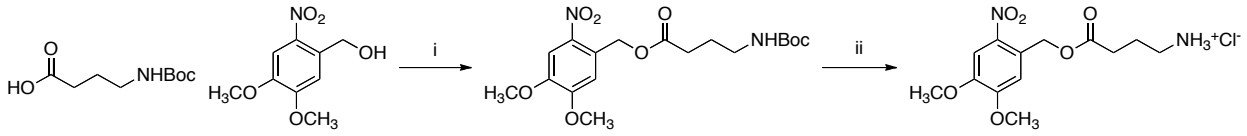
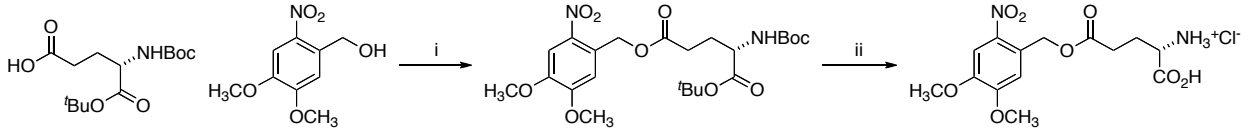
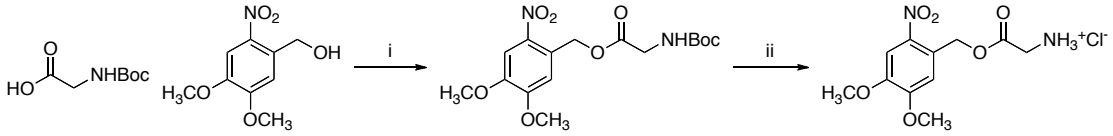
Synthesis of caged compounds



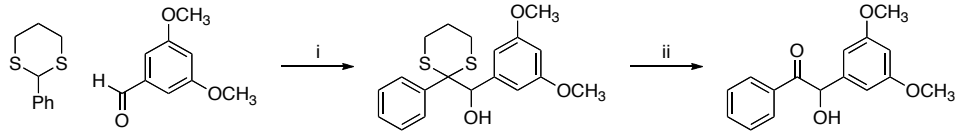
Scheme S1. Synthesis of DMNB-GABA. *Reagents and conditions*. i. DCC, DMAP, CH_2Cl_2, 70%. ii. 1 M HCl in dioxane, 97%.



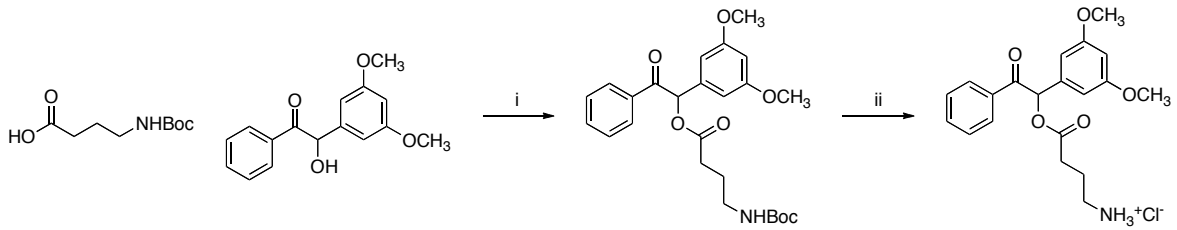
Scheme S2. Synthesis of DMNB-glutamate. *Reagents and conditions*. i. DCC, DMAP, CH_2Cl_2, 69%. ii. 1 M HCl in acetic acid, 45 °C, 50%.



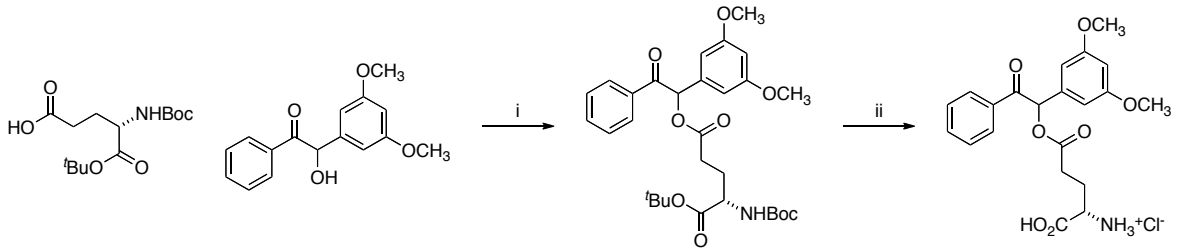
Scheme S3. Synthesis of DMNB-glycine. *Reagents and conditions*. i. DCC, DMAP, CH_2Cl_2, 75%. ii. 1 M HCl in dioxane, 100%.



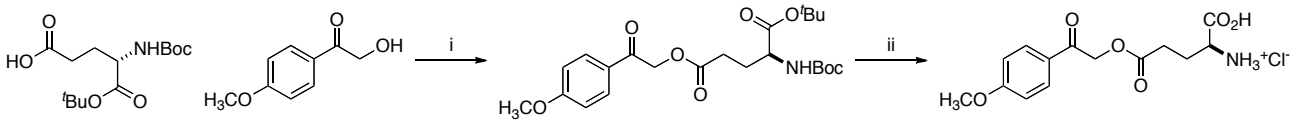
Scheme S4. Synthesis of 3',5'-dimethoxybenzoin. *Reagents and conditions*. i. ^{n}BuLi, THF, 77%. ii. $PhI(OCOCF_3)_2$, CH_3CN, 43%.



Scheme S5. Synthesis of benzoin-GABA. *Reagents and conditions*. i. DCC, DMAP, CH_2Cl_2, 85%. ii. 1 M HCl in acetic acid, 82%.



Scheme S6. Synthesis of benzoin-glutamate. *Reagents and conditions*. i. DCC, DMAP, CH_2Cl_2, 82%. ii. 1 M HCl in acetic acid, 45 °C, 62%.



Scheme S7. Synthesis of MPA-glutamate. *Reagents and conditions*. i. EDC·HCl, HBTU, Hunig's base, CH_2Cl_2, 45%. ii. 1 M HCl in acetic acid, 45 °C, 68%.

Dimethoxybenzoin discussion

Extending the work of Bochet, who demonstrated the principle of wavelength-orthogonal photolysis, we investigated the use of the DMNB and 3,5-dimethoxybenzoin caging groups as wavelength-orthogonal caging groups for neurotransmitters. To investigate wavelength-orthogonal photolysis of DMNB- and 3,5-dimethoxybenzoin-caged pairs of compounds, laser photolysis studies were conducted and analysed by ^{1}H NMR. The results showed that when a mixture of DMNB-glycine (**2**) and 3,5-dimethoxybenzoin-GABA (**4**) were irradiated at 250 nm, only GABA was photoreleased and the DMNB-glycine remained intact. When an identical mixture was irradiated at 405 nm, only photolysis of the DMNB-glycine was observed (data not shown).

Having demonstrated that it was possible to release transmitters from the DMNB and 3,5-dimethoxybenzoin caging groups in a wavelength-orthogonal manner, we next attempted to perform *in vitro* wavelength-orthogonal photolysis on neurons. In this case, DMNB-caged and 3,5-dimethoxybenzoin-caged Glu (**3**, **5**) and GABA (**1**, **4**) were employed. DMNB-caged Glu (**3**) and GABA (**1**) were both shown to have minimal activity on neurons until photolysed and both produced the expected responses when photolysed with a 405nm diode (data not shown). However, both 3,5-dimethoxybenzoin-caged Glu (**5**) and GABA (**4**) produced unexpected toxicity when applied to neurons. Given that both caged Glu and GABA produced similar activity, we hypothesised that the effect was non-selective and not mediated by a receptor-ligand interaction. Hence, we applied 3,5-dimethoxybenzoin to the neurons and observed similar toxicity.

It is not clear what the basis for this toxicity is, or whether this is a general phenomenon. The 3,5-dimethoxybenzoin group has been used in a biological system previously by Thirlwell *et al.*,[1] and no toxicity was reported. However, there are many variations between our work and that of Thirlwell. None-the-less, our data suggest that the 3,5-dimethoxybenzoin group is not suitable for use, at least in our system.

General chemistry experimental

^{1}H NMR spectra were recorded on Bruker Avance II 400 (400 MHz) or Bruker Avance III (500 MHz) using deuteromethanol (unless indicated otherwise) as a reference for internal deuterium lock. The chemical shift data for each signal are given as δH in units of parts per million (ppm) relative to tetramethylsilane (TMS) where δ (TMS) = 0.00 ppm. The multiplicity of each signal is indicated by: s (singlet); br s (broad singlet); d (doublet); t (triplet); q (quartet); dd (doublet of doublets); or m (multiplet). The number of protons (n) for a given resonance signal is indicated by nH. Coupling constants (*J*) are quoted in Hz and are recorded to the nearest 0.1 Hz. Identical proton coupling constants (*J*) are averaged in each spectrum and reported to the nearest 0.1 Hz. The coupling constants are determined by analysis using Bruker TopSpin software.

^{13}C NMR spectra were recorded on Bruker Avance III (125 MHz) spectrometers using the PENDANT or DEPT Q pulse sequences with broadband proton decoupling and internal deuterium lock. The chemical shift data for each signal are given as δ in units of parts per million (ppm) relative to tetramethylsilane (TMS) where δC (TMS) = 0.00 ppm. ^{1}H and ^{13}C spectra were assigned using 2D NMR experiments including COSY, HSQC, HMBC, DEPT-135, HMQC, and DEPT Q.

Mass spectra were acquired on a VG platform spectrometer. Electrospray ionisation spectra were obtained on Micromass LCT; Micromass LCT Premier; and Bruker MicroTOF spectrometers, operating in positive or negative mode, from solutions of methanol, acetonitrile or water. *m/z* values are reported in Daltons and followed by their percentage abundance in parentheses.

Melting points were determined using an Electrothermal 9100 melting point apparatus or Kofler hot stage microscope and are uncorrected.

Microanalyses were obtained by the Elemental Analysis Service, London Metropolitan University, London.

Infrared spectra were obtained as potassium bromide discs. The spectra were recorded on a Bruker Tensor 27 spectrometer. Absorption maxima are reported in wavenumbers (cm^{-1}).

Specific optical rotations were measured using Perkin Elmer Model 241 polarimeter, in cells with a path length of 1 dm. The light source was maintained at 589 nm. The concentration (*c*) is expressed in g/100 mL (equivalent to g/0.1 dm^3). Specific rotations are denoted $[\alpha]_D^T$ and are given in implied units of 10^{-1} deg cm^2 g^{-1} (T = ambient temperature in °C).

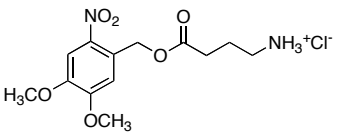
Optical absorption spectra were obtained using a PerkinElmer LAMBDA 950 UV/Vis/NIR Spectrophotometer. Spectra were obtained using the PerkinElmer high grade quartz UV/VIS spectroscopy cells provided with the spectraphotometer. A reference sample of the appropriate solvent was placed in the reference arm.

Analytical thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} aluminum-supported thin layer chromatography sheets. Visualisation was by absorption of UV light (λ_{max} 254 or 365 nm), or thermal development after dipping in one of: **a** ethanolic solution of phosphomolybdic acid (PMA); **b** aqueous solution of potassium permanganate, potassium carbonate and sodium hydroxide.

Reverse phase silica gel column chromatography was carried out on C_{18} Fluka silica gel eluting with the appropriate solvents as supplied, under a positive pressure of compressed air.

Chemicals were purchased from Sigma Aldrich UK. Where appropriate and if not stated otherwise, all non aqueous reactions were performed in a flame-dried flask under an inert atmosphere of nitrogen or argon, using a double vacuum manifold with the inert gas passing through a bed of activated 4 Å molecular sieves and self indicating silica gel. *In vacuo* refers to the use of a rotary evaporator attached to a diaphragm pump.

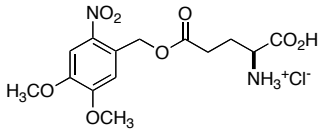
4,5-Dimethoxy-2-nitrobenzyl 4-aminobutanoate hydrochloride (1)



4,5-Dimethoxy-2-nitrobenzyl 4-(*tert*-butoxycarbonylamino)butanoate (230 mg, 0.577 mmol) was dissolved in a solution of 1 M HCl in dioxane (15 mL) and stirred for 5 h. The dioxane was removed *in vacuo* and the crude mixture precipitated in Et_2O to afford 4,5-dimethoxy-2-nitrobenzyl 4-aminobutanoate hydrochloride

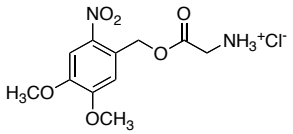
(163 mg, 97%) as a yellow solid: mp 160-161 °C (from Et_2O) (lit.[2] 168-169 °C); δ_H (500 MHz; D_2O) 7.64 (1H, s), 7.02 (1H, s), 5.33 (2H, s), 3.87 (6H, s), 3.00 (2H, t, *J* 7.8), 2.57 (2H, t, *J* 7.4), 1.98-1.90 (2H, m); δ_C (125 MHz; D_2O) 174.5 (C=O), 153.1 (C), 147.9 (C), 140.0 (C), 126.0 (C), 112.0 (C-H), 108.6 (C-H), 64.2 (Ar-CH_2), 56.3 (OMe), 56.1 (OMe), 38.6 (CH_2), 30.6 (CH_2), 22.0 (CH_2); *m/z* (ES^+) 299 ($[M+H]^+$, 100%); *m/z* (ES^-) 427 (20%), 369 ($[M+HCl+Cl]^-$, 100%), 333 ($[M+Cl]^-$, 10%). These data are in good agreement with the literature values.[2]

(*S*)-2-Amino-5-(4,5-dimethoxy-2-nitrobenzyloxy)-5-oxopentanoic acid hydrochloride (3)



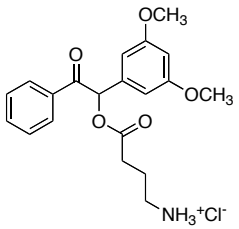
(*S*)-1-*tert*-Butyl 5-(4,5-dimethoxy-2-nitrobenzyl) 2-(*tert*-butoxycarbonylamino)pentanedioate (200 mg, 0.402 mmol) was dissolved in a solution of 1 M HCl in acetic acid (10 mL) and stirred at 45 °C for 6 h. An azeotrope of acetic acid and cyclohexane was removed *in vacuo* and the crude mixture precipitated in MeOH/Et_2O to afford (*S*)-2-amino-5-(4,5-dimethoxy-2-nitrobenzyloxy)-5-oxopentanoic acid hydrochloride (69 mg, 50%) as a yellow solid: $[\alpha]_D^{25}$ -4.4 (*c* 0.25 in DMSO); mp 119-121 °C (from MeOH/Et_2O); ν_{max}/cm^{-1} (KBr) 3440.6 (m)(N-H), 2951.6 (m)(C-H), 1736.4 (s)(C=O), 1523.5 (s)(NO_2), 1281.8 (s)(NO_2), 1069.7 (s)(C-O); δ_H (500 MHz; MeOD) 7.74 (1H, s), 7.16 (1H, s), 5.51 (1H, d, *J* 14.0), 5.47 (1H, d, *J* 14.0), 4.05 (1H, br s), 3.96 (3H, s), 3.91 (3H, s), 2.68-2.74 (2H, m), 2.19-2.28 (2H, m); δ_C (125 MHz; MeOD) 173.2 (C=O), 155.1 (C), 150.1 (C), 141.7 (C), 127.5 (C), 112.7 (C-H), 109.5 (C-H), 64.9 (Ar-CH_2), 56.9 (OMe), 53.4 (NH_2-CH), 30.6 (CH_2), 26.9 (CH_2); HRMS *m/z* (ESI^+) [Found: $(M+Na)^+$ 365.0957. $C_{14}H_{18}N_2O_8Na$ requires M^+ 365.0955]; *m/z* (ES^+) 401 ($[M+HCl+Na]^+$, 100%), 365 ($[M+Na]^+$, 45%); *m/z* (ES^-) 341 ($[M-H]^-$, 100%).

4,5-Dimethoxy-2-nitrobenzyl 2-aminoacetate hydrochloride (2)



4,5-Dimethoxy-2-nitrobenzyl 2-(*tert*-butoxycarbonylamino)acetate (500 mg, 1.350 mmol) was dissolved in a solution of 1 M HCl in dioxane (25 mL) and stirred for 24 h. The dioxane was removed *in vacuo* and the crude mixture precipitated in Et_2O to afford 4,5-dimethoxy-2-nitrobenzyl 2-aminoacetate hydrochloride (413 mg, 100%) as a yellow solid**:** mp 168-170 °C (from Et_2O); ν_{max}/cm^{-1} (KBr) 3442.1 (m)(N-H), 2959.9 (m)(C-H), 1741.6 (s)(C=O), 1529.5 (s)(NO_2), 1280.3 (s)(NO_2), 1066.6 (s)(C-O); δ_H (400 MHz; D_2O) 7.78 (1H, s), 7.15 (1H, s), 5.55 (2H, s), 4.00 (2H, s), 3.93 (6H, s); δ_C (125 MHz; D_2O) 168.1 (C=O), 153.5 (C), 148.3 (C), 140.1 (C), 125.4 (C), 112.3 (C-H), 108.8 (C-H), 65.5 (Ar-CH_2), 56.6 (OMe), 56.4 (OMe), 40.3 (N-CH_2); HRMS *m/z* (EI^+) [Found: $(M)^+$ 270.0847. $C_{11}H_{14}N_2O_6$ requires M^+ 270.0852]; *m/z* (EI^+) 270 ($[M]^+$, 100%); *m/z* (ES^-) 401 (35%), 363 (60%), 341 ($[M+HCl+Cl]^-$, 40%), 305 ($[M+Cl]^-$, 30%).

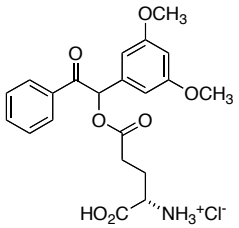
(*R,S*)-1-(3,5-Dimethoxyphenyl)-2-oxo-2-phenylethyl 4-aminobutanoate hydrochloride (4)



(*R,S*)-1-(3,5-Dimethoxyphenyl)-2-oxo-2-phenylethyl 4-(*tert*-butoxycarbonylamino)butanoate (500 mg, 1.093 mmol) was dissolved in a solution of 1 M HCl in acetic acid (10 mL) and stirred at RT for 4 h. An azeotrope of acetic acid and cyclohexane was removed *in vacuo* and the crude mixture precipitated in MeOH/Et_2O to afford (*R,S*)-1-(3,5-dimethoxyphenyl)-2-oxo-2-phenylethyl 4-aminobutanoate hydrochloride (350 mg, 82%) as a yellow gum: ν_{max}/cm^{-1} (KBr) 3440.7 (s)(N-H), 2962.9 (m)(C-H), 1736.3 (m)(C=O), 1694.6

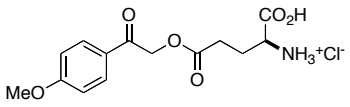
(m)(C=O), 1597.4 (s), 1160.3 (m)(C-O); δ_H (500 MHz; MeOD) 7.99 (2H, dd, *J* 7.9, 1.3), 7.57-7.60 (1H, m), 7.46 (2H, dd, *J* 7.9, 7.4), 6.93 (1H, s), 6.61 (2H, d, *J* 2.2), 6.46 (1H, dd, *J* 2.2, 2.2), 3.73 (6H, s), 3.06 (2H, t, *J* 7.7), 2.58-2.72 (2H, m), 2.03 (2H, tt, *J* 7.7, 7.3); δ_C (125 MHz; MeOD) 195.9 (C=O), 173.5 (OC=O), 163.0 (C), 136.6 (C), 135.9 (C), 135.0 (C-H), 129.9 (C-H , C-H), 108.1 (C-H), 101.8 (C-H), 79.4 (C-H), 56.0 (OMe), 40.0 (CH_2), 31.6 (CH_2), 23.8 (CH_2); HRMS *m/z* (ESI^+) [Found: $(M+H)^+$ 358.1657. $C_{20}H_{24}NO_5$ requires M^+ 358.1649]; *m/z* (ES^+) 416 ($[M+HCl+Na]^+$, 85%), 358 ($[M+H]^+$, 100%).

(2*S*)-Amino-5-[(*R,S*)-1-(3,5-dimethoxyphenyl)-2-oxo-2-phenylethoxy]-5-oxopentanoic acid hydrochloride (5)



(*S*)-1-*tert*-Butyl 5-[(*R,S*)-1-(3,5-dimethoxyphenyl)-2-oxo-2-phenylethyl] 2-(*tert*-butoxycarbonylamino)pentanedioate (150 mg, 0.269 mmol) was dissolved in a solution of 1 M HCl in acetic acid (10 mL) and stirred at 45 °C for 3 h. An azeotrope of acetic acid and cyclohexane was removed *in vacuo* and the crude mixture purified by reverse phase silica gel chromatography, eluting with CH_3CN and water (1:3), to afford (2*S*)-amino-5-[(*R,S*)-1-(3,5-dimethoxyphenyl)-2-oxo-2-phenylethoxy]-5-oxopentanoic acid hydrochloride (73 mg, 62%) as a colourless solid: $[\alpha]_D^{25}$ +9.0 (*c* 0.5 in DMSO); mp 85-87 °C (from MeOH/Et_2O); ν_{max}/cm^{-1} (KBr) 3441.3 (s)(N-H), 2926.6 (m)(C-H), 1751.0 (s)(C=O), 1697.8 (s)(C=O), 1598.1 (s)(C=O), 1160.6 (s)(C-O); δ_H (500 MHz; MeOD) 7.99 (2H, dd, *J* 7.8, 1.0), 7.56-7.59 (1H, m), 7.46 (2H, dd, *J* 7.8, 7.6), 6.91 (1H, d, *J* 1.8), 6.61 (2H, dd, *J* 2.2, 1.8), 6.44 (1H, dd, *J* 2.2, 2.2), 3.73 (6H, s), 3.62-3.66 (1H, m), 2.66-2.79 (2H, m), 2.10-2.27 (2H, m); δ_C (125 MHz; MeOD) 195.8 (C=O), 195.6 (OC=O), 173.54 (OC=O), 173.46 (C), 162.8 (C), 136.8 (C), 135.9 (C), 134.4 (C-H), 129.1 (C-H), 128.9 (C-H), 107.1 (C-H), 101.2 (C-H), 78.5 (C-H), 55.3 (OMe), 54.7 (NH_2-CH), 30.6 (CH_2), 26.9 (CH_2); HRMS *m/z* (ES^+) [Found: $(M+H)^+$ 402.1536. $C_{21}H_{24}NO_7$ requires M^+ 402.1547]; *m/z* (ES^+) 460 ($[M+HCl +Na]^+$, 30%), 402 ($[M+H]^+$, 100%).

(*S*)-2-Amino-5-(2-(4-methoxyphenyl)-2-oxoethoxy)-5-oxopentanoic acid hydrochloride (6)



(*S*)-1-*tert*-Butyl 5-(2-(4-methoxyphenyl)-2-oxoethyl) 2-(*tert*-butoxycarbonylamino)pentanedioate (200 mg, 0.443 mmol) was dissolved in a solution of 1 M HCl in acetic acid (10 mL) and stirred at 45 °C for 3 h. An azeotrope of acetic acid and cyclohexane was removed *in vacuo* to afford (*S*)-2-amino-5-(2-(4-methoxyphenyl)-2-oxoethoxy)-5-oxopentanoic acid hydrochloride (89 mg, 68%) as a light yellow solid: $[\alpha]_D^{25}$ +10.2 (*c* 0.5 in DMSO); mp 174-176 °C (from MeOH); ν_{max}/cm^{-1} (KBr) 3437.3 (m)(N-H), 2930.7 (s)(C-H), 2344.4 (s), 1752.2 (s)(C=O), 1684.7 (s)(C=O), 1605.2 (s)(C=O), 1245.0 (s)(C-O), 1176.8 (s)(C-O); δ_H (500 MHz; DMSO) 7.95 (2H, d, *J* 8.9), 7.08 (2H, d, *J* 8.9), 5.45 (2H, s), 3.86 (3H, s), 3.72-3.76 (1H, m), 2.69-2.75 (1H, m), 2.57-2.64 (1H, m), 1.99-2.15 (2H, m); δ_C (125 MHz; DMSO) 190.9 (C=O), 171.6 (OC=O), 170.7 (OC=O), 163.7 (C), 130.1 (C-H), 126.7 (C), 114.2 (C-H), 66.3 (OCH_2), 55.7 (OMe), 52.0 (NH_2-CH), 29.4 (CH_2), 25.8 (CH_2); *m/z* (ESI^+) [Found: $(M+H)^+$ 318.0947. $C_{14}H_{17}NO_6Na$ requires M^+ 318.0948]; *m/z* (ES^+) 296 ($[M + H]^+$, 100%); *m/z* (ES^-) 367 ($[M+HCl+Cl]^-$, 20%), 330 ($[M+Cl]^-$, 50%), 294 ($[M-H]^-$, 100%).

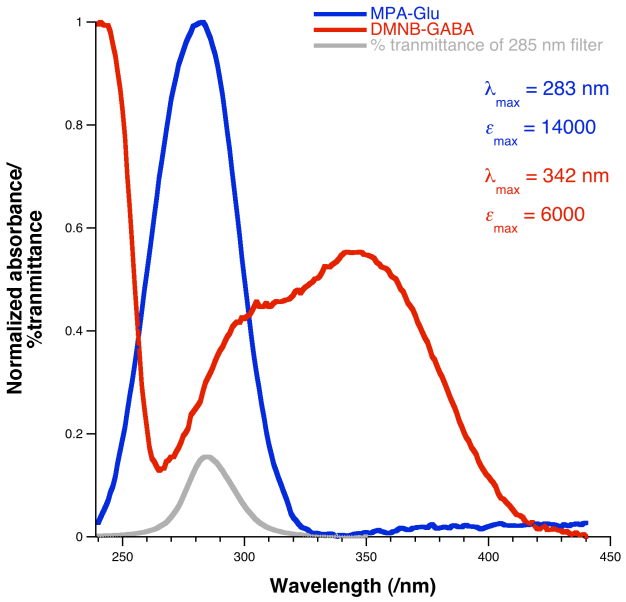


Figure S1. The normalised UV/vis spectra, in artificial cerebral spinal fluid, for DMNB-GABA (red) and MPA-Glu (blue) overlayed with the transmission spectrum of the filter employed when conducting photolysis for ^{1}H NMR analysis (grey). The maximum transmission (15%) is at 285 nm.

^{1}H NMR analysis of photolysis

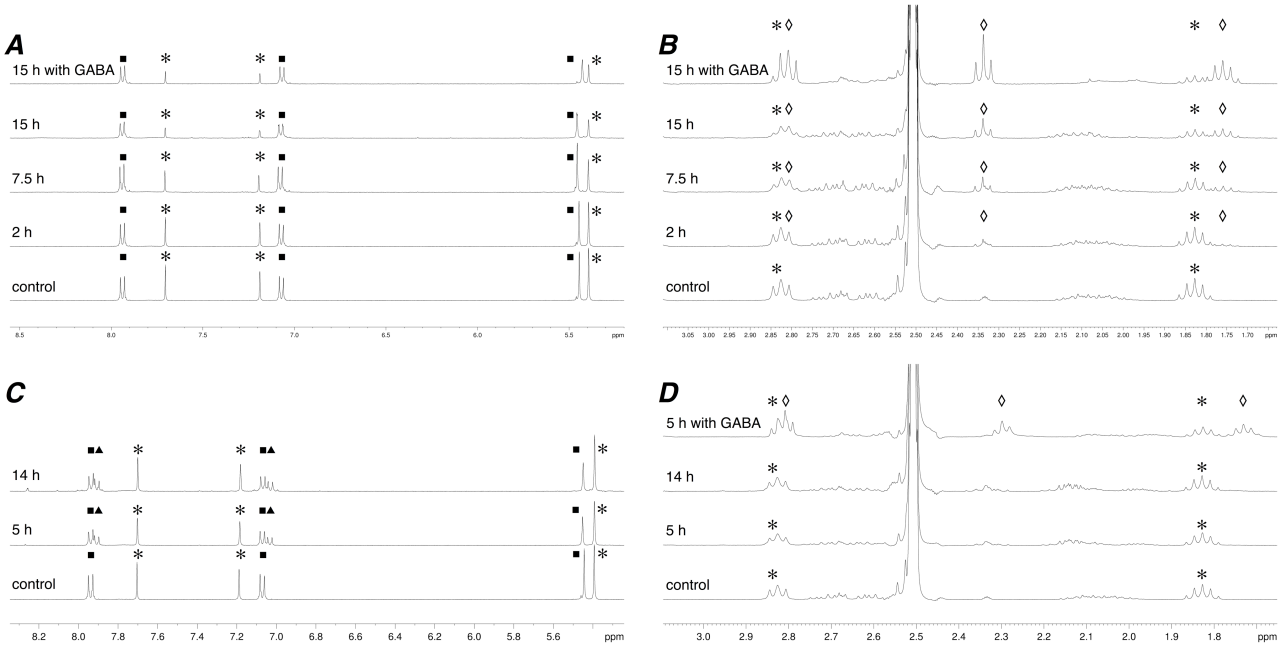


Figure S2. Partial ^{1}H NMR spectra (400 MHz, D_6-DMSO) demonstrating the selective photolysis of DMNB-GABA (*) to give free GABA (◊) in the presence of MPA-Glu (■) (3A and 3B) following irradiation at 405 nm and the selective photolysis of MPA-Glu (■) in the presence of DMNB-GABA (*) (3C and 3D) following irradiation at 285 nm. The appearance of a photolysis by-product (▲) can be observed (3C). Spiking the sample, which had been irradiated at 285 nm for 5 h, with free GABA (◊) confirms that no free GABA is formed following irradiation at this wavelength.

Irradiation at 405 nm			
Time	**% MPA-Glu**	**% DMNB-GABA**	**% GABA**
Control	100	100	0
2 h	100	89	9
7.5 h	100	58	25
15 h	100	47	48

Table S1. Quantification of the photolysis observed when an equimolar mixture of MPA-Glu and DMNB-GABA was irradiated at 405 nm for the time shown. It is demonstrated that only photolysis of DMNB-GABA is observed. The figures are based on integration (using Topspin) relative to an internal standard of hexamethyldisiloxane.

Irradiation at 285 nm			
Time	**% MPA-Glu**	**% DMNB-GABA**	**% GABA**
Control	100	100	0
5 h	80	100	0
14 h	50	100	0

Table S2. Quantification of the photolysis observed when an equimolar mixture of MPA-Glu and DMNB-GABA was irradiated at 285 nm for the time shown. It is demonstrated that only photolysis of MPA-Glu is observed. The figures are based on integration (using Topspin) relative to an internal standard of hexamethyldisiloxane. As the ^{1}H NMR spectra of the MPA-Glu and free Glu are complex and overlap, accurate analysis of the quantity of Glu released was not possible.

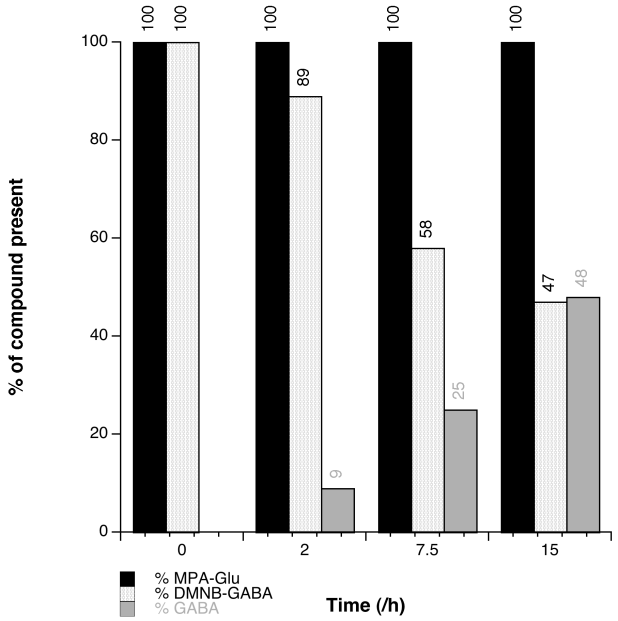


Figure S3. A graphical representation of the photolysis achieved when an equimolar mixture of MPA-Glu and DMNB-GABA was irradiated at 405 nm for the time shown.

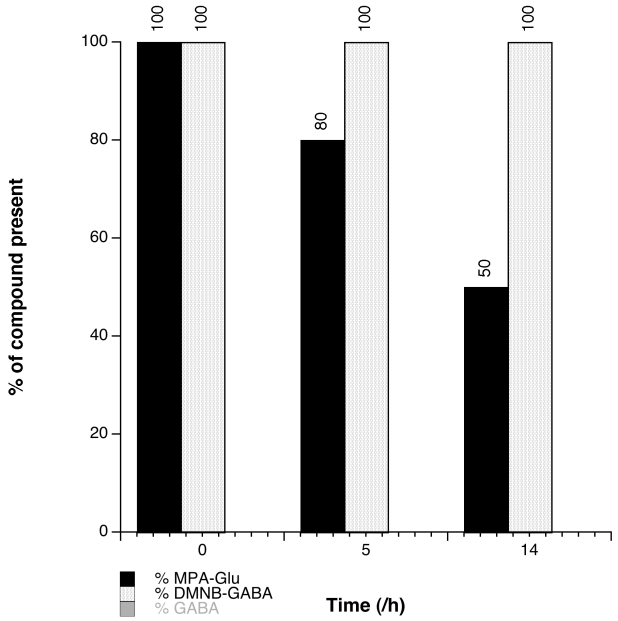


Figure S4. A graphical representation of the photolysis achieved when an equimolar mixture of MPA-Glu and DMNB-GABA was irradiated at 285 nm for the time shown.

General Photolysis Experimental

In order to confirm that wavelength-orthogonal photolysis of MPA-Glu and DMNB-GABA could be achieved, solutions of these compounds were irradiated at 405 nm and 285 nm and the resulting solutions analysed by ^{1}H NMR (with an internal standard included).

DMNB-GABA (0.5 mL, 7.5 mM solution in D_6-DMSO) and MPA-Glu (0.5 mL, 7.5 mM solution in D_6-DMSO) were added to a quartz cuvette. This sample was photolysed for 2, 7.5 and 15 h using a 405 nm 30 mW laser diode. The sample was placed 15 cm from the irradiation source. The sample was positioned so that the irradiation beam passed through the centre of the solution. A control sample, which had not been exposed to laser irradiation, but was exposed to the same conditions as the photolysis samples, was also analysed. A standard solution of hexamethyldisiloxane (125 μL, 30 mM solution in D_4-MeOD) was added to the photolysis sample. This solution was analysed by ^{1}H NMR using the Bruker Avance II 400 (400 MHz) using D_6-DMSO as a reference for internal deuterium lock. Bruker TopSpin software was used to analyze the data.

DMNB-GABA (0.5 mL, 7.5 mM solution in D_6-DMSO) and MPA-Glu (0.5 mL, 7.5 mM solution in D_6-DMSO) were added to a quartz cuvette. This sample was placed inside a metal box equipped with a 205 nm bandpass filter (Figure S1) and photolysed for 5 h and 14 h using a 300 W ultraviolet lamp. The sample was placed 10 cm from the irradiation source. A control sample, which had not been exposed to irradiation, but was exposed to the same conditions as the photolysis samples, was also analysed. A standard solution of hexamethyldisiloxane (125 μL, 30 mM solution in D_4-MeOD) was added to the photolysis sample. This solution was analysed by ^{1}H NMR using the Bruker Avance II 400 (400 MHz) using D_6-DMSO as a reference for internal deuterium lock. Bruker TopSpin software was used to analyze the data.

***in vitro* photolysis experimental section**

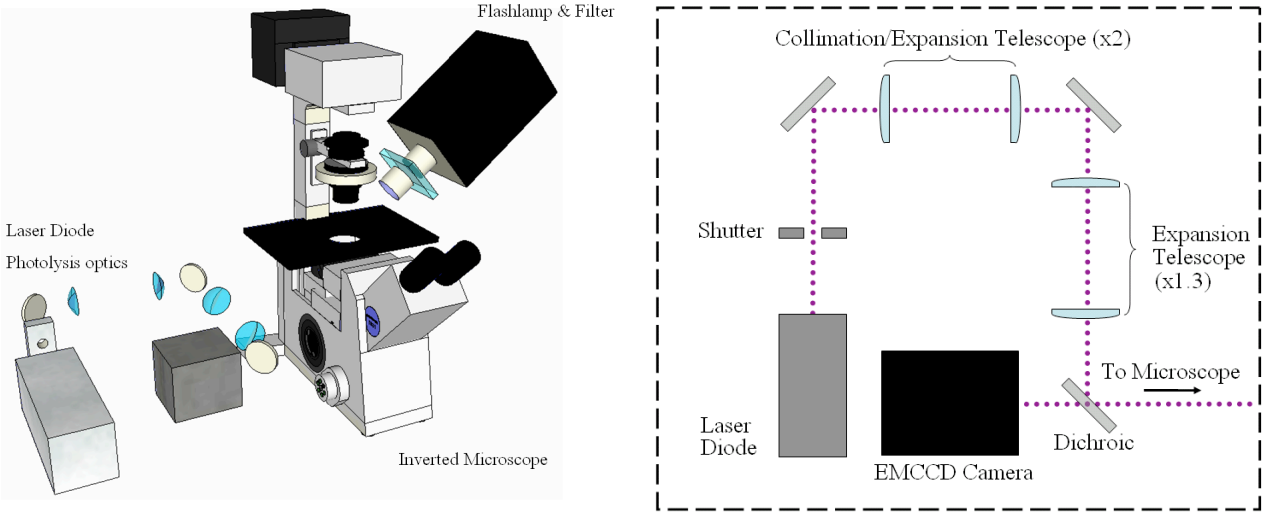


Figure S5. 3D schematic of the *in vitro* photolysis apparatus. Orthogonal photolysis was achieved in an inverted microscope. Selective photolysis of the MPA-Glu was accomplished using a flash lamp system (JML-C1, Rapp Optoelectronic, Hamburg, Germany). The flash lamp was focused to cover the entire sample region using the built in focusing lens. The output from the flash lamp was filtered using a bandpass filter (254FIB25, Knight Optical, Harrietsham, UK) which had a central transmission wavelength of 254 nm with a bandwidth of 8 nm. Orthogonal photolysis of DMNB-GABA was achieved using a 405 nm laser diode (Topica Photonics PxLS, Hamburg, Germany) the output of which could be controlled through the use of the mechanical shutter (Uniblitz VS25, Rochester, NY). The laser diode was coupled into the microscope through the use of beam steering mirrors and a dichroic mirror (530 BK 16, Comar, Cambridge, UK) which was placed in the beam path of the electron multiplying charge coupled device (EMCCD) imaging camera. The external optics were designed to achieve efficient coupling of the laser into the microscope and comprised a x2 telescope for initial beam expansion and collimation and a second x1.3 expansion telescope to ensure that the back aperture of the microscope objective was fully filled.

The *in vitro* orthogonal photolysis of the caged compounds was performed with an inverted microscope (IX71, Olympus) incorporating a high numerical aperture (NA) oil immersion objective (UPlanSApo 100x/1.4, Olympus) and a custom-built electrophysiology stage. Biological samples were held in a perfusion chamber which provided the necessary physiological environment to maintain biological viability for the duration of the experiment while permitting access for the high NA objective and electrophysiological equipment. Selective orthogonal photolysis of the caged compounds in solution was achieved by means of two independent light

sources. Selective photolysis of the MPA-Glu (**6**) was obtained with a mercury flash lamp (JML-C1, Rapp Optoelectronic) in combination with a 254 nm interference bandpass filter (254FIB25, Knight Optical) mounted externally to the microscope assembly and focussed onto the sample plane by an internal focusing lens. Orthogonal photolysis of the DMNB-GABA (**1**) was accomplished with a 405 nm UV laser diode (PxLS, Toptica photonics) co-aligned to the optical path of the microscope through the camera port of the microscope utilising a custom built optical cage plate system (Thorlabs) which collimated the output of the diode laser and expanded the beam to match the back aperture of the high NA objective. A dichroic mirror (530 BK 16, Comar) permitted the introduction of the photolysis beam without interrupting image capture with a high quality EMCCD camera (iXon DV887DCS-BV, Andor technology). A mechanical shutter (Uniblitz VS25, Rochester) provided temporal control of the 405 nm beam. The arrangement provided delivery of both wavelengths at the sample plane of the microscope with exquisite control over exposure duration and intensity.

Tissue Culture

Cultured hippocampal neurons were prepared as previously described (Kaech & Banker Nature Protocols 2006). Briefly, rat hippocampi were isolated at E18 and mechanically dissociated in 2.5% trypsin. Hippocampal neurons were then plated (at a density of 20000 cells per coverslip) on coverslips previously coated with poly-D-lysine and fibronectin to aid cell adherence. The coverslips were suspended over astroglia feeder layers and incubated in Neurobasal based conditioned medium at 35 °C and 5% CO_2. Neurons were maintained in culture for up to three weeks.

Conditioned media was a supplemented Neurobasal solution containing: 8% B27 supplement, 0.25 μM GlutaMAX, 0.25 μM Glutamine, 25 μg/mL insulin, 100 μg/mL transferrin, 5 ng/mL bFGF, 25 mM 2-mercaptoethanol, 100 μg/mL primocin antibiotic. Antibiotic was removed from the culture media by supplementing with a supplemented Neurobasal solution that was antibiotic free.

Electrophysiology

Giga-seal whole-cell patch clamp recordings were obtained from visually identified hippocampal pyramidal neurons. Patch pipettes (4.0-7.0 MΩ) were pulled from borosilicate glass capillary tubes using a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments). The internal patch pipette solution contained 140 mM KGluconate, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM Na_2ATP and 0.4 mM Na_3GTP; pH 7.2-7.3 with KOH; osmolarity 290-300 mOsM. Recordings were performed at room temperature ~25 °C in a static bath. The bath solution consisted of a Tyrode's solution containing 128 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM HEPES, 4 mM $NaHCO_3$; pH 7.25 with NaOH; osmolarity 290-300 mOsM. Caged neurotransmitters were dissolved in DMSO (final concentration 0.1-1%) and added to the bath at the beginning of the experiment. Once a whole-cell recording configuration had been established, the access resistance was monitored throughout the experiment. Cells, in which access varied > 20% over the course of the experiment were discarded. No series resistance compensation and no corrections for liquid junction potentials were made.

Neurons were clamped at a membrane holding potential of –50 mV for the duration of the experiment. Data were generated and acquired using an Axopatch 200 A amplifier (Axon Instruments) and the software WinWCP (Strathclyde). Data was sampled at 7.5 kHz and filtered by a 5 kHz Bessel filter. Data were further filtered offline by a low pass filter with a cutoff at 819.3 Hz.

Dose-response analysis

Wavelength-orthogonal photolysis experiments were conducted using 125 μM MPA-glutamate and 50 μM DMNB-GABA, caged neurotransmitter concentrations were chosen that elicited the maximal current response. The charge of the current elicited is calculated by determining the integral of the deviation from baseline in response to light presentation. MPA-Glutamate (125 μM: 132.79±27.50 pC, n=6; 50 μM: 130.15±58.25 pC, n=7; 25μM: 31.80±11.11 pC, n=5). DMNB-GABA (25 μM: 79.61±41.14 pC, n=4; 50 μM: 71.44±19.98, n=9; 100 μM: 50.42±11.05, n=6).

Discussion of the potential role of the caged compounds as antagonists during orthogonal photolysis of MPA-Glu and DMNB-GABA

MPA-Glu does not act as a GABA receptor antagonist because a GABA response is observed upon irradiation at 405 nm. DMNB-GABA does not act as a Glu receptor antagonist because a Glu response is observed upon irradiation at 250-260 nm. MPA-Glu does not act as a Glu receptor antagonist because only a proportion of it is photolysed at 250-260 nm and a Glu response is observed. Similarly, DMNB-GABA does not act as a GABA receptor antagonist because only a proportion of it is photolysed at 405 nm and a GABA response is observed.

Example of larger Glu and GABA currents

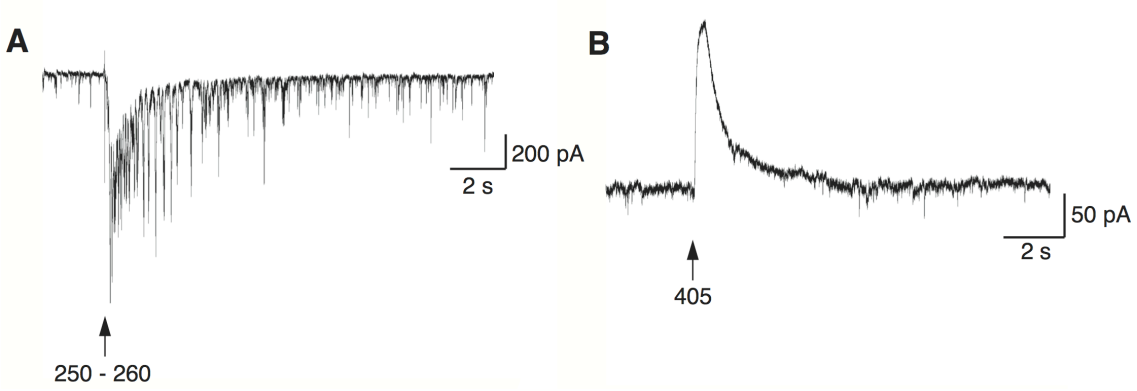


Figure S6. Examples of large currents elicited by photolysis of MPA-glutamate (panel **A**, 50 μM) and DMNB-GABA (panel **B**, 50 μM). Arrows indicated point of light illumination at 250-260nm and 405 nm respectively.

Photolysis of MPA-Glu and DMNB-GABA under current clamp

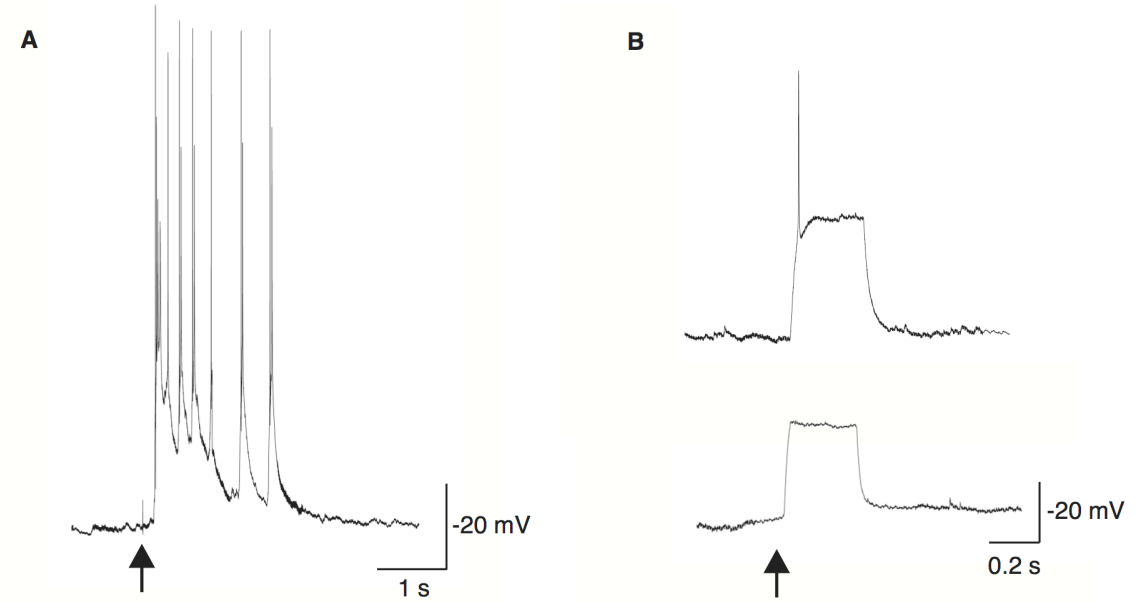


Figure S7. The effect of photolysing caged Glu or GABA on action potential firing in hippocampal neurons under current clamp conditions. **A**. Representative recording, illustrating the effect of photolysis of MPA-glutamate (125 μM) by exposure to light (250-260 nm, arrow indicates point of light presentation). Photoylsis of MPA-glutamate causes membrane potential depolarisation resulting in action potential firing in hippocampal neurons held in current clamp. **B**. Direct current injection results in the firing of an action potential (**top panel**) that is shunted by the photolysis of DMNB-GABA (50 μM) by light presentation of 405 nm immediately prior to current injection (indicated by the arrow**, bottom panel**).

HPLC assessment of caged compound stability in water

The hydrolytic stability of MPA-Glu (**6**) and DMNB-GABA (**1**) was assessed. A Dionex Acclaim 120 C_{18} column was flushed with MeCN:H_2O, 7:3, and then MeCN:H_2O, 25:75 + 1% (v/v) TFA. Elution of each sample was achieved using a mobile phase of MeCN:H_2O, 25:75 + 1% (v/v) TFA for 30 minutes. Detection was achieved by 225 nm and 254 nm lamps. A blank sample of MeCN:H_2O, 25:75 + 1% (v/v) TFA (20 µL) was run before each batch of samples.

MPA-Glu at a concentration of 1 mg/mL was prepared in a solution of MeCN:H_2O, 15:85 + 10% (v/v) TFA. This solution was loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 5.9 minutes was observed, corresponding to MPA-Glu (Figure S8A). To determine the hydrolytic stability of MPA-Glu, Millipore water (0.85 mL) was added to a sample of MPA-Glu (1 mg) and left in the dark at RT overnight. After this time, MeCN (0.15 mL) and TFA (0.10 mL) were added to the sample, the solution loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 6.3 minutes, corresponding to MPA-Glu, was observed. No significant new peaks were observed (Figure S8B). Identical results were obtained when this experiment was repeated.

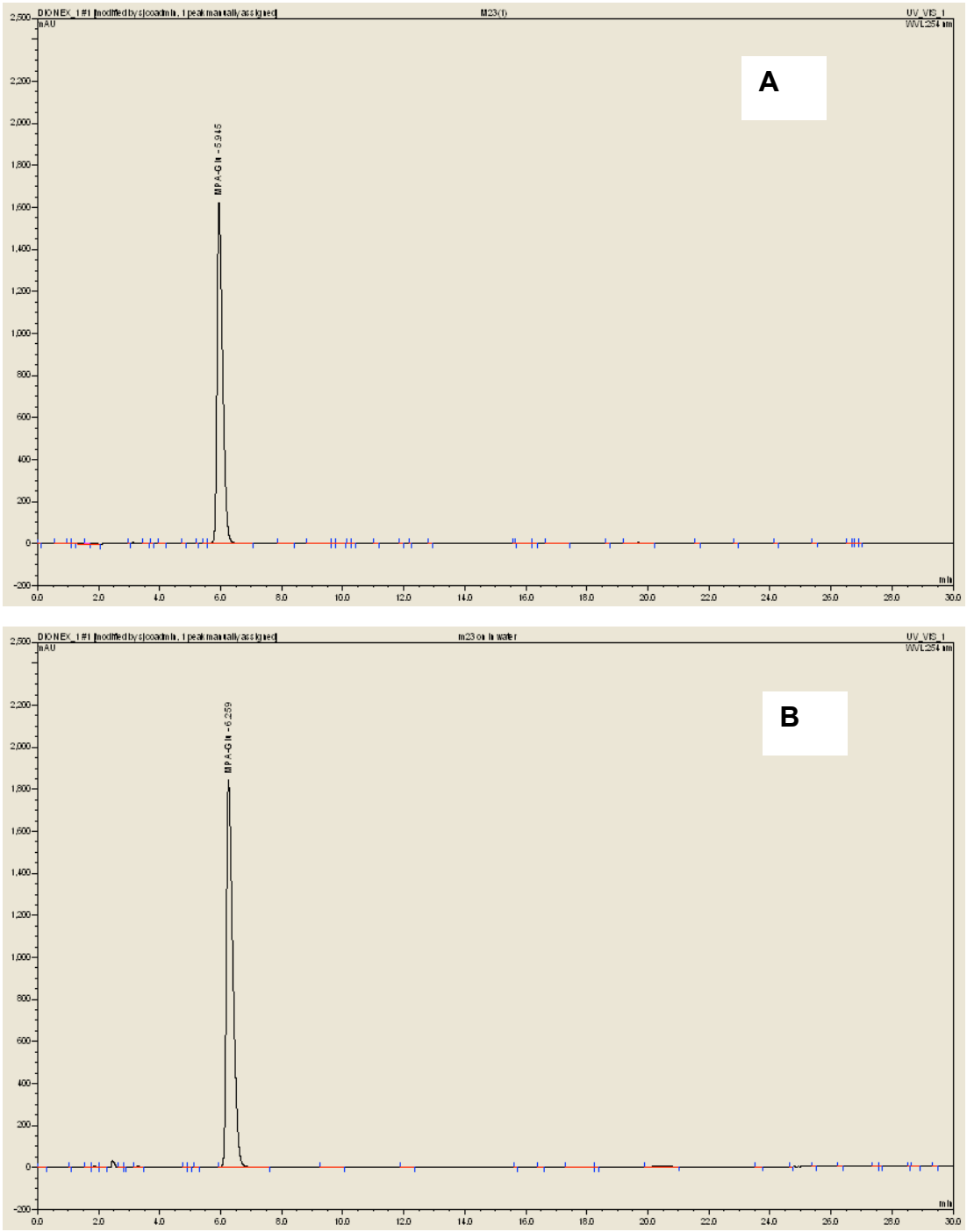


Figure S8. HPLC analysis of MPA-Glu hydrolytic stability. **A**. Control, MPA-Glu prepared from frozen solid sample and run immediately. **B**. MPA-Glu left overnight in water.

DMNB-GABA at a concentration of 1 mg/mL was prepared in a solution of MeCN:H_2O, 15:85 + 10% (v/v) TFA. This solution was loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 8.6 minutes was observed, corresponding to DMNB-GABA (Figure S9A). A minor impurity (1.4%) with a retention time of 8.0 minutes was also observed. It was confirmed by HPLC that this impurity was not 4,5-dimethoxy-2-nitrobenzyl alcohol, the DMNB-GABA precursor (data not shown). To determine the hydrolytic stability of DMNB-GABA, Millipore water (0.85 mL) was added to a sample of DMNB-GABA (1 mg) and left in the dark at RT overnight. After this time, MeCN (0.15 mL) and TFA (0.10 mL) were added to the sample, the solution loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak, with a retention time of 8.9 minutes, corresponding to DMNB-GABA, was observed. The impurity (1.3%) with a retention time of 8.2 minutes was observed. No significant new peaks were observed (Figure S9B).

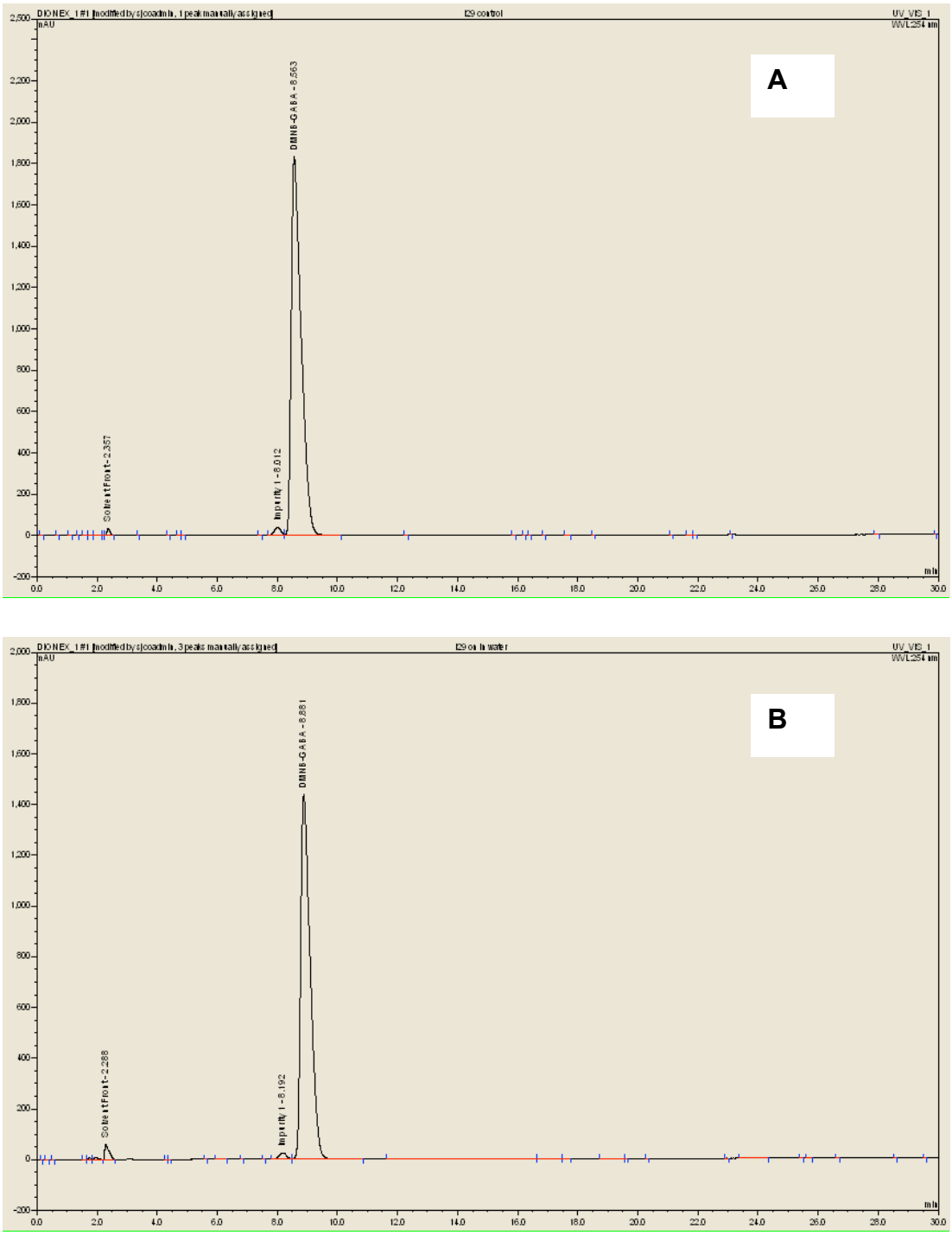


Figure S9. HPLC analysis of DMNB-GABA hydrolytic stability. **A**. Control, DMNB-GABA prepared from frozen solid sample and run immediately. **B**. DMNB-GABA left overnight in water.

HPLC assessment of caged compound stability in Tyrode's solution

A Dionex Acclaim 120 C_{18} column was flushed with MeCN:H_2O, 7:3, and then MeCN:H_2O, 25:75 + 1% (v/v) TFA. Elution of each sample was achieved using a mobile phase of MeCN:H_2O, 25:75 + 1% (v/v) TFA for 30 minutes. Detection was achieved by 225 nm and 254 nm lamps. A blank sample of Tyrode's solution + 10% (v/v) TFA (20 µL) was run before each batch of samples.

MPA-Glu at a concentration of 1 mg/mL was prepared in a solution of Tyrode's solution + 10% (v/v) TFA. This solution was loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 6.1 minutes, corresponding to MPA-Glu, was observed (Figure S10A). In order to assess the stability of MPA-Glu, Tyrode's solution (0.90 mL) was added to a sample of MPA-Glu (1 mg) and left in the dark at RT overnight. After this time TFA (0.10 mL) was added to the sample, the solution loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 5.7 minutes, corresponding to MPA-Glu, was observed. No significant new peaks were observed (Figure S10B).

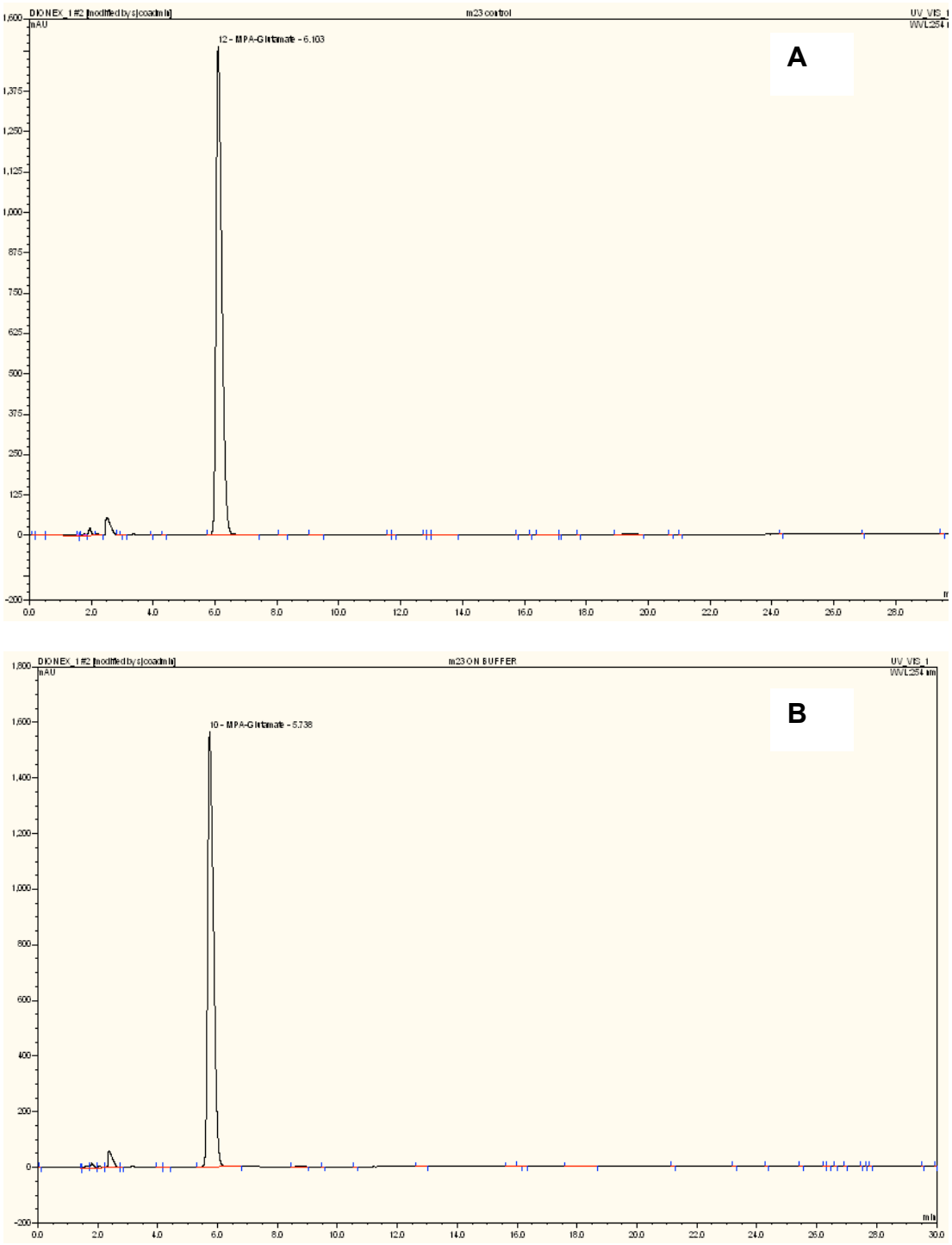


Figure S10. HPLC traces of MPA-Glu to assess hydrolytic stability in Tyrode's solution. **A**. Control, MPA-Glu prepared from frozen solid sample and run immediately. **B**. MPA-Glu left overnight in Tyrode's solution.

DMNB-GABA at a concentration of 1 mg/mL was prepared in a solution of Tyrode's solution + 10% (v/v) TFA. This solution was loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 8.3 minutes, corresponding to DMNB-GABA, was observed. An impurity (3.5%) with a retention time of 7.8 minutes was observed (Figure S11A). It was confirmed by HPLC that this impurity was not 4,5-dimethoxy-2-nitrobenzyl alcohol, the DMNB-GABA precursor (data not shown). In order to assess the hydrolytic stability of DMNB-GABA, Tyrode's solution (0.90 mL) was added to a sample of DMNB-GABA (1 mg) and left in the dark at RT overnight. After this time TFA (0.10 mL) was added to the sample, the solution loaded onto the column (20 µL) and eluted with MeCN:H_2O, 25:75 + 1% (v/v) TFA. A peak with a retention time of 8.0 minutes, corresponding to DMNB-GABA, was observed. The impurity (4.0%) with a retention time of 7.5 minutes was observed. No significant new peaks were observed (Figure S11B).

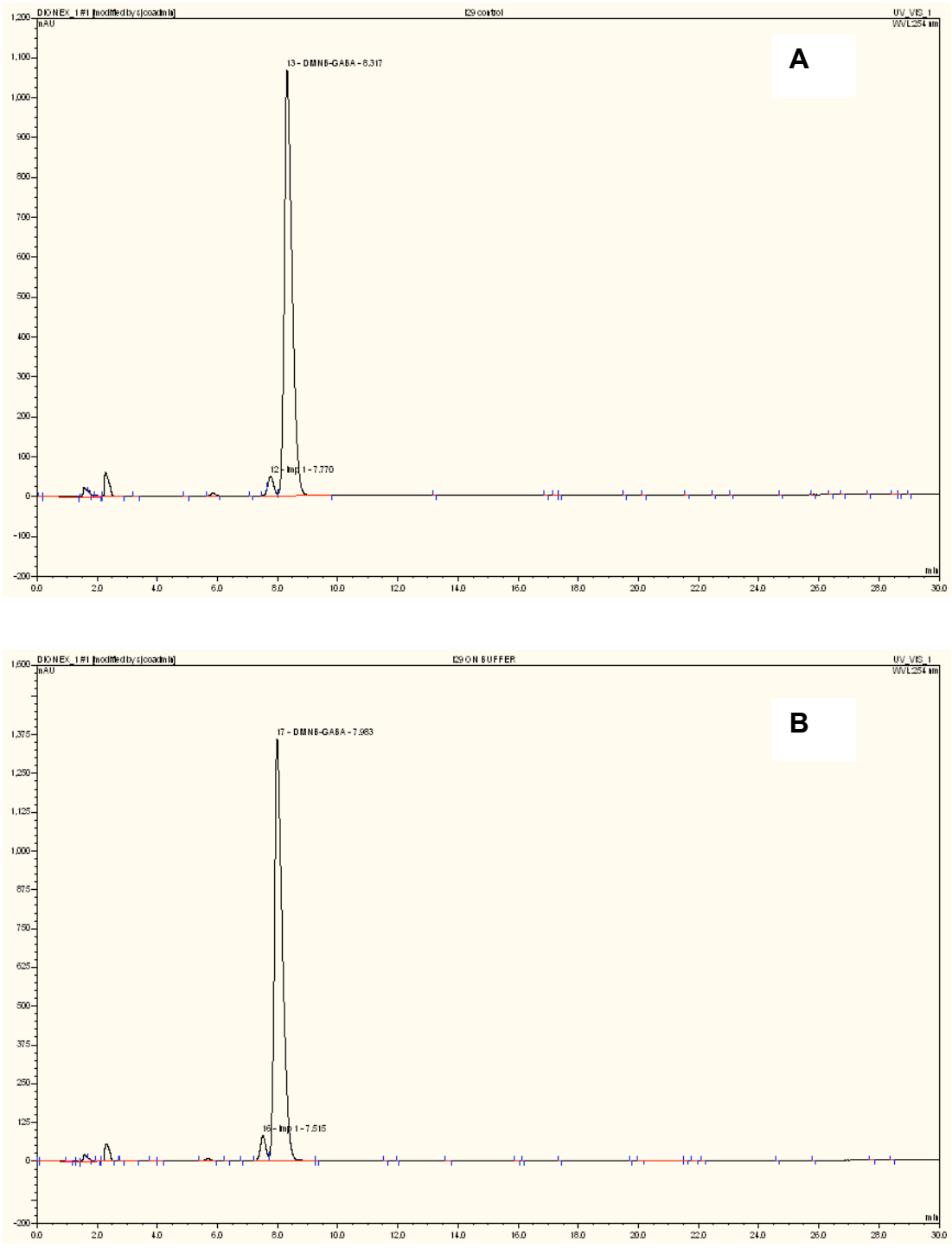
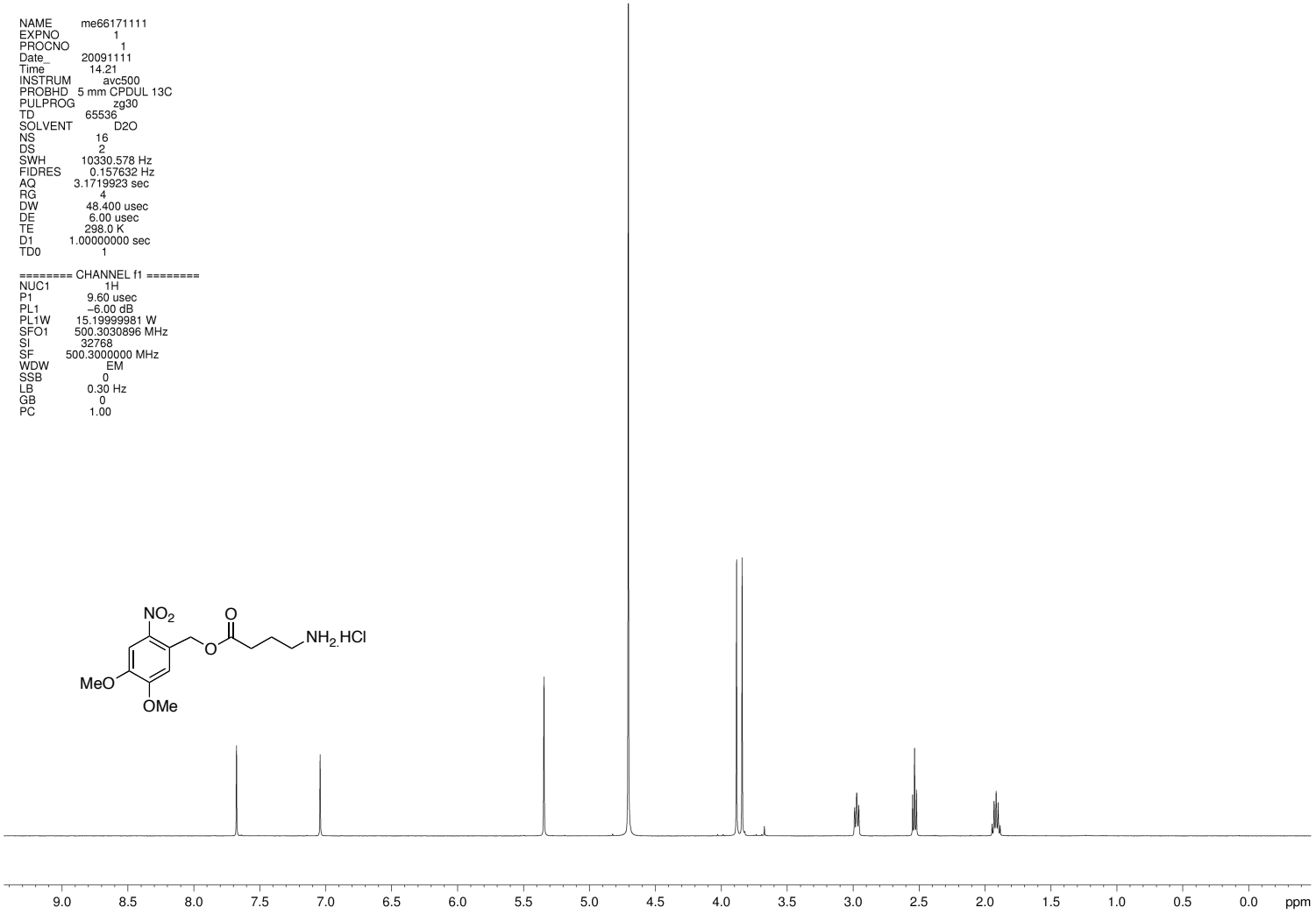


Figure S11. HPLC traces of DMNB-GABA to assess hydrolytic stability in Tyrode's solution. **A**. Control, DMNB-GABA prepared from frozen solid sample and run immediately. **B**. DMNB-GABA left overnight in Tyrode's solution.

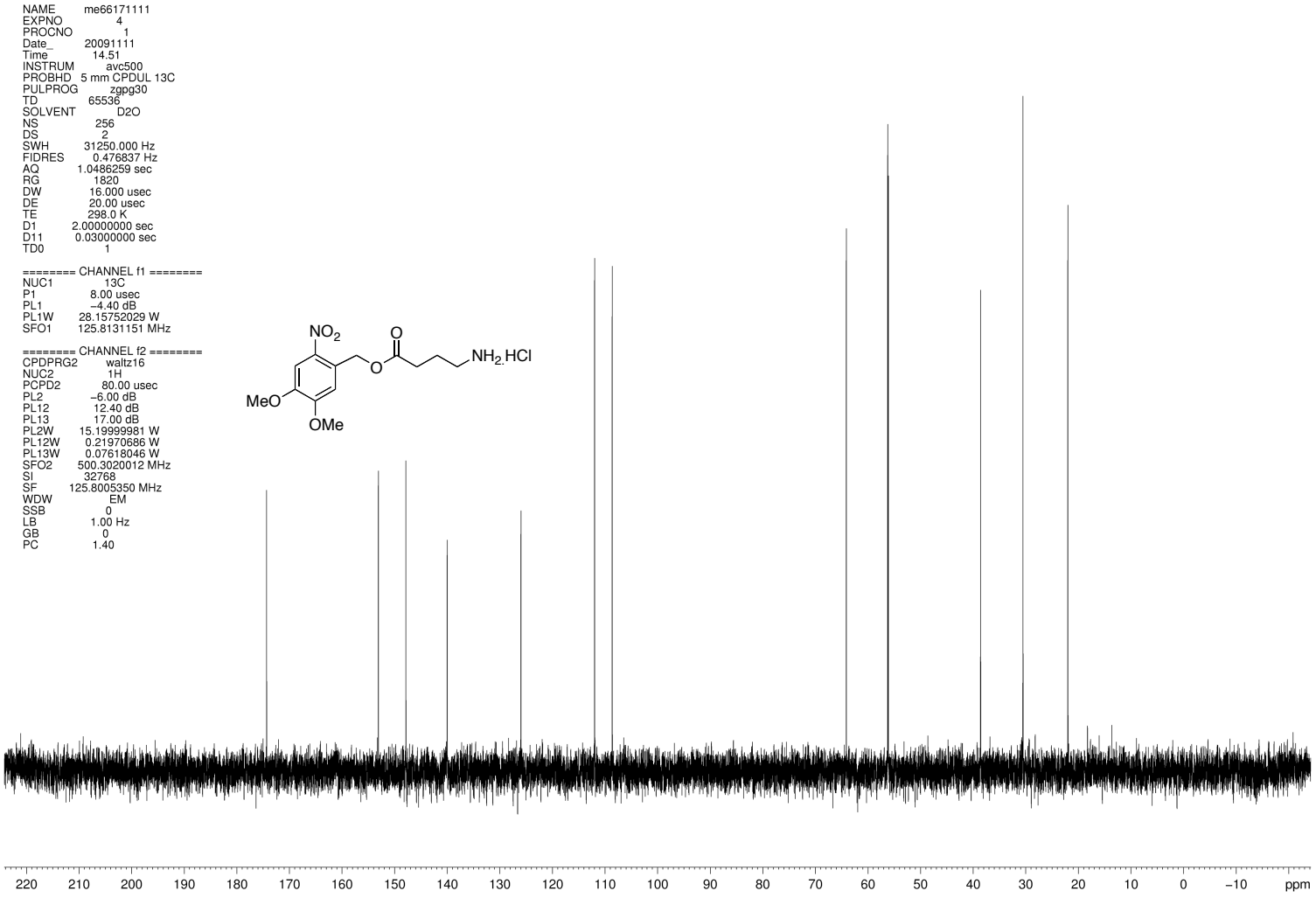
References

(1) Thirlwell, H.; Corrie, J.; Reid, G.; Trentham, D.; Ferenczi, M. *Biophys. J.* **1994**, *67*, 2436-2447.
(2) Wilcox, M.; Viola, R. W.; Johnson, K. W.; Billington, A. P.; Carpenter, B. K.; McCray, J. A.; Guzikowski, A. P.; Hess, G. P. *J. Org. Chem.* **1990**, *55*, 1585-1589.

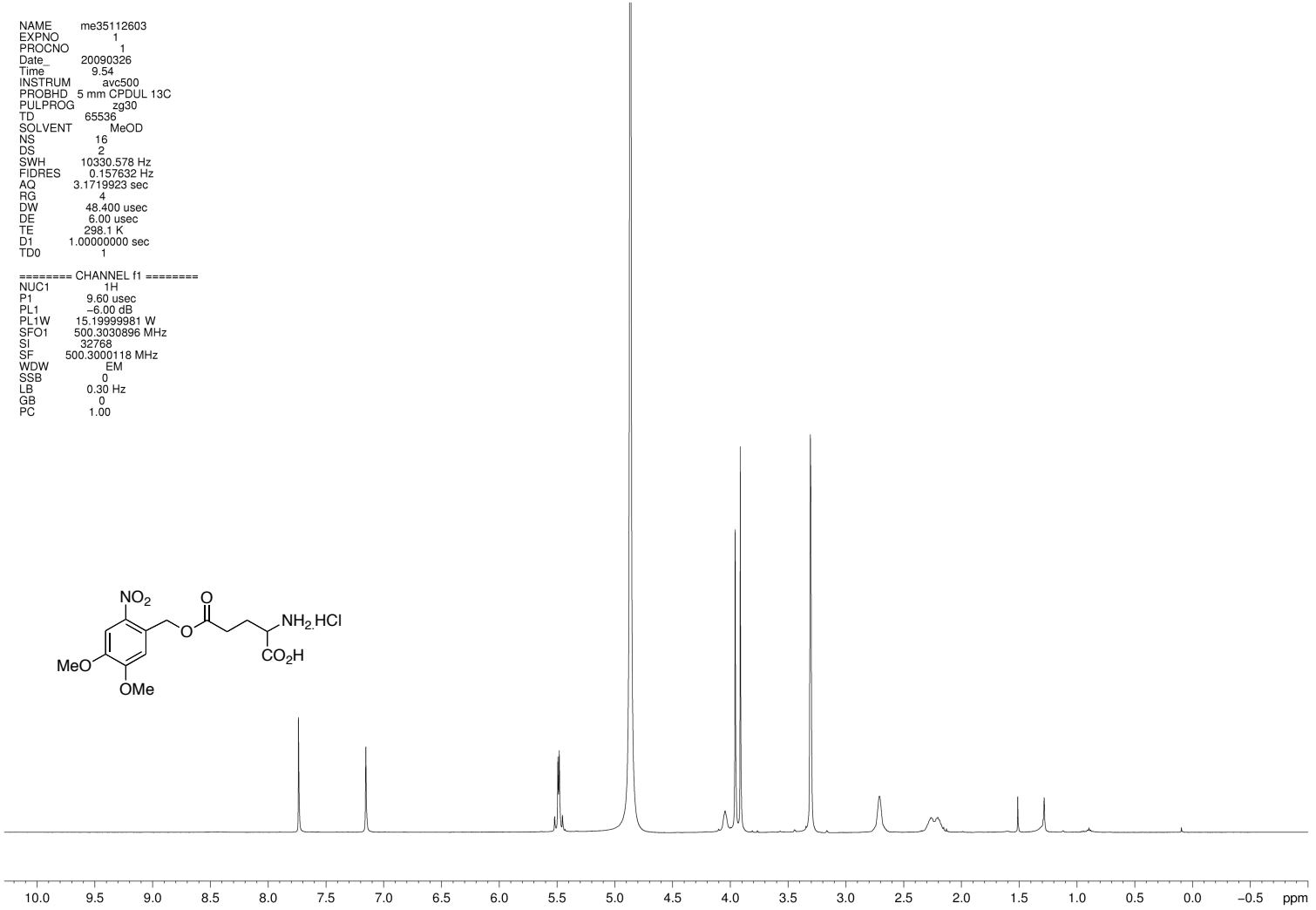
4,5-Dimethoxy-2-nitrobenzyl 4-aminobutanoate hydrochloride (1) - ^{1}H NMR spectrum



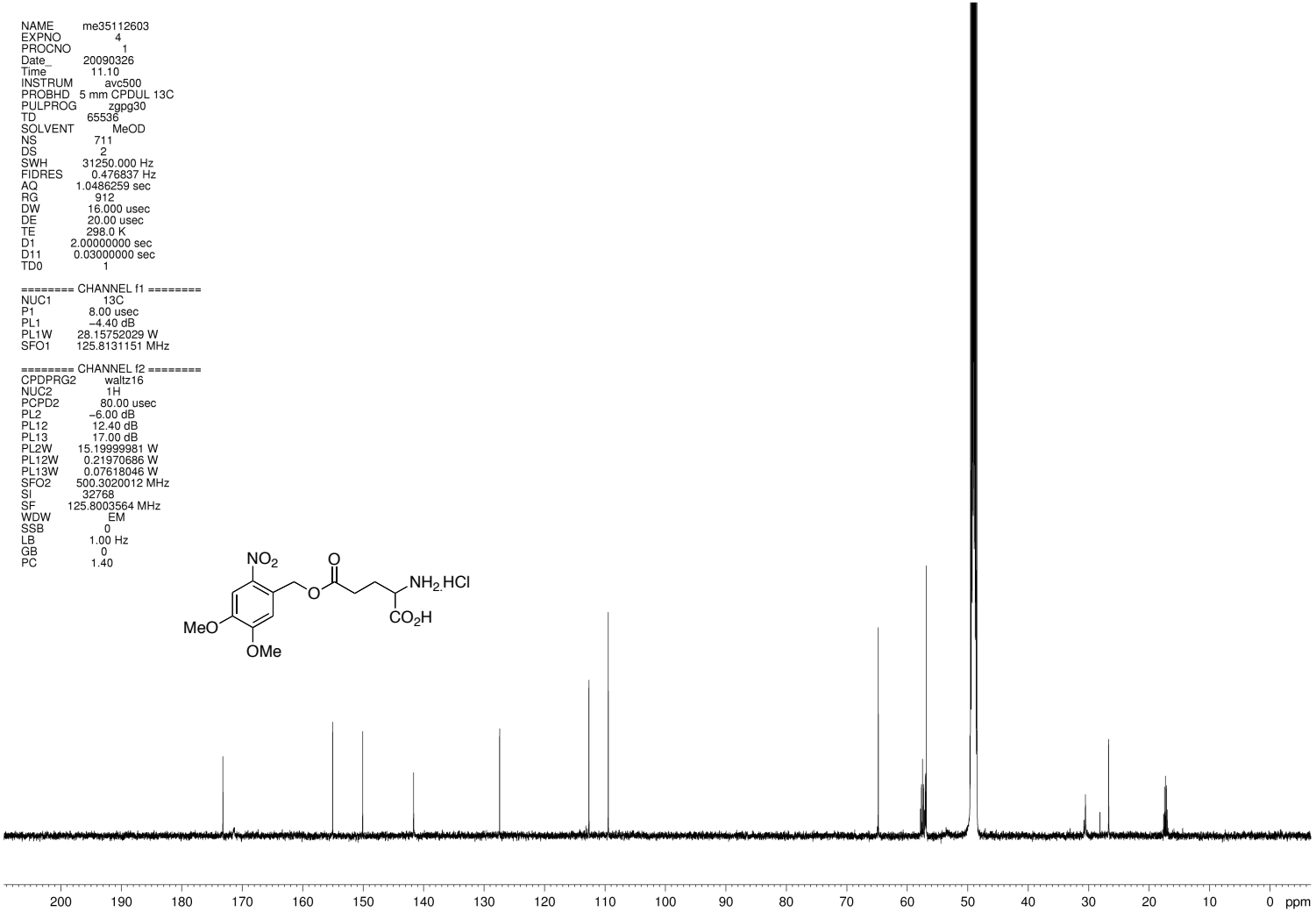
4,5-Dimethoxy-2-nitrobenzyl 4-aminobutanoate hydrochloride (1) - ^{13}C NMR spectrum



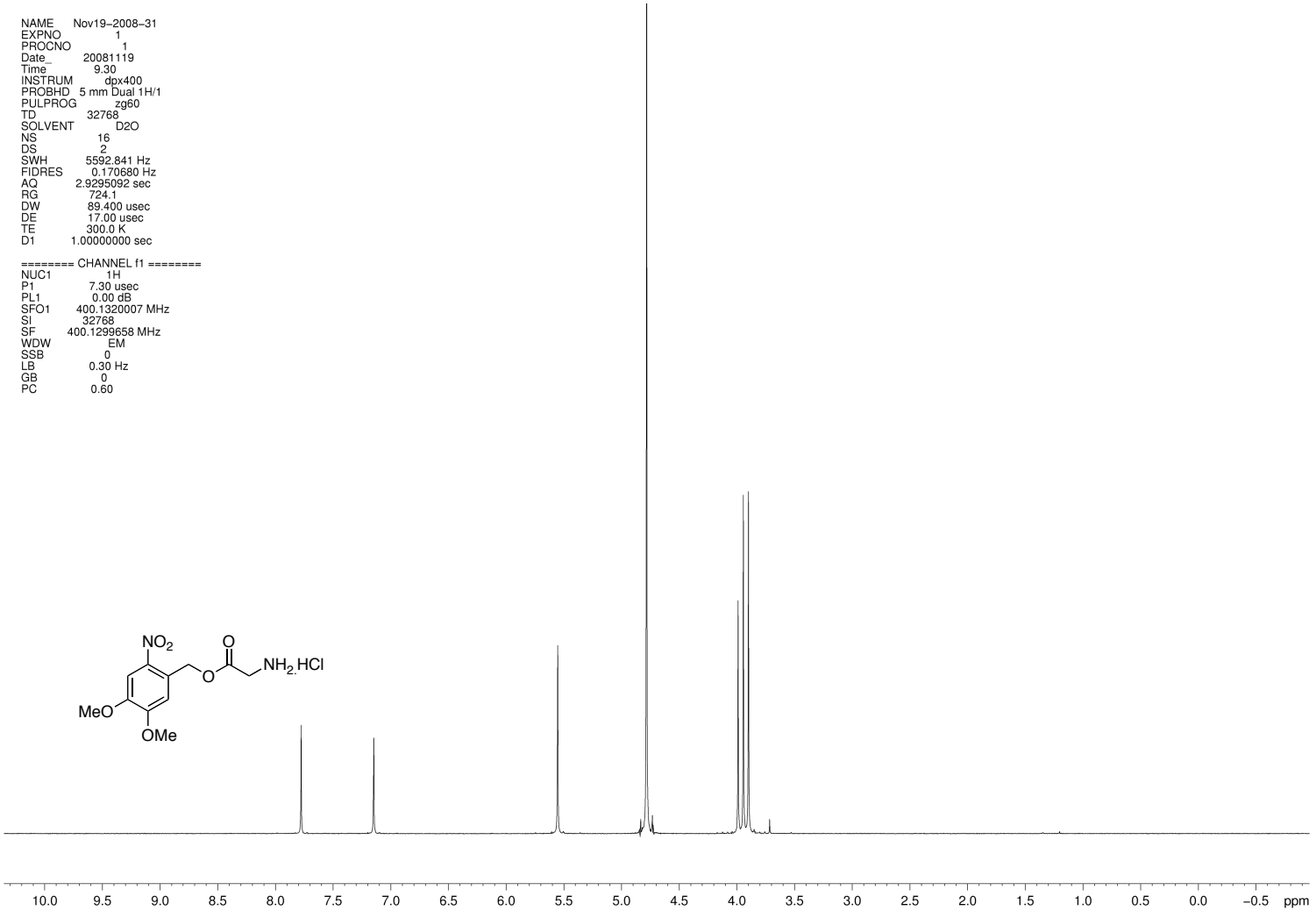
2-Amino-5-(4,5-dimethoxy-2-nitrobenzyloxy)-5-oxopentanoic acid hydrochloride (3) - ^{1}H NMR spectrum



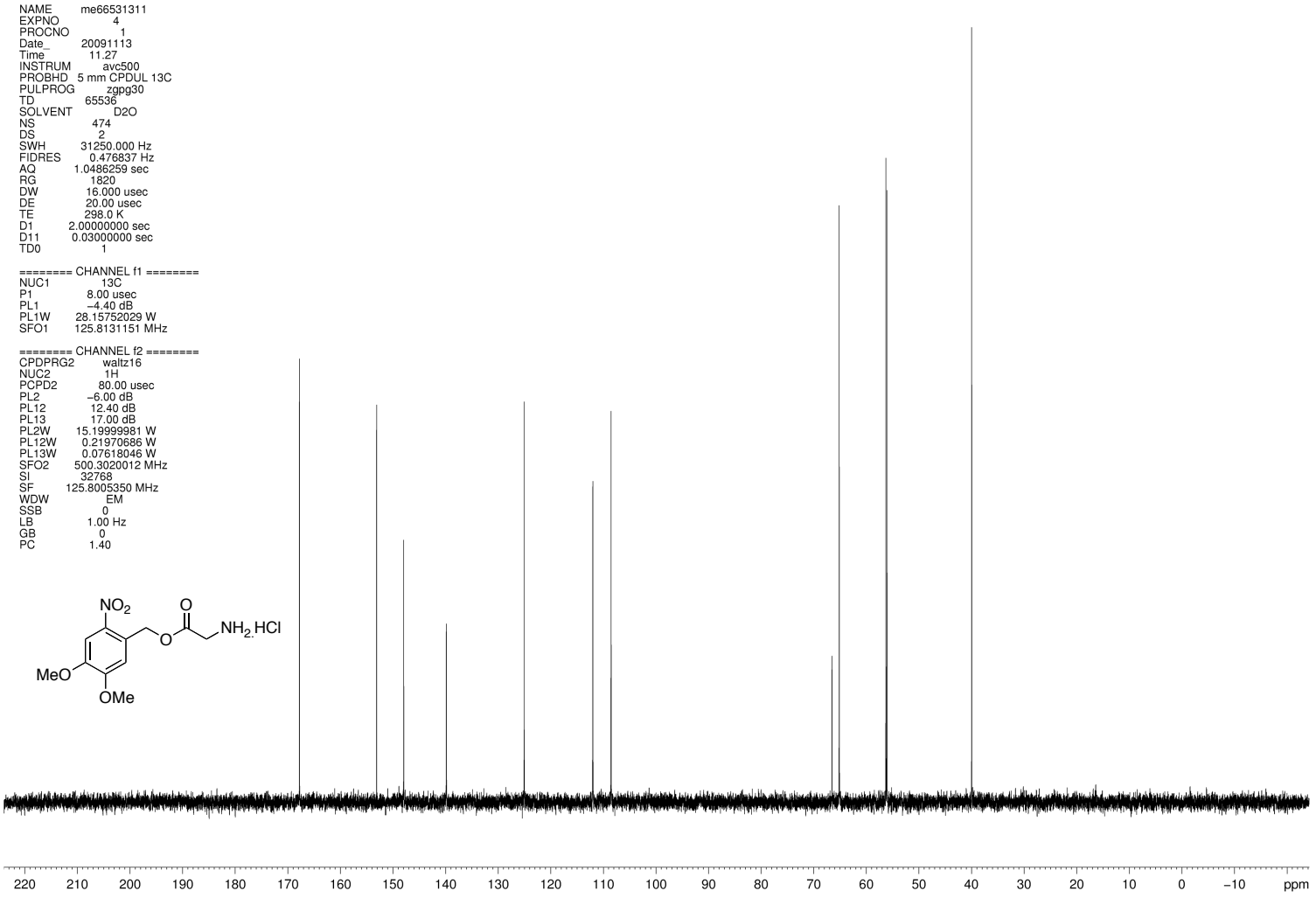
2-Amino-5-(4,5-dimethoxy-2-nitrobenzyloxy)-5-oxopentanoic acid hydrochloride (3) - ^{13}C NMR spectrum



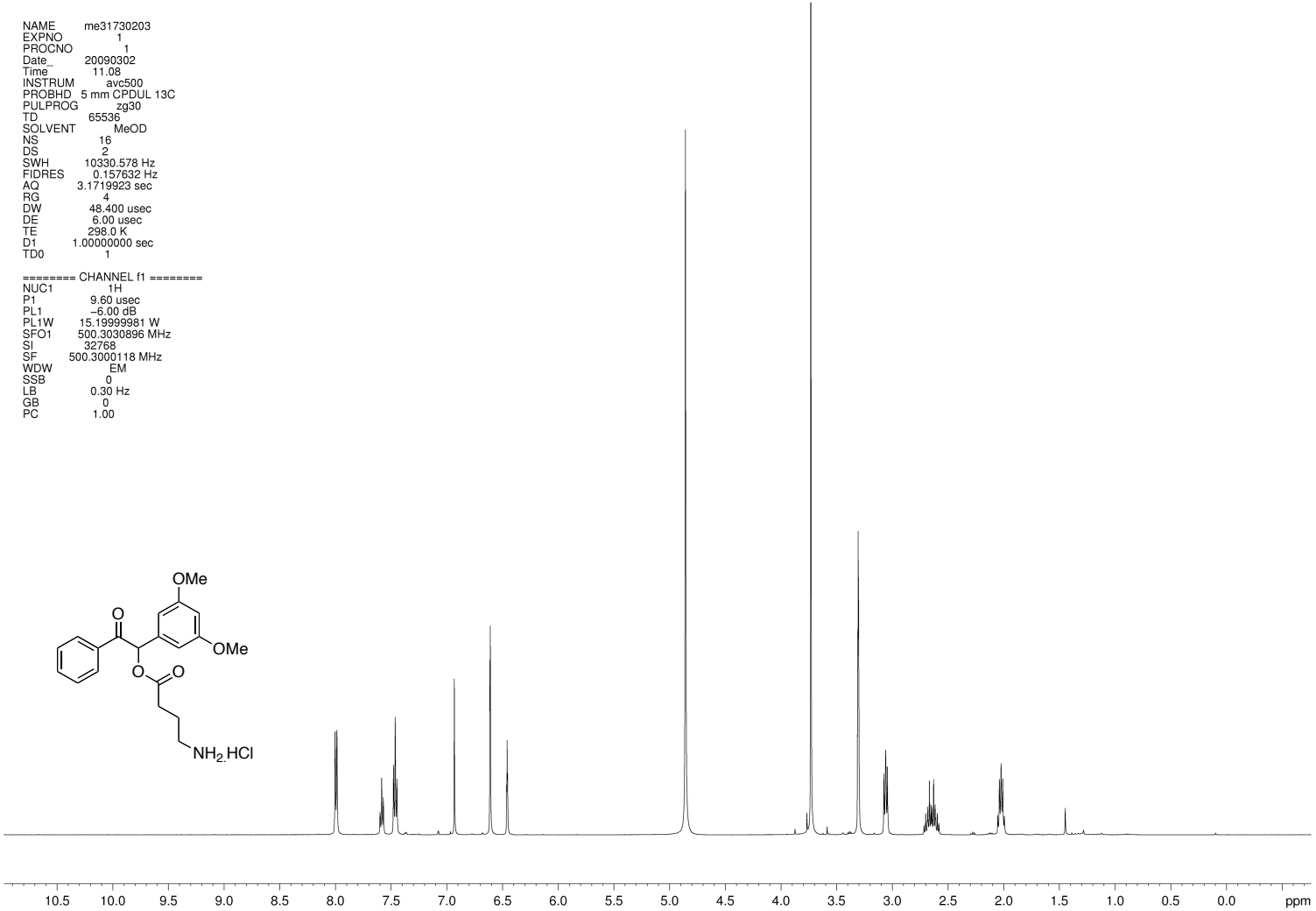
4,5-Dimethoxy-2-nitrobenzyl 2-aminoacetate hydrochloride (2) - ^{1}H NMR spectrum



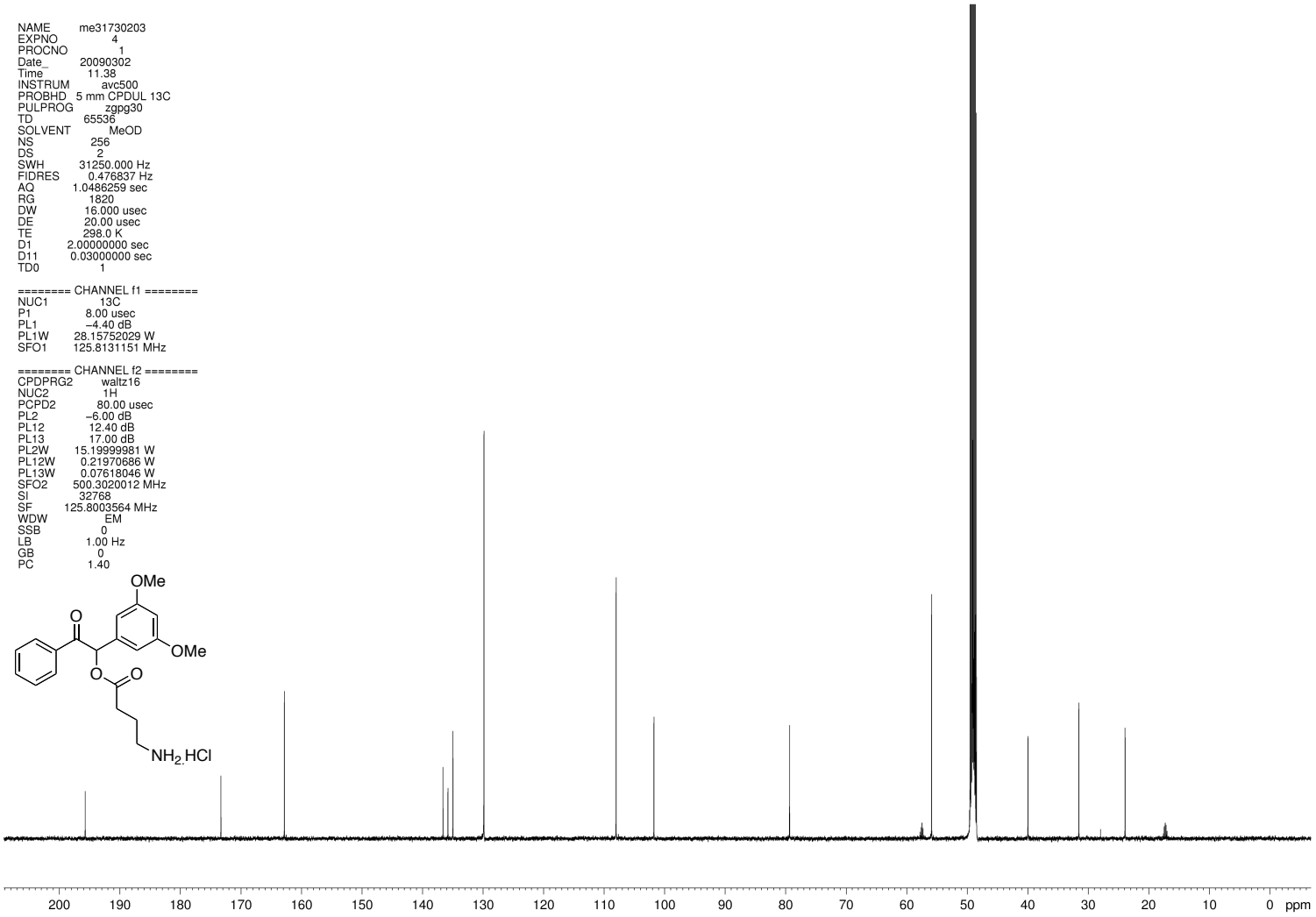
4,5-Dimethoxy-2-nitrobenzyl 2-aminoacetate hydrochloride (2) - ^{13}C NMR spectrum



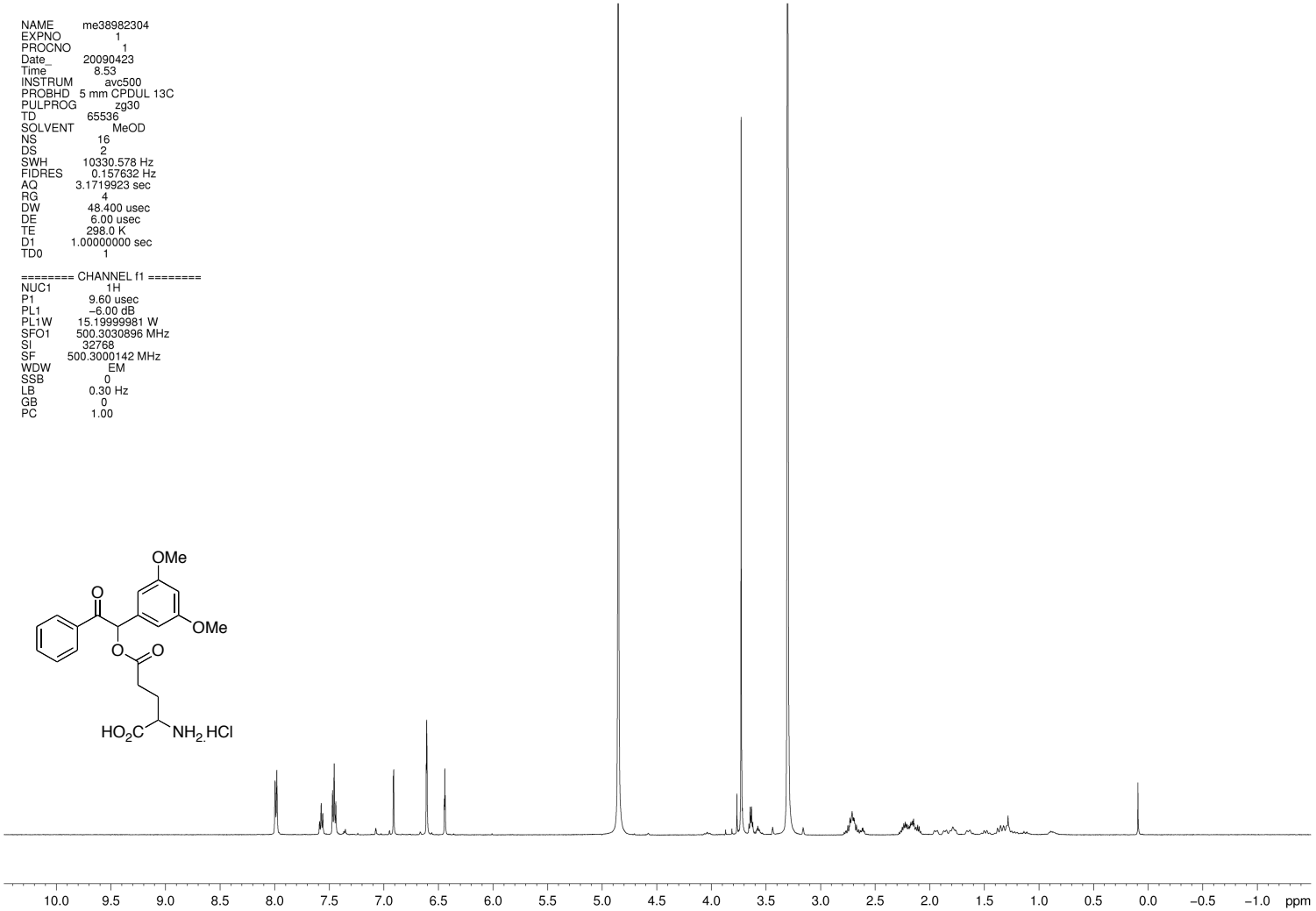
1-(3,5-Dimethoxyphenyl)-2-oxo-2-phenylethyl 4-aminobutanoate hydrochloride (4) - ^{1}H NMR spectrum



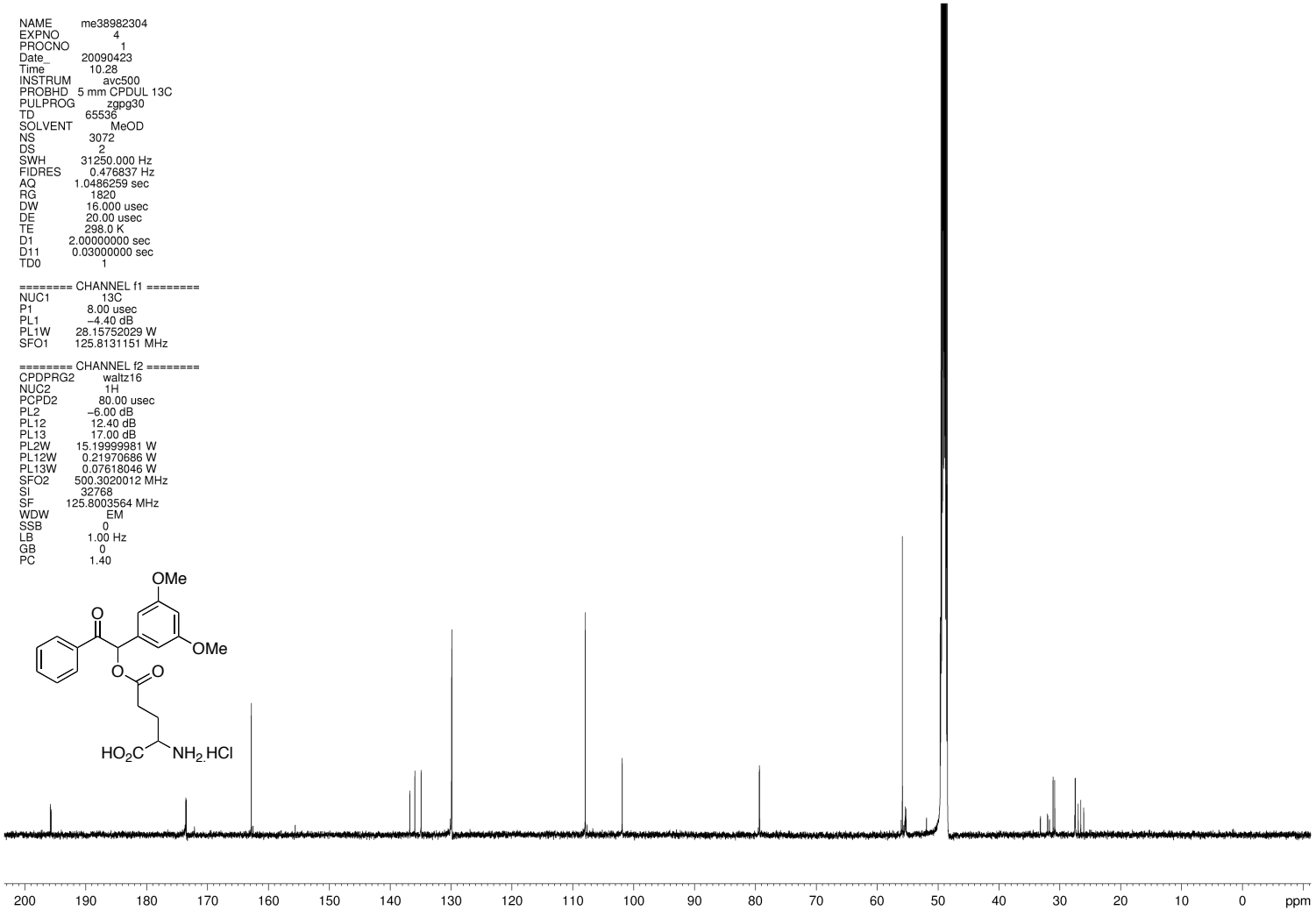
1-(3,5-Dimethoxyphenyl)-2-oxo-2-phenylethyl 4-aminobutanoate hydrochloride (4) - ^{13}C NMR spectrum



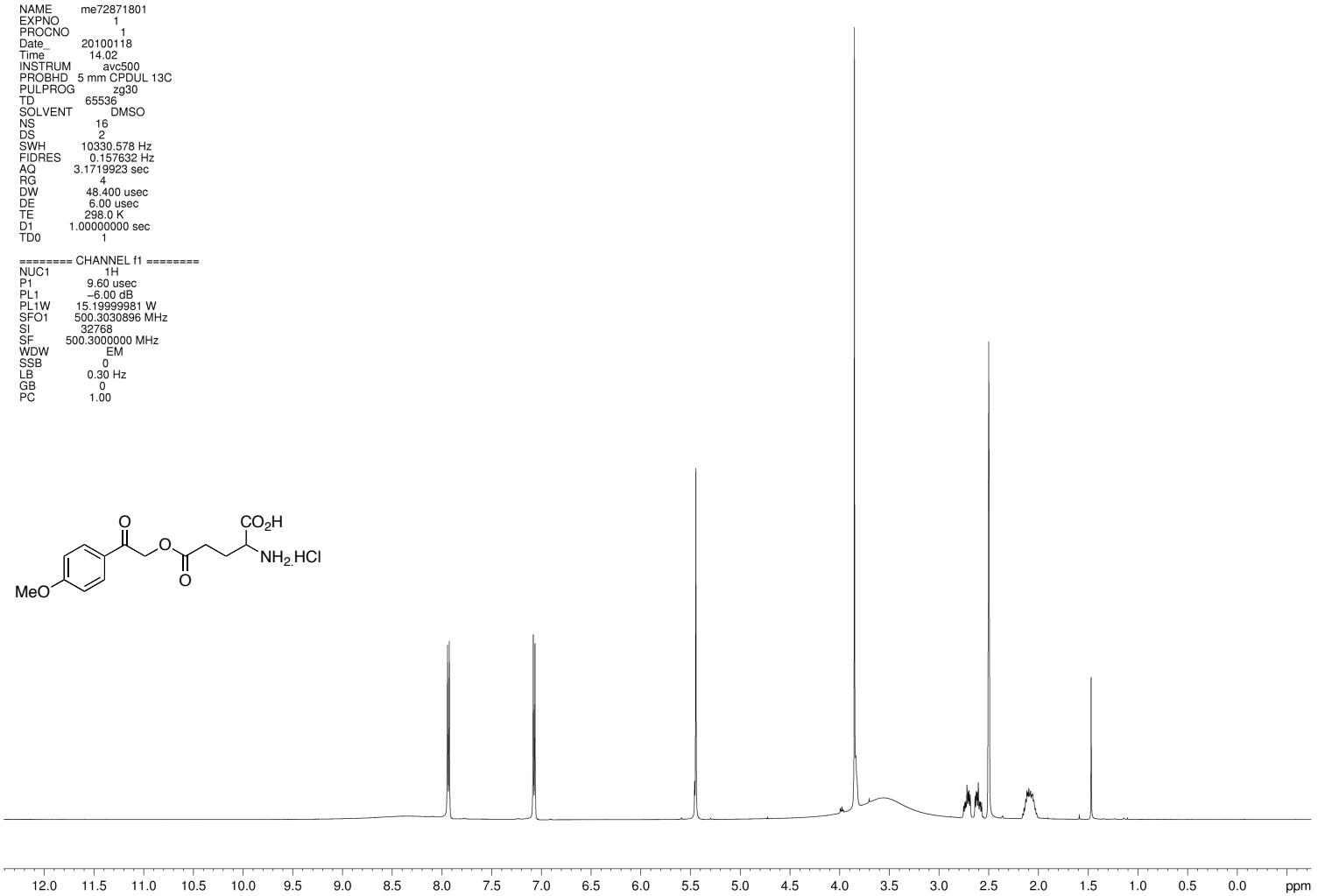
2-Amino-5-(1-(3,5-dimethoxyphenyl)-2-oxo-2-phenylethoxy)-5-oxopentanoic acid hydrochloride (5) - ^{1}H NMR spectrum



2-Amino-5-(1-(3,5-dimethoxyphenyl)-2-oxo-2-phenylethoxy)-5-oxopentanoic acid hydrochloride (5) - ^{13}C NMR spectrum



2-Amino-5-(2-(4-methoxyphenyl)-2-oxoethoxy)-5-oxopentanoic acid hydrochloride (6) - ^{1}H NMR spectrum



2-Amino-5-(2-(4-methoxyphenyl)-2-oxoethoxy)-5-oxopentanoic acid hydrochloride (6) - ^{13}C NMR spectrum