Supplementary Material (ESI) for Photochemical & Photobiological Sciences

Decarboxylation is a significant reaction pathway for photolabile calcium chelators and related compounds

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Electronic Supplementary Information

The ESI (13 pages) contains synthetic details for compounds **4-6**, **8** and **9** (pages 1-7), Figure S1 (difference spectrum for photolysis of **1**; page 8) and details of the equations underlying the calcium titrations, which include Figure S2 (pages 9-12). Additional references, not in the main text, are on page 13.

Synthetic Details

Compounds **4-6** and **8**, **9** were all prepared by appropriate variations of the methods described in the main text for synthesis of **1** and **7**, i.e. by alkylation of glycine ethyl ester with the appropriate 2- or 4-nitrobenzyl bromide,[1] followed where required for synthesis of the iminodiacetate compounds **6** and **9** by a second alkylation of the product with ethyl bromoacetate. In all cases, the resulting mono- or di-esters were saponified and purified by anion-exchange chromatography. Compounds **4** and **6-9** (although not in the isotopically-labelled form used here) have been described previously, almost always by a slightly less convenient route based on alkylation of the appropriate nitrobenzylamine.[2-5] None of these previous references gives

spectroscopic data although compounds have generally been characterised by elemental analysis. The synthesis of known compounds is therefore reported here, with ^{1}H NMR spectroscopic data included.

General Experimental Details, including details of NMR solvents used, are as given in the main text. TEAB refers to triethylammonium bicarbonate, prepared by passing CO_2 into a 1 M solution of redistilled triethylamine in ice-cold water, until the pH stabilised at ~7.4.

***N*-(2-Nitrobenzyl)glycine, 4.** A mixture of glycine ethyl ester hydrochloride (5.2 g, 37 mmol) and $NaHCO_3$ (3.88 g, 46.2 mmol) in 95 % aq. EtOH (150 mL) was treated with 2-nitrobenzyl bromide (2.0 g, 9.25 mmol) and heated under reflux for 20 h. The solution was cooled and filtered, and the filtrate was evaporated under reduced pressure. The residue was washed with water, dried and evaporated, and the residual oil was flash chromatographed (EtOAc–petroleum ether 35:65) to give ethyl *N*-(2-nitrobenzyl)glycinate (1.72 g, 78 % based on the nitrobenzyl bromide). The ^{1}H NMR spectrum (in $CDCl_3$) was identical to that previously reported.[1]

A solution of the above ester (238 mg, 1 mmol) in MeOH (5 mL) was mixed with 2 M aq. NaOH (1.2 mL) and kept overnight at room temperature, then neutralised with dilute H_3PO_4 and concentrated under reduced pressure. [Note that H_3PO_4 was used to ensure adequate separation of the phosphate anion from the zwitterionic compound **4** on anion-exchange chromatography. Similar deliberate choices of the acid for neutralisation were made elsewhere in this work in the light of appropriate considerations for each compound.] The residue was dissolved in water (100 mL) and the UV spectrum was measured to provide an estimate of the extinction coefficient: UV [λ_{max} (H_2O)/ nm (ε/$M^{-1}cm^{-1}$) 264 (6300)]. The solution was purified by anion-exchange chromatography on DEAE cellulose (column as described in the

main text for synthesis of compound **7**). After application of the solution to the column, the column was washed with 10 mmol TEAB (200 mL), then eluted with a linear gradient of 10-200 mM TEAB (total volume 2 L). Fractions were analysed by reverse-phase HPLC [mobile phase 25 mM Na phosphate, pH 6–acetonitrile (20:1 v/v)], t_R 5.0 min, and those containing the required compound were combined, evaporated and re-evaporated (× 3) from MeOH to remove residual TEAB. The recovered material (0.90 mmol) was dissolved in water (concentration 79 mM) and stored at –20 °C; ^{1}H NMR (90 MHz): 8.27 (1H, d, J = 8 Hz, Ar-H3), 5.43-5.66 (3H, m, Ar-H), 4.56 (2H, s, ArCH_2), 3.75 (2H, s, CH_2CO_2). The compound has previously been reported as its hydrochloride salt.[2]

***N*-(3,4-Dimethoxy-6-nitrobenzyl)glycine, 5.** A mixture of glycine ethyl ester hydrochloride (299 mg, 2.13 mmol) and $NaHCO_3$ (0.22 g, 2.62 mmol) in 95% aq. EtOH (8.5 mL) was treated with 3,4-dimethoxy-6-nitrobenzyl bromide[6] (147 mg, 0.53 mmol) and heated under reflux for 16 h. The solution was cooled, filtered and evaporated and a solution of the residue in EtOAc was washed with water, dried and evaporated. Flash chromatography (EtOAc–petroleum ether 4:1) gave a major product (90 mg) identified as ethyl *N*-(3,4-dimethoxy-6-nitrobenzyl)glycinate: ^{1}H NMR (δ, 90 MHz, $CDCl_3$) 7.62 (1H, s, Ar-H5), 7.15 (1H, s, Ar-H3), 4.18 (2H, q, J = 7 Hz, OCH_2), 4.09 (2H, s, ArCH_2), 3.98 (3H, s, OMe), 3.93 (3H, s, OMe), 3.45 (2H, s, ArCH_2), 1.26 (3H, t, J = 7 Hz, CH_3).

This ester was dissolved in MeOH (1.6 mL) and treated with aq. 2 M NaOH (0.4 mL) and the solution was kept at room temperature overnight, then neutralised with dilute H_3PO_4 and evaporated. The residue was dissolved in water and purified by anion-exchange chromatography as described for **4** above. Fractions were analysed by

reverse-phase HPLC [mobile phase 25 mM Na phosphate, pH 6–acetonitrile (85:15) v/v)], t_R 4.4 min and processed as described for **4**. Solutions were quantified using ε_{347} 6500 $M^{-1}cm^{-1}$ as described for **1** in the main text. The residue was stored at –20 °C at 10.5 mM concentration in water: HRMS (positive ion ESI) (*m/z*): found: 271.0912. calcd. for $(C_{11}H_{15}N_2O_6)^+$ 271.0925; ^{1}H NMR (δ, 500 MHz) 7.90 (1H, s, Ar-H5), 7.21 (1H, s, Ar-H2), 4.53 (2H, s, ArCH_2), 4.01 (3H, s, OMe), 3.97 (3H, s, OMe), 3.75 (2H, s, CH_2CO_2).

***N*-(2-Nitrobenzyl)iminodiacetic acid, 6.** A solution of ethyl *N*-(2-nitrobenzyl)glycinate (476 mg, 2 mmol; prepared as described above) in dry MeCN (10 mL) was stirred at 0 °C and treated with disiopropylethylamine (310 mg, 2 mmol) and ethyl bromoacetate (334 mg, 2 mmol). After 1 h, the solution was allowed to warm to room temperature and kept for 48 h, then diluted with Et_2O and washed with water, aq. $NaHCO_3$ and brine, dried and evaporated under reduced pressure. Flash chromatography (EtOAc–petroleum ether 15:85, then 20:80) gave the expected diethyl *N*-(2-nitrobenzyl)iminodiacetate as a pale yellow oil (320 mg, 50 %); ^{1}H NMR (δ, 90 MHz, $CDCl_3$) 7.27-7.93 (4H, m, Ar-H), 4.26 (2H, s, ArCH_2), 4.14 (4H, q, J = 7 Hz, OCH_2), 3.54 (4H, s, CH_2CO_2), 1.25 (6H, t, J = 7 Hz, CH_3).

The whole of this diester was dissolved in MeOH (10 mL) and mixed with 2 aq. 2 M NaOH (5 mL) and the solution was kept at room temperature overnight, then neutralised with dilute aq. HCl and concentrated under reduced pressure. The residue was dissolved in water (500 mL) and pumped onto a DEAE cellulose column (as above). After application, the column was washed with 10 mM TEAB (200 mL0 and eluted with a 10–250 mM linear gradient of TEAB (total volume 2 L). Fractions were analysed by anion-exchange HPLC [mobile phase 10 mM Na phosphate, pH7–MeCN

(9:1)], t_R 2.2 min. Fractions containing the product were combined and processed as described for **4**. The recovered **6** (0.75 mmol) was quantified using ε_{268} 6300 $M^{-1}cm^{-1}$ as determined for **4**, and was dissolved in water and stored at –20 °C at a concentration of 119 mM. An aliquot was converted to the sodium salt with Dowex 50 (Na^+ form); ^{1}H NMR (δ, 500 MHz) 8.30 (1H, dd, J = 8.1 and 1.3 Hz, Ar-H3), 7.82 (1H, td, J = 8.1 and 1.3 Hz, Ar-H), 7.77 (1H, td, J = 8.1 and 1.3 Hz, Ar-H), 7.71 (1H, dd, J = 8.1 and 1.3 Hz, Ar-H6), 4.78 (2H, s, ArCH_2), 3.95 (4H, s, CH_2CO_2). The compound has been previously reported as its free acid.[3]

***N*-(4-Nitrobenzyl)glycine, 8.** A mixture of glycine ethyl ester hydrochloride (5.2 g, 37 mmol) and $NaHCO_3$ (3.16 g, 37.6 mmol) in 95 % aq. EtOH (120 mL) was treated with 4-nitrobenzyl bromide (1.63 g, 7.5 mmol) and heated under reflux for 20 h. The solution was cooled and filtered, and the filtrate was evaporated under reduced pressure. The residue was washed with water, dried and evaporated, and the residual oil was flash chromatographed (EtOAc–petroleum ether 35:65) to give ethyl *N*-(4-nitrobenzyl)glycinate (1.30 g, 73 % based on the nitrobenzyl bromide): ^{1}H NMR (δ, 90 MHz, $CDCl_3$) 8.18 (2H, d, J = 8.4 Hz, Ar-H3,5), 7.52 (2H, d, J = 8.4 Hz, Ar-H2,6), 4.21 (2H, q, J = 7 Hz, OCH_2), 3.92 (2H, s, ArCH_2), 3.41 (2H, s, CH_2CO_2).

A portion of this material (238 mg, 1 mmol) was dissolved in MeOH (5 mL) and treated with aq. 2 M NaOH (1.2 mL). After overnight standing at room temperature, the solution was neutralised with dilute aq. H_3PO_4 and evaporated. The solution was diluted to 100 mL with water and the UV spectrum was measured to provide an estimate of the extinction coefficient: UV [λ_{max} (H_2O)/ nm (ε/$M^{-1}cm^{-1}$) 264 (10,000)]. The solution was purified by anion-exchange chromatography on DEAE cellulose (column as described in the main text for synthesis of compound **7**). After

application of the solution to the column, the column was washed with 10 mmol TEAB (200 mL), then eluted with a linear gradient of 10-200 mM TEAB (total volume 2 L). Fractions were analysed by reverse-phase HPLC [mobile phase 25 mM Na phosphate, pH 6–acetonitrile (20:1 v/v)], t_R 8.4 min, and those containing the required compound were combined and processed as described for **4**. The recovered material (0.88 mmol) was dissolved in water (concentration 20 mM) and stored at –20 °C; ^{1}H NMR (δ, 500 MHz) 8.32-8.35 (2H, m, Ar-H3,5), 7.71-7.74 (2H, m, Ar-H2,6), 4.41 (2H, s, ArCH_2), 3.72 (CH_2CO_2). The compound has been reported previously as its hydrochloride salt.[2]

[carboxy-$^{13}C_1$]*N*-(4-Nitrobenzyl)iminodiacetic acid, 9. The procedure described for **8** was repeated using [1-^{13}C]glycine ethyl ester hydrochloride and the corresponding labelled ethyl *N*-(4-nitrobenzyl)glycinate was used without further characterisation. Thus the labelled ethyl ester (1.0 g, 4.2 mmol) was dissolved in dry MeCN (21 mL), cooled to 0 °C and mixed with diisopropylethylamine (651 mg, 4.2 mmol) and ethyl bromoacetate (702 mg, 4.2 mmol). After 1 h, the solution was allowed to warm to room temperature and was kept overnight, then diluted with Et_2O and washed with water, aq. $NaHCO_3$ and brine, dried and evaporated. Flash chromatography (EtOAc–petroleum ether 15:85, then 20:80) gave diethyl [carboxy-$^{13}C_1$]*N*-(4-nitrobenzyl)iminodiacetate as a pale yellow oil (0.82 g, 60 %):^{1}H NMR (δ, $CDCl_3$, 500 MHz): 8.17-8.20 (2H, m, Ar-H3,5), 7.59-7.63 (2H, m, Ar-H2,6), 4.18 (2H, q, J = 7.1 Hz, OCH_2) superimposed on 4.18 (2H, qd, J = 7.1 Hz and $^3J_{^{13}C,H}$ = 3.0 Hz, $^{13}CO_2CH_2$), 4.04 (2H, s, ArCH_2), 3.55 (2H, s, CH_2CO_2) superimposed on 3.55 (2H, d, $^2J_{^{13}C,H}$ = 4.9 Hz, $CH_2{}^{13}CO_2$), 1.27 (6H, t, J = 7.1 Hz, CH_3).

A portion of the diester (324 mg, 1 mmol) was dissolved in EtOH (10 mL) and aq. 2 M NaOH (4.9 mL) was added. The solution was kept at room temperature overnight, then neutralised with dilute HCl and concentrated under reduced pressure. The residue was dissolved in water (50 mL) and purified by anion-exchange chromatography on DEAE cellulose (column as described in the main text for synthesis of compound **7**), using a 10-250 mM linear gradient of TEAB. Fractions containing the product were analysed by anion-exchange HPLC [mobile phase 10 mM Na phosphate, pH 5.5–acetonitrile (9:1 v/v)], t_R 2.0 min, and those containing the required compound were combined and processed as described for **4**. The recovered material (0.77 mmol) was dissolved in water (concentration 75 mM) and stored at –20 °C. A portion was converted to the sodium salt (Dowex 50, Na^+ form); ^{1}H NMR (δ, 500 MHz, D_2O + DCl, acetone ref.) 8.33-8.36 (2H, m, Ar-H3,5), 7.80-7.83 (2H, m, Ar-H2,6), 4.74 (2H, s, ArCH_2), 4.27 (2H, s, CH_2CO_2) superimposed on 4.27 (2H, d, $^2J_{^{13}C,H}$ = 6.0 Hz, $CH_2{}^{13}CO_2$). The hydrochloride of the non-isotopic compound has been described previously.[3]

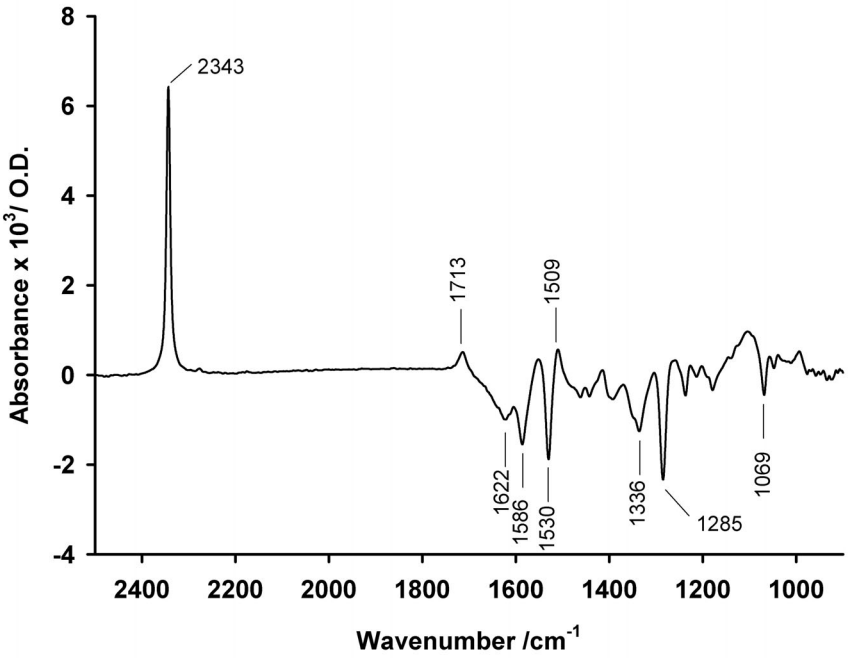

Figure S1. IR difference spectrum for photolysis of **1** (68 mM) in 500 mM MOPS, pH 7 (in H_2O) at 20 °C. The data are averaged from 4 samples for spectra recorded in the time interval 0-3.4 s after the light flash.

Theoretical basis of Ca^{2+} titrations with 5-nitro-BAPTA

A mixture of two competing ligands, 5-nitro BAPTA (N) and pentadentate chelator, such as MEDTA or structures **14/15** (L), is titrated with calcium (C). If either K_d is known then the other can be determined.

The calcium (C) interacts with the two ligands (N and L) according to the following scheme:

$C + N \rightleftharpoons CN$ with $K_{dN} = ([C][N])/[CN]$

$C + L \rightleftharpoons CL$ with $K_{dL} = ([C][L])/[CL]$

Total concentrations of calcium, 5-nitro BAPTA (N), and pentadentate chelator (L) (C_T, N_T, and L_T) are given by:

$C_T = [C] + [CN] + [CL]$

$N_T = [N] + [CN]$

$L_T = [L] + [CL]$

Then

$[N] = K_{dN}[CN]/[C]$ and $N_T = K_{dN}[CN]/[C] + [CN]$

Then $N_T = [CN](K_{dN}/[C] + 1)$

$[CN] = N_T/(K_{dN}/[C] + 1) = N_T[C]/(K_{dN} + [C])$

In the same way one can show that

$[CL] = L_T[C]/(K_{dL} + [C])$

Substituting these expressions for [CN] and [CL] in the equation for C_T gives:

$$C_T = [C] + N_T[C]/(K_{dN} + [C]) + L_T[C]/(K_{dL} + [C])$$

Expanding this equation gives:

$$C_T(K_{dN} + [C])(K_{dL} + [C]) = [C](K_{dN} + [C])(K_{dL} + [C]) + N_T[C](K_{dL} + [C]) + L_T[C](K_{dN} + [C])$$

$$C_T K_{dN} K_{dL} + C_T K_{dN}[C] + C_T K_{dL}[C] + C_T[C]^2 = K_{dN} K_{dL}[C] + K_{dN}[C]^2 + K_{dL}[C]^2 + [C]^3 + N_T K_{dL}[C] + N_T[C]^2 + L_T K_{dN}[C] + L_T[C]^2$$

$$[C]^3 + [C]^2(- C_T + K_{dN} + K_{dL} + N_T + L_T) + [C](- C_T K_{dN} - C_T K_{dL} + K_{dN} K_{dL} + N_T K_{dL} + L_T K_{dN}) - C_T K_{dN} K_{dL} = 0$$

The free calcium concentration is then obtained as the root of the following equation, using bisection combined with the Newton-Raphson method.[7]

$$C_3[C]^3 + C_2[C]^2 + C_1[C] + C_0 = 0$$

where

$$C_3 = 1$$

$$C_2 = - C_T + K_{dN} + K_{dL} + N_T + L_T$$

$$C_1 = - C_T K_{dN} - C_T K_{dL} + K_{dL} K_{dN} + N_T K_{dL} + L_T K_{dN}$$

$$C_0 = - C_T K_{dL} K_{dN}$$

The remaining concentrations are calculated using

$$[CN] = N_T[C]/(K_{dN} + [C])$$

$[CL] = L_T[C]/(K_{dL} + [C])$

$[N] = K_{dN}[CN]/[C]$

$[L] = K_{dL}[CL]/[C]$

The absorbance is fit to the following equation:

$\text{Absorbance} = \varepsilon_N[N] + \varepsilon_{CN}[CN]$

where ε_N and ε_{CN} are the extinction coefficients of N and CN

In the fitting procedure, K_{dN} is fixed at the value determined under our solution conditions (7.7 μM, see main paper). K_{dL} and the concentration of the pentadentate chelator (N) are varied simultaneously to obtain the best fit to the data points.

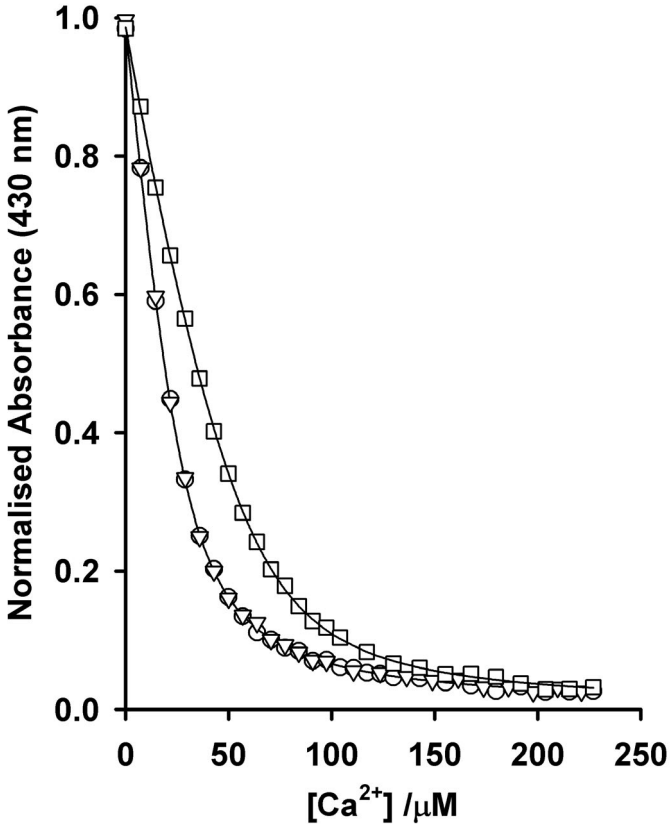

Figure S2. Calcium titrations of 27.5 μM 5-nitro-BAPTA with no addition (○); 100 μM IDA (▽);40 μM MEDTA (□).

References

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