Electronic Supplementary Information

Responsive MR-imaging probes for *N*-methyl-D-aspartate receptors and direct visualisation of the cell-surface receptors by optical microscopy

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General Experimental

Materials and Methods

All solvents used were laboratory grade and anhydrous solvents, when required, were freshly distilled over the appropriate drying agent. Water was purified by the 'PuriteSTILLplus' system, with conductivity of ≤ 4 μS cm^{-1}. All reagents used were purchased from commercial suppliers (Acros, Aldrich, Fluka, Merck and Strem) and were used without further purification unless otherwise stated. Reactions requiring anhydrous conditions were carried out using Schlenk line techniques under an atmosphere of argon.

Thin layer chromatography was performed on neutral aluminium sheet silica gel plates (Merck Art 5554) and visualised under UV irradiation (254 nm), or using Dragendorff reagent staining. Preparative column chromatography was performed using silica gel (Merck Silica Gel 60, 230-400 mesh).

Reverse phase analytical HPLC traces were recorded at 298 K using Waters Mass Directed Auto Preparation (MDAP) system. XBridge C18 4.6 x 100 mm, i.d. 5 μm analytical column and XBridge C18 OBD 19 x 100 mm, i.d. 5 μm semi-preparative columns were used to analyse and purify the complexes. A gradient elution with a solvent system composed of H_2O + 0.1% HCOOH/MeOH + 0.1% HCOOH was

performed for a total run time of 16.5 min. The separation details are tabulated below;

Time	**% Water (+ 0.1 % Formic acid)**	**% MeOH (+ 0.1 % Formic acid)**	**Curve**
0	90	10	0
10	5	95	6
13	5	95	6
13.5	90	10	6
16.5	90	10	1

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in commercially available deuterated solvents on a Varian Mercury-400 (^{1}H 399.960, ^{13}C 100.572), Bruker Avance-400 (^{1}H 400.052, ^{13}C 100.603 and ^{31}P 161.91), Varian Inova-500 (^{1}H 499.722, ^{13}C 125.671,), Appleby VNMRS-600 (^{1}H 599.832, ^{13}C 150.828), or Varian VNMRA-700 (^{1}H 699.731, ^{13}C 175.948 and ^{31}P 283.26) spectrometer. All chemical shifts are given in ppm and all coupling constants are reported in Hz.

Low resolution electrospray mass spectra (LR-MS) were recorded on a Fisons VG Platform II, Waters Micromass LCT or Thermo-Finnigan LTQ FT instrument operating in positive or negative ion mode as stated, with MeOH as the carrier solvent. Accurate mass spectra [High resolution electrospray mass spectra (HR-MS)] were recorded using the Thermo-Finnigan LTQ FT mass spectrometer.

Melting points were recorded using a Gallenkamp (Sanyo) apparatus and are uncorrected.

Relaxivity measurements were carried out at 310 K, 60 MHz (1.4 T) on a Bruker Minispec mq60 instrument. The mean value of three independent measurements was recorded and averaged. The relaxivities of the compounds were calculated as the slope of the function shown in equation (1),

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1,d}} + r_1[Gd.L^n] \quad (1)$$

where $T_{1,\mathrm{obs}}$ is the measured T_1, $T_{1,\mathrm{d}}$ is the diamagnetic contribution of the solvent (calculated to be 4000 ms) and [Gd.L^{n}] is the concentration in mM of the

appropriate Gd^{3+} complex (n = 1 - 5). Error for all relaxivity values was less then 0.3 $mM^{-1}s^{-1}$.

The apparent binding constant for the interaction of the Gd^{3+} complex with Human Serum Albumin (HSA) was calculated using equation (2):

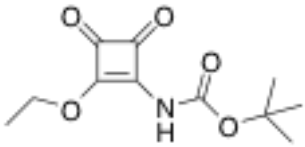
$$[X] = \frac{\frac{(R-R_0)/(R_1-R_0)}{K} + [Gd.L^n] * \frac{(R-R_0)}{(R_1-R_0)} - [Gd.L^n] * \left(\frac{(R-R_0)}{(R_1-R_0)}\right)^2}{1 - \frac{(R-R_0)}{(R_1-R_0)}} \quad (2)$$

$$K = \frac{[GdX]}{[X_f][Gd_f]}$$

where: [X]: the total concentration of HSA in the solution; [Gd.L^n]: the total concentration of the complex; K: the binding constant; R: relaxation rate of a given concentration of X; R_0: the initial relaxation rate; R_1: final relaxation rate; [Gd.X]: the concentration of the HSA- coordinated complex; [X_f]: the concentration of free HSA in the mixture; [Gd_f]: the concentration of the free complex.

Synthetic Methods: ligand and complex synthesis

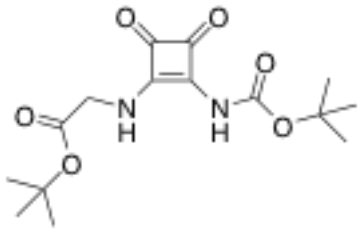
***tert*-Butyl (2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)carbamate, 1.**



3-Amino-4-ethoxy-3-cyclobutene-1,2-dione[1] (4.76 g, 33.7 mmol) and di-*tert*-butyl-dicarbonate (7.40 mg, 33.9 mmol) were dissolved in dichloromethane (500 mL) to which triethylamine (4.70 mL, 33.7 mmol) was added and the resulting solution stirred at room temperature for 24 h, when further portions of di-*tert*-butyl-dicarbonate and triethylamine were added and stirred again for 24 h. Once no further change was observed by TLC the volatiles were removed under reduced pressure to yield a crude orange residue which was purified by column chromatography (100% DCM R_f=0.6) to give a light yellow solid (4.46g, 55%). ^{1}H

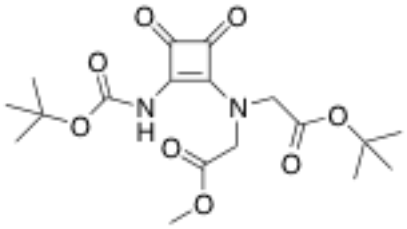
NMR ($CDCl_3$, 400 MHz) δ 1.51 (3H, t, ^3J = 7, C**H**$_3$CH$_2$), 1.54 (9H, s, (C**H**$_3$)$_3$), 4.89 (2H, q, ^3J = 7, CH$_3$C**H**$_2$), 7.53 (1H, s, br, N**H**) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 16.0 (CH$_2$**C**H$_3$), 28.1 ((C**C**H$_3$)$_3$), 70.8 (**C**H$_2$CH$_3$), 84.6 ((**C**CH$_3$)$_3$), 149.0 (**C**NH), 167.2 (**C**OEt), 182.3, 187.1, 187.8 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{11}H_{15}NO_5$ requires 242.1022 $[M+H]^+$; found 242.1023 $[M+H]^+$. M. Pt = 163-165 °C.

tert-Butyl 2-((2-((_tert_-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetate, 2.



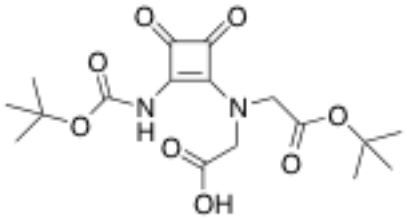
To a solution of *tert*-butyl (2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)carbamate, **1** (200 mg, 0.83 mmol) in anhydrous ethanol (10 mL) was added a solution of glycine *tert*-butyl ester hydrochloride (140 mg, 0.83 mmol) and diisopropylethylamine (145 μL, 0.83 mmol) in anhydrous ethanol (2 mL), over a two hour period. The resulting mixture was stirred at room temperature and reaction progress monitored by TLC. Upon consumption of starting materials, the ethanol was removed under reduced pressure and the crude residue purified by column chromatography (100 % DCM to 95:5 DCM/MeOH; R_f=0.62) to give a light yellow solid (220 mg, 82%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.49 (9H, s, (C**H**$_3$)$_3$), 1.51 (9H, s, (C**H**$_3$)$_3$), 4.40 (2H, d, ^2J = 6.0, C**H**$_2$), 7.77 (1H, s, br, N**H**) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 28.1, 28.2 ((C**C**H$_3$)$_3$), 45.7 (**C**H$_2$), 83.2, 84.6 ((**C**CH$_3$)$_3$), 151.6 (**C**NHCO$_2$^{t}Bu), 159.8 (**C**NHCH$_2$), 168.3, 171.4, 182.5, 187.2 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{15}H_{22}N_2O_6Na$ requires 349.1387 $[M+Na]^+$; found 349.1376 $[M+Na]^+$. M.Pt >250 °C.

tert-Butyl 2-((2-((_tert_-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-methoxy-2-oxoethyl)amino)acetate, 3.



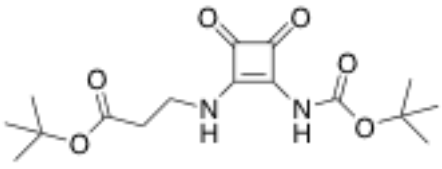
To a solution of *tert*-butyl 2-((2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetate, **2** (100 mg, 0.31 mmol) and NaH (20 mg, 0.50 mmol) in anhydrous DMF (5 mL) was added methylbromoacetate (50 μL, 0.50 mmol). The resulting solution was stirred at 40 °C for 48h when the mixture was cooled to room temperature and the solvent removed under reduced pressure. The crude residue was then taken up into EtOAc (20 mL) and H_2O (15 mL) added. The layers were separated and the aqueous portion extracted with EtOAc (3 x 50 mL). The organic portions were combined, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (100% DCM to 95:5 DCM/MeOH using 0.5% increments; R_f=0.75) to give a light green viscous oil (116 mg, 94%). ^{1}H NMR ($CDCl_3$, 700 MHz) δ 1.48 (9H, s, (C**H**$_3$)$_3$), 1.49 (9H, s, (C**H**$_3$)$_3$, 3.76 (3H, s, C**H**$_3$), 4.42 (2H, d, ^{2}J = 5.5, C**H**$_2$CO$_2$^{t}Bu), 4.82 (2H, s, C**H**$_2$CO$_2$Me), 7.84 (1H, s, br, N**H**) ppm. ^{13}C NMR ($CDCl_3$, 176 MHz): δ 27.9, 28.2 ((**C**H$_3$)$_3$), 45.7 (**C**H$_2$CO$_2$^{t}Bu), 47.6 (**C**H$_2$CO$_2$Me), 52.6 (**C**H$_3$), 83.1, 86.7 (**C**(CH$_3$)$_3$), 151.6 (**C**NHCO$_2$^{t}Bu), 161.3 (**C**N(CH$_2$)$_2$), 168.2, 168.9, 171.2, 182.7, 188.0 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{18}H_{26}N_2O_8Na$ requires 421.1584 $[M+Na]^+$; found 421.1587$[M+Na]^+$.

2-((2-(*tert*-Butoxy)-2-oxoethyl)(2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetic acid, 4.



tert-Butyl 2-((2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-methoxy-2-oxoethyl)amino)acetate, **3** (116 mg, 0.29 mmol) was dissolved in a mixture of THF (3 mL) and methanol (2 mL). To the solution was added LiOH powder (7 mg, 0.29 mmol) as a solution in H_2O (1 mL). The reaction was stirred at room temperature for 15 hours with loss of methyl ester verified by ESI-MS, ^{1}H NMR (loss of methyl singlet at 3.76 ppm) and TLC. At this point, the solvent cocktail was evaporated under reduced pressure and the glassy yellow solid taken up into purite H_2O. The pH was adjusted to pH 7 and then lyophilized. This revealed the title compound, which was used directly in the next step without further purification. ^{1}H NMR (D_2O, 400 MHZ) δ 1.36-1.42 (18H, C(C**H**$_3$)$_3$), 3.92-4.92 (4H, C**H**$_2$NC**H**$_2$), 7.48 (1H, N**H**), 7.52 (1H, O**H**) ppm. LR-MS (ES$^-$) *m/z* $C_{17}H_{22}N_2O_8Li_3$ requires 403.19 $[M-2H+3Li]^+$; found 403.2 $[M-2H+3Li]^+$.

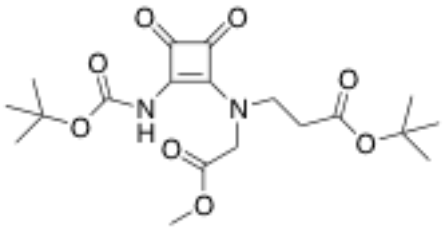
***tert*-Butyl 2-((2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoate, 5.**



A solution of *tert*-butyl (2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)carbamate, **1** (300 mg, 1.24 mmol) in anhydrous ethanol (12 mL) was treated with a solution of *β*-alanine *tert*-butyl ester hydrochloride (226 mg, 1.24 mmol) and diisopropylethylamine (216 μL, 1.24 mmol) in anhydrous ethanol (3mL) over a

two hour period. The reaction was then stirred at room temperature and monitored by TLC. Upon consumption of starting materials, excess ethanol was removed under reduced pressure and the crude residue purified by column chromatography (100 % DCM to 95:5 DCM/MeOH; R_f=0.52) to give the title compound as a light yellow solid (332 mg, 79%). ^{1}H NMR ($CDCl_3$, 700 MHz) δ 1.45 (9H, s, (C**H**$_3$)$_3$), 1.46 (9H, s, (C**H**$_3$)$_3$, 2.57 (2H, t, ^{3}J = 7 Hz, CH_2C**H**$_2$$CO_2$^{t}Bu), 3.93 (2H, q, ^{3}J = 7Hz, C**H**$_2$$CH_2$$CO_2$^{t}Bu), 7.85 (1H, s, br, N**H**), 8.90 (1H, s, br, N**H**) ppm. ^{13}C NMR ($CDCl_3$, 176 MHz) δ 28.0, 28.1 ((C**C**H$_3$)$_3$), 36.9, 39.9 ((**C**H$_2$)$_2$), 81.7, 83.9 ((**C**CH$_3$)$_3$), 152.0 (**C**NHCO$_2$^{t}Bu), 160.1 (**C**NHCH$_2$), 170.4, 171.6, 182.3, 187.0 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{16}H_{24}N_2O_6Na$ requires 363.1548 [M+Na]$^+$; found 363.1532 [M+Na]$^+$. M.Pt >250 °C.

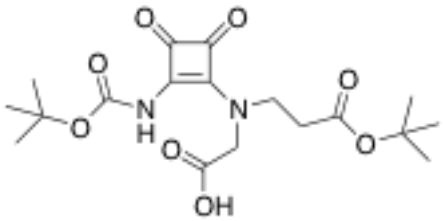
***tert*-Butyl 2-((2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-methoxy-2-oxoethyl)amino)propanoate, 6.**



To a solution of *tert*-butyl 2-((2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoate, **5** (330 mg, 0.97 mmol) and NaH (28 mg, 1.16 mmol) in anhydrous DMF (8 mL) was added methylbromoacetate (110 μL, 1.16 mmol) dropwise. The resulting solution was stirred at 40 °C for 48 hours at which point the mixture was cooled to room temperature and the solvent removed under reduced pressure. The crude residue was then taken up into EtOAc (20 mL) and H_2O added (15 mL). The layers were separated and the aqueous extracted with EtOAc (3 x 50 mL), with the combined organic portions dried over $MgSO_4$. Filtration and removal of the volatiles under reduced pressure gave a crude residue which was purified by column chromatography (100% DCM to 95:5 DCM/MeOH using 0.5% increments; R_f=0.74) to give the title compound as a light green viscous oil (280 mg, 71%). ^{1}H NMR ($CDCl_3$, 700 MHz) δ 1.45 (s, 9H, (C**H**$_3$)$_3$), 1.46 (s, 9H, (C**H**$_3$)$_3$), 2.57 (t, 2H, ^{3}J = 7 Hz, C**H**$_2$), 3.75 (s, 3H,

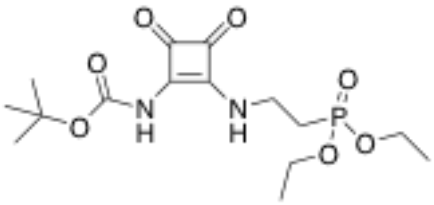
C**H**$_3$), 3.96 (q, 2H, ^{3}J = 7 Hz, C**H**$_2$), 4.81 (s, 2H, C**H**$_2$CO$_2$Me), 7.93 (s, br, 1H, N**H**) ppm. ^{13}C NMR ($CDCl_3$, 176 MHz): δ 27.8, 28.1 ((**C**H$_3$)$_3$), 36.8, 39.8 (**C**H$_2$**C**H$_2$), 47.6 (**C**H$_2$CO$_2$Me), 52.6 (**C**H$_3$), 81.7, 85.4 (**C**(CH$_3$)$_3$), 151.5 (**C**NHCO$_2$^{t}Bu), 160.9 (**C**N(CH$_2$)$_2$), 169.0, 170.6, 171.2, 182.3, 188.0 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{19}H_{28}N_2O_8Na$ requires 435.1743 $[M+Na]^+$; found 435.1548 $[M+Na]^+$.

2-((3-(*tert*-Butoxy)-3-oxopropyl)(2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetic acid, 7.



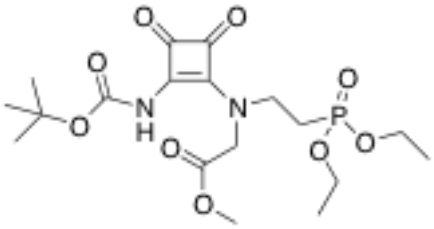
tert-Butyl 2-((2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-methoxy-2-oxoethyl)amino)propanoate, **6** (154 mg, 0.37 mmol) was dissolved in a mixture of THF (3 mL) and methanol (2 mL). To the solution was added LiOH powder (9 mg, 0.37 mmol) as a solution in H_2O (1 mL). The reaction was stirred at room temperature for 15 hours, with loss of methyl ester verified by ESI-MS, ^{1}H NMR (loss of methyl singlet at 3.75 ppm) and TLC. At this point, the solvent cocktail was evaporated under reduced pressure and the glassy yellow solid taken up into purite H_2O. The pH was adjusted to pH 7 and then lyophilized. This revealed the title compound, which was used directly in the next step without further purification. ^{1}H NMR (D_2O, 400 MHZ) δ 1.36-1.42 (18H, C(C**H**$_3$)$_3$), 2.54-2.66 (2H, C**H**$_2$), 3.50-3.65 (2H, C**H**$_2$), 4.08-4.27 (2H, C**H**$_2$), 7.48 (1H, N**H**), 7.52 (1H, O**H**) ppm. LR-MS (ES$^+$) *m/z* $C_{18}H_{24}N_2O_8Li_3$ $[M-2H+3Li]^+$ requires 417.21; found 415.3 $[M-2H+3Li]^+$.

tert-Butyl (2-((diethoxyphosphoryl)ethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)carbamate, 8.



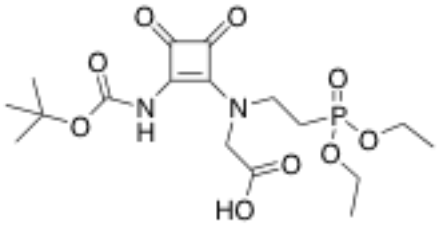
A solution of *tert*-butyl (2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)carbamate, **1** (236 mg, 0.98 mmol) in anhydrous ethanol (12 mL) was treated with a solution of diethyl-2-aminoethyl phosphonate oxalate (443 mg, 1.64 mmol) and diisopropylethylamine (570 μL, 3.27 mmol) in anhydrous ethanol (4 mL) over a one hour period. The resulting mixture was allowed to stir at room temperature for 22 hours. After this time, ethanol was removed under reduced pressure to give an orange residue which was purified by column chromatography (100% DCM to 95:5 DCM/MeOH using 0.5% increments; R_f=0.37) to give a light green oil (223 mg, 60%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.30 (6H, t, ^3J = 7, $P(OCH_2C\mathbf{H}_3)_2$), 1.45 (9H, s, $(C\mathbf{H}_3)_3$), 2.07-2.15 (2H, m, $PC\mathbf{H}_2CH_2$), 3.93-4.03 (2H, m, $PCH_2C\mathbf{H}_2$), 4.05-4.17 (4H, qd, $^3J_{H\text{-}H}$ = 7, $^3J_{H\text{-}P}$ = 3, $P(OC\mathbf{H}_2CH_3)_2$), 7.76 (1H, t, ^3J = 4, $N\mathbf{H}CH_2$), 9.23 (1H, s, br, N**H**) ppm. ^{31}P NMR ($CDCl_3$, 162 MHz) δ 27.06 ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 16.5 (d, ^3J = 6, $P(OCH_2\mathbf{C}H_3)_2$), 27.8 (d, ^1J = 141, $NHCH_2\mathbf{C}H_2P$), 27.9 ($C(\mathbf{C}H_3)_3$), 38.5 (d, ^2J = 6, $NH\mathbf{C}H_2CH_2P$), 62.1 (d, ^2J = 6, $P(O\mathbf{C}H_2CH_3)_2$), 83.7 ($\mathbf{C}(CH_3)_3$), 152.1, 160.4 (**C**NH), 171.3, 182.3, 187.1 (**C**O) ppm. HR-MS (ES^+) *m/z* $C_{15}H_{25}N_2O_7PNa$ requires 399.1297 $[M+Na]^+$; found 399.1276 $[M+Na]^+$.

Methyl 2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-(diethoxyphosphoryl)ethyl)amino)acetate, 9



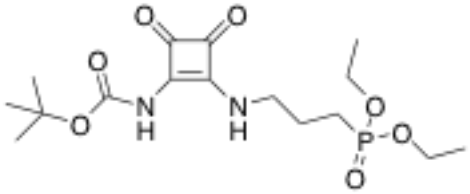
To a solution of methylbromoacetate (75 μL, 0.80 mmol) in anhydrous DMF (3 mL) was added a solution of *tert*-butyl (2-((diethoxyphosphoryl)ethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)carbamate, **8** (200 mg, 0.53 mmol) and NaH (19 mg, 0.80 mmol) in DMF (4 mL) dropwise over a period of 10 minutes. The resulting solution was then stirred at 40 °C for 48 hours. Upon no further reaction, DMF was removed under reduced pressure and the resulting residue taken up into EtOAc (10 mL). H_2O (10 mL) was added and the layers separated. The aqueous layer was extracted with EtOAc (3 x 40 mL) and the combined organic fractions dried over $MgSO_4$. The solution was filtered and EtOAc removed under reduced pressure to give the crude residue, which was purified by column chromatography (100% DCM to 95:5 DCM/MeOH using 0.2% increments; R_f=0.31) to give a light green oil (115 mg, 50%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.34 (6H, t, ^{3}J = 8, P($OCH_2C\mathbf{H}_3$)$_2$), 1.47 (9H, s, C($C\mathbf{H}_3$)$_3$), 2.09-2.16 (2H, m, P$C\mathbf{H}_2CH_2$), 3.76 (3H, s, $C\mathbf{H}_3$), 3.98-4.08 (2H, m, $PCH_2C\mathbf{H}_2$), 4.09-4.21 (4H, qd, $^3J_{H\text{-}H}$ = 7, $^3J_{H\text{-}P}$ = 3, P($OC\mathbf{H}_2CH_3$)$_2$), 4.83 (2H, s, $C\mathbf{H}_2CO_2Me$), 7.89 (1H, s, br, N**H**) ppm. ^{31}P NMR ($CDCl_3$, 283 MHz) δ 27.83 ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 16.6 (d, ^{3}J = 6, P($OCH_2\mathbf{C}H_3$)$_2$), 27.9 (d, ^{1}J = 141, $NHCH_2\mathbf{C}H_2P$), 27.9 (C($\mathbf{C}H_3$)$_3$), 38.5 (d, ^{2}J = 6, $NH\mathbf{C}H_2CH_2P$), 47.6 ($\mathbf{C}H_2CO_2Me$), 52.6 ($\mathbf{C}H_3$), 62.2 (d, ^{2}J = 7, P($O\mathbf{C}H_2CH_3$)$_2$), 85.2 ($\mathbf{C}(CH_3)_3$), 151.5, 161.0 (**C**NH), 168.9, 171.1, 182.4, 187.9 (**C**O) ppm. HR-MS (ES^+) *m/z* $C_{18}H_{29}N_2O_9PNa$ requires 471.1508 $[M+Na]^+$; found 471.1513 $[M+Na]^+$.

2-((2-((Tert-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-(diethoxyphosphoryl)ethyl)amino)acetic acid, 10



Methyl 2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-(diethoxyphosphoryl)ethyl)amino)acetate, **9** (185 mg, 0.41 mmol) was dissolved in MeOD (3 mL), to which KOD (39 mg, 0.68 mmol) was added as a solution in D_2O (4 mL). The resulting mixture was stirred at room temperature and reaction profile monitored by TLC, ^1H (loss of methyl singlet at 3.76 ppm) and ^{31}P NMR spectroscopy (no shift in ^{31}P signal). After 24 h, complete conversion of starting material was achieved and the solvent removed under reduced pressure. The solid residue was taken up in purite H_2O (5 mL) and the pH adjusted to about 7.6 using HCl (0.1 M). The resulting solution was lyophilized to give the product as a light green solid which was used directly in the next step without further purification. ^1H NMR (D_2O, 400 MHz) δ 1.27-1.35 (6H, ($OCH_2C\mathbf{H}_3)_2$), 1.37-1.42 (9H, $C(C\mathbf{H}_3)_3$), 2.17-2.31 (2H, $C\mathbf{H}_2$), 3.11-3.37 (2H, N**H** +O**H**) 3.53-3.75 (2H, $C\mathbf{H}_2$), 4.05-4.20 (4H, ($OC\mathbf{H}_2CH3)_2$), 4.88 (2H, $C\mathbf{H}_2$) ppm. ^{31}P NMR (D_2O, 162 MHz) δ 30.59 ppm. LR-MS (ES^+) *m/z* $C_{17}H_{25}N_2O_9PKD_2$ requires 475.12 $[M+K]^+$; found 475.2 $[M+K]^+$ (after deuterium exchange).

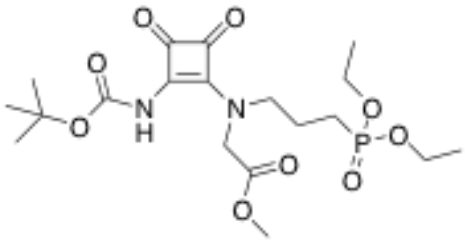
***tert*-Butyl (2-((diethoxyphosphoryl)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)carbamate, 11**



A solution of *tert*-butyl (2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)carbamate, **1** (392 mg, 1.62 mmol) in anhydrous ethanol (19 mL) was treated with a solution of diethyl (3-aminopropyl) phosphonate[2] (317 mg, 1.62 mmol) and

diisopropylethylamine (283 μL, 1.62 mmol) in anhydrous ethanol (4 mL) over a one hour period. The resulting mixture was allowed to stir at room temperature for 48 hours. After this time, ethanol was removed under reduced pressure to give an orange residue which was then purified by column chromatography (100% DCM to 95:5 DCM/MeOH using 0.5% increments; R_f=0.35) to give a yellow oil (266 mg, 42%). ^{1}H NMR ($CDCl_3$, 700 MHz) δ 1.33 (6H, t, ^{3}J = 7, $P(OCH_2C\mathbf{H}_3)_2$), 1.51 (9H, s, $(C\mathbf{H}_3)_3$), 1.79-1.83 (2H, m, $PC\mathbf{H}_2(CH_2)_2$), 1.93-1.99 (2H, m, $PCH_2C\mathbf{H}_2CH_2$), 3.79 (2H, AB quartet, $P(CH_2)_2C\mathbf{H}_2$), 4.06-4.15 (4H, qd, $^3J_{H\text{-}H}$ = 7, $^3J_{H\text{-}P}$ = 3, $P(OC\mathbf{H}_2CH_3)_2$), 7.51 (1H, s, br, N**H**), 7.96 (1H, s, br, N**H**) ppm. ^{31}P NMR ($CDCl_3$, 283 MHz) δ 30.70 ppm. ^{13}C NMR ($CDCl_3$, 176 MHz): δ 16.6 (d, ^{3}J = 5, $P(OCH_2\mathbf{C}H_3)_2$), 22.7 (d, ^{1}J = 143, $NH(CH_2)_2\mathbf{C}H_2P$), 24.6 (d, ^{2}J = 5, $NHCH_2\mathbf{C}H_2CH_2P$), 28.1 ($C(\mathbf{C}H_3)_3$), 44.4 (d, ^{3}J = 18, $NH\mathbf{C}H_2(CH_2)_2P$), 61.9 (d, ^{2}J = 7, $P(O\mathbf{C}H_2CH_3)_2$), 84.5 ($\mathbf{C}(CH_3)_3$), 151.8, 159.4 (**C**NH), 171.6, 182.1, 187.1 (**C**O) ppm. HR-MS (ES^+) *m/z* $C_{16}H_{28}N_2O_7P$ requires 391.1634 $[M+H]^+$; found 391.1639 $[M+H]^+$.

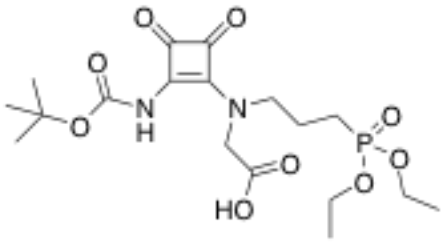
Methyl 2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-(diethoxyphosphoryl)propyl)amino)acetate, 12



tert-Butyl (2-((diethoxyphosphoryl)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)carbamate, **11** (169 mg, 0.43 mmol) and NaH (15 mg, 0.65 mmol) were dissolved in anhydrous DMF (5 mL) and stirred at room temperature for 15 minutes. After this period, methylbromoacetate (61 μL, 0.65 mmol) was added and the resulting solution stirred at 40 °C for 48 hours. The resulting solution was cooled to room temperature and the solvent removed under reduced pressure. The residue was then taken up in EtOAc (50 mL) and H_2O (30 mL) added. The layers were separated and the aqueous washed with EtOAc (3 x 50 mL). The combined organic portions were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give the crude residue, which was

then purified by column chromatography (100% DCM to 95:5 DCM/MeOH using 0.2% increments; R_f=0.43) to give a viscous yellow oil (126 mg, 63%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.31 (6H, t, ^{3}J = 8, $P(OCH_2C\mathbf{H}_3)_2$), 1.47 (9H, s, $C(C\mathbf{H}_3)_3$), 1.75-1.84 (2H, m, $PC\mathbf{H}_2(CH_2)_2$), 1.89-1.99 (2H, m, $PCH_2C\mathbf{H}_2CH_2$), 3.76 (3H, s, $C\mathbf{H}_3$), 3.80 (2H, AB quartet, $P(CH_2)_2C\mathbf{H}_2$), 4.02-4.16 (4H, qd, $^3J_{H\text{-}H}$ = 7, $^3J_{H\text{-}P}$ = 3, $P(OC\mathbf{H}_2CH_3)_2$), 4.82 (2H, s, $C\mathbf{H}_2CO_2Me$), 7.66 (1H, s, br, N**H**) ppm. ^{31}P NMR ($CDCl_3$, 162 MHz) δ 29.89 ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 16.6 (d, ^{3}J = 6, $P(OCH_2\mathbf{C}H_3)_2$), 22.7 (d, ^{1}J = 143, $N(CH_2)_2\mathbf{C}H_2P$), 24.6 (d, ^{2}J = 5, $NCH_2\mathbf{C}H_2CH_2P$), 27.9 ($C(\mathbf{C}H_3)_3$), 44.2 (d, ^{3}J = 17, $N\mathbf{C}H_2(CH_2)_2P$), 47.6 ($\mathbf{C}H_2CO_2Me$), 52.6 ($\mathbf{C}H_3$), 61.9 (d, ^{2}J = 7, $P(O\mathbf{C}H_2CH_3)_2$), 85.6 ($\mathbf{C}(CH_3)_3$), 151.6, 160.9 (**C**NH), 168.9, 171.5, 182.3, 187.9 (**C**O) ppm. HR-MS (ES^+) *m/z* $C_{19}H_{31}N_2O_9PNa$ requires 485.1665 $[M+Na]^+$; found 485.1647$[M+Na]^+$.

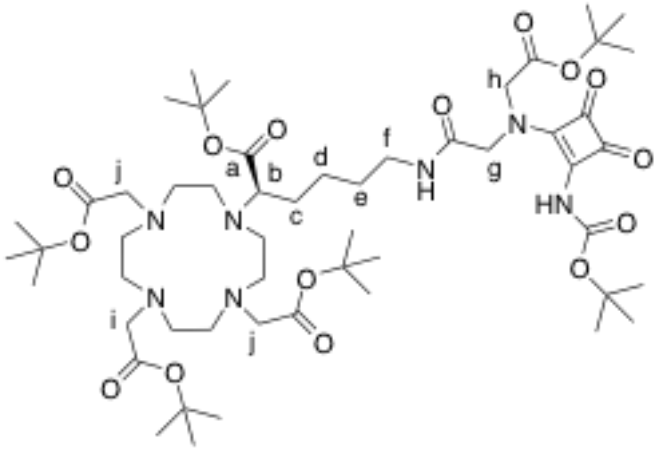
2-((2-((tert-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(3-(diethoxyphosphoryl)propyl)amino)acetic acid, 13



Methyl 2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-(diethoxyphosphoryl)propyl)amino)acetate, **12** (126 mg, 0.27 mmol) was dissolved in MeOD (3 mL), to which KOD (39 mg, 0.68 mmol) was added as a solution in D_2O (4 mL). The resulting mixture was stirred at room temperature and reaction profile monitored by TLC, ^{1}H (loss of methyl singlet at 3.76 ppm) and ^{31}P NMR spectroscopy (no shift in ^{31}P signal). After 24 h, complete conversion of starting material was achieved and the solvent removed under reduced pressure. The solid residue was taken up in purite H_2O (5 mL) and the pH adjusted to about 7.6 using HCl (0.1 M). The resulting solution was lyophilized to give the product as a light green solid which was used directly in the next step without further purification. ^{1}H NMR (D_2O, 400 MHz) δ 1.20-1.34 (6H, $(OCH_2C\mathbf{H}_3)_2$), 1.40-1.44 (9H, $C(C\mathbf{H}_3)_3$), 1.78-2.06 (4H, $(C\mathbf{H}_2)_2$), 3.01-3.32

(2H, N**H** +O**H**) 3.36-3.57 (2H, C**H**$_2$), 4.07-4.32 (4H, (OC**H**$_2$CH3)$_2$), 4.81 (2H, C**H**$_2$) ppm. ^{31}P NMR (D_2O, 162 MHz) δ 34.24 ppm. LR-MS (ES^+) *m/z* $C_{18}H_{28}N_2O_9PKD_2$ requires 490.52 $[M+K]^+$; found 490.3 $[M+K]^+$ (Deuterium exchange).

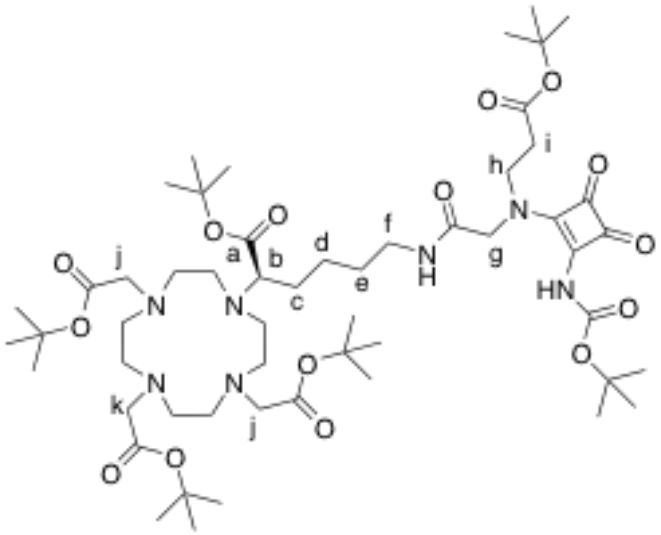
[Conjugate 1]



2-((2-(*Tert*-butoxy)-2-oxoethyl)(2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetic acid, **4** (50 mg, 0.13 mmol), EDC (27 mg, 0.14 mmol) and HOBt (19 mg, 0.14 mmol) were dissolved in anhydrous DMF (4 mL) and stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of *(R)* –tri-*tert*-butyl 2,2',2''-(10-(6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, **14**[3] (91 mg, 0.13 mmol) and NMM (29 μL, 0.26 mmol) in anhydrous DMF (2 mL) was added dropwise and the resulting solution stirred at room temperature for a total of 4 days (second addition of EDC and HOBt after 2 days). After this period, DMF was removed under reduced pressure and the crude oil taken up into EtOAc (50 mL). $NaHCO_3$ (30 mL) was added, the layers separated and the aqueous washed with EtOAc (3 x 50 mL). The combined organic portions were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH 100% to 90:10 in 1% increments; R_f =0.26) to yield a viscous yellow oil (26 mg, 19%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 1.29-1.38 (4H, m, br, **H**$^{d+e}$), 1.40, 1.40, 1.41, 1.41, 1.44 (54H, (C(C**H**$_3$)$_3$), 1.51-1.57 (2H, m, br, **H**^{c}), 2.04-2.14 (4H, m, cyclen), 2.21-2.37 (4H, m, cyclen), 2.43-2.55 (4H, m, cyclen), 2.67-2.90 (4H, m, cyclen), 2.98-3.78 (11H, m, **H**$^{b+f+j+i+g}$), 4.16-4.35 (2H, m, **H**^{h}) ppm.^{13}C NMR

($CDCl_3$, 151 MHz): δ 24.2 (**C**^{d}), 27.7(**C**^{e}), 27.7, 27.8, 27.8, 27.9, 27.9, 27.9 (C(**C**H_3)$_3$), 39.0 (**C**^{f}), 47.2 (**C**^{h}), 48.0 (**C**^{cy}), 48.4 (**C**^{cy}), 52.4 (**C**^{g}), 52.61 (**C**^{cy}) 53.3 (**C**^{cy}), 55.4 (**C**^{j}), 55.7 (**C**^{i}), 61.2 (**C**^{b}), 81.9, 82.1, 82.6, 82.7, 83.1 (**C**$(CH_3)_3$), 151.7 (C**C**N), 151.8 (C**C**N), 160.4, 160.6, 166.2, 167.6, 170.4,172.7, 172.7, 172.8 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{53}H_{92}N_7O_{15}$ requires 1066.665 $[M+H]^+$; found 1066.670 $[M+H]^+$.

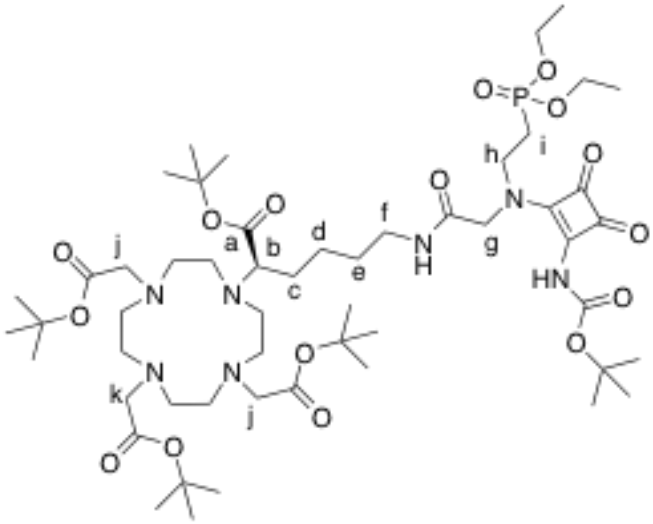
[Conjugate 2]



2-((3-(*Tert*-butoxy)-3-oxopropyl)(2-((*tert*-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetic acid, **7** (46 mg, 0.12 mmol), EDC (28 mg, 0.14 mmol) and HOBt (19 mg, 0.14 mmol) were dissolved in anhydrous DMF (3 mL) and stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of *(R)* –tri-*tert*-butyl 2,2',2''-(10-(6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, **14**[3] (84 mg, 0.12 mmol) and NMM (24 μL, 0.24 mmol) in anhydrous DMF (1.2 mL) was added dropwise and the resulting solution stirred at room temperature for a total of 4 days (second addition of EDC and HOBt after 2 days). After this period, DMF was removed under reduced pressure and the crude oil taken up into EtOAc (50 mL). $NaHCO_3$ (30 mL) was added, the layers separated and the aqueous washed with EtOAc (3 x 50 mL). The combined organic portions were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH 100% to 90:10 using 1% increments; R_f =0.26) to yield a viscous

yellow oil (17 mg, 14%). ^{1}H NMR ($CDCl_3$, 700 MHz): δ 1.29-1.38 (4H, m, br, **H**$^{d+e}$), 1.40, 1.40, 1.41, 1.41, 1.44 (54H, ($C(C\mathbf{H}_3)_3$), 1.51-1.57 (2H, m, br, **H**^{c}), 2.04-2.14 (4H, m, cyclen), 2.21-2.37 (4H, m, cyclen), 2.43-2.55 (4H, m, cyclen), 2.59 (2H, t, J = 7, **H**^{i}), 2.67-2.90 (4H, m, cyclen), 2.98-3.78 (11H, m, **H**$^{b+f+j+k+g}$), 4.16-4.35 (2H, m, **H**^{h}) ppm ^{13}C NMR ($CDCl_3$, 151 MHz): δ 24.4 (**C**^{d}), 27.9(**C**^{e}), 27.9, 28.0, 28.0, 28.1, 28.1, 28.1 ($C(\mathbf{C}H_3)_3$), 34.6 (**C**^{i}) 39.2 (**C**^{f}), 47.4 (**C**^{h}), 48.2 (**C**^{cy}), 48.6 (**C**^{cy}), 52.6 (**C**^{g}), 52.63 (**C**^{cy}) 53.5 (**C**^{cy}), 55.6 (**C**^{j}), 55.9 (**C**^{k}), 61.4 (**C**^{b}), 81.9, 82.1, 82.8, 82.9, 83.3 ($\mathbf{C}(CH_3)_3$), 151.9 (C**C**N), 152.0 (C**C**N), 160.6, 160.8, 166.4, 167.8, 170.6, 172.9, 172.9, 173.0 (**C**O) ppm. HR-MS (ES^{+}) *m/z* $C_{54}H_{94}N_7O_{15}$ requires 1080.681 $[M+H]^{+}$; found 1080.677 $[M+H]^{+}$.

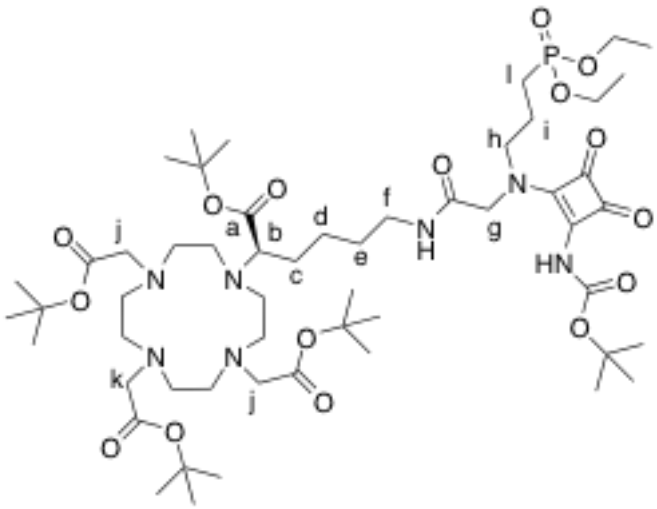
[Conjugate 3]



2-((2-((Tert-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(2-(diethoxyphosphoryl)ethyl)amino)acetic acid, **10** (182 mg, 0.42 mmol), EDC (100 mg, 0.50 mmol) and HOBt (70 mg, 0.50 mmol) were dissolved in anhydrous DMF (4 mL) and stirred at room temperature under an atmosphere of argon for 20 minutes. After this period, a pre-stirred solution of *(R)* –tri-*tert*-butyl 2,2',2''-(10-(6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, **14**[3] (292 mg, 0.42 mmol) and NMM (91 μL, 0.83 mmol) in anhydrous DMF (1.5 mL) was added drop wise and the resulting solution stirred at room temperature for a total of 4 days (second addition of EDC and HOBt after 2 days). After this period, DMF was removed under reduced pressure and the crude oil taken up into EtOAc (40 mL). $NaHCO_3$ (30 mL) was added, the layers

separated and the aqueous washed with EtOAc (3 x 50 mL). The combined organic portions were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH 100% to 93:7 using 1% increments; R_f =0.24) to yield a viscous yellow-brown oil (89 mg, 19%). ^{1}H NMR ($CDCl_3$, 700 MHz) δ 1.23-1.27 (2H, m, **H**^{d}), 1.29 (3H, t, ^{3}J = 7, $OCH_2C\mathbf{H}_3$), 1.32 (3H, t, ^{3}J = 7, $OCH_2C\mathbf{H}_3$), 1.40 (9H, s, $C(C\mathbf{H}_3)_3$), 1.41 (9H, s, $C(C\mathbf{H}_3)_3$), 1.42 (9H, s, $C(C\mathbf{H}_3)_3$), 1.42 (9H, s, $C(C\mathbf{H}_3)_3$), 1.42 (9H, s, $C(C\mathbf{H}_3)_3$), 1.51-1.68 (6H, m, **H**$^{e+c+i}$), 2.06-2.15 (4H, m, cyclen), 2.25-2.39 (4H, m, cyclen), 2.41-2.61 (4H, m, cyclen), 2.70-2.87 (5H, m, cyclen+**H**^{b}), 3.07-3.43 (10H, m, **H**$^{f+g+j+k}$), 3.65-3.86 (2H, m, **H**^{h}), 3.99-4.16 (4H, m, $(OC\mathbf{H}_2CH_3)_2$) ppm. ^{31}P NMR ($CDCl_3$, 283 MHz): δ 27.28 ppm. ^{13}C NMR ($CDCl_3$, 176 MHz): δ 16.52 (d, ^{3}J = 7, $POCH_2\mathbf{C}H_3$), 24.6 (**C**^{d}), 27.7(d, ^{1}J = 141, $NCH_2\mathbf{C}H_2P$), 27.8 ($C(\mathbf{C}H_3)_3$), 27.8(**C**^{e}), 27.8 27.9, ($C(\mathbf{C}H_3)_3$), 28.1 (**C**^{c}), 29.2 ($C(\mathbf{C}H_3)_3$), 39.1 (**C**^{f}), 45.2 (d, ^{2}J = 6, $N\mathbf{C}H_2CH_2P$), 47.3, 48.1 (**C**^{cy}), 48.5 (**C**^{g}), 52.5, 52.6 (**C**^{cy}), 55.5, 55.7 ($\mathbf{C}H_2O$), 61.9 (**C**^{b}), 62.1 (d, ^{2}J = 7, $P(O\mathbf{C}H_2CH_3)_2$), 81.9, 82.0, 82.1, 82.7 ($\mathbf{C}(CH_3)_3$), 151.8, 151.9 (C**C**N), 160.5, 160.7, 165.2, 172.8, 172.8, 172.9, 174.9 (**C**O) ppm. HR-MS (ES^+) *m/z* $C_{53}H_{96}N_7O_{16}P$ requires 1117.665 $[M+H]^+$; found 1117.663 $[M+H]^+$.

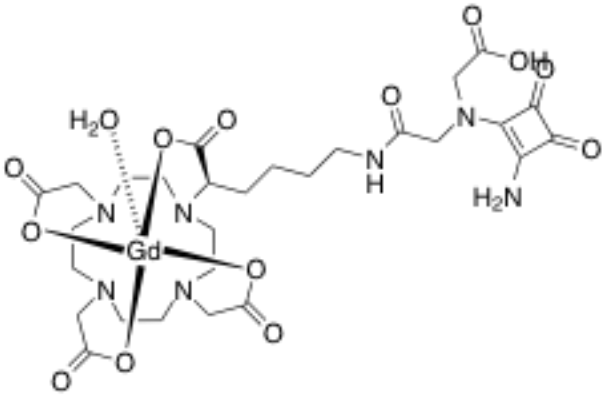
[Conjugate 4]



2-((2-((Tert-butoxycarbonyl)amino)-3,4-dioxocyclobut-1-en-1-yl)(3-(diethoxyphosphoryl)propyl)amino)acetic acid, **13** (75 mg, 0.17 mmol), EDC (40 mg, 0.20 mmol) and HOBt (28 mg, 0.20 mmol) were dissolved in anhydrous DMF (3 mL) and stirred at room temperature under an atmosphere of argon for 20

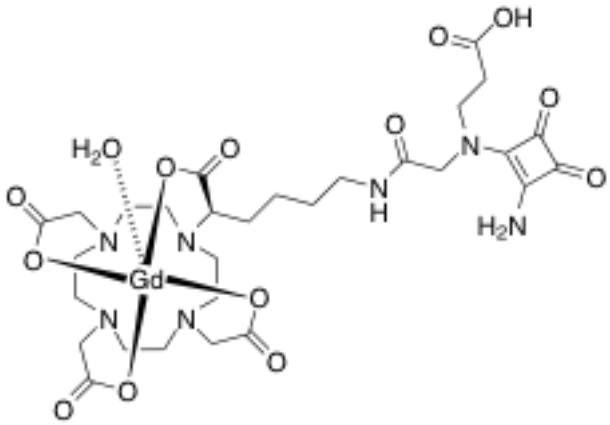
minutes. After this period, a pre-stirred solution of *(R)* –tri-*tert*-butyl 2,2',2''-(10-(6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, **14**[3] (117 mg, 0.17 mmol) and NMM (37 μL, 0.34 mmol) in anhydrous DMF (1.5 mL) was added drop wise and the resulting solution stirred at room temperature for a total of 4 days (second addition of EDC and HOBt after 2 days). After this period, DMF was removed under reduced pressure and the crude oil taken up into EtOAc (40 mL). $NaHCO_3$ (30 mL) was added, the layers separated and the aqueous washed with EtOAc (3 x 50 mL). The combined organic portions were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH 100% to 93:7 using 1% increments; R_f =0.30) to yield a viscous yellow-brown oil (23 mg, 12%). ^{1}H NMR ($CDCl_3$, 700 MHz): δ 1.23-1.27 (2H, m, **H**^{d}), 1.29 (3H, t, ^{3}J = 7, $OCH_2C\mathbf{H}_3$), 1.32 (3H, t, ^{3}J = 7, $OCH_2C\mathbf{H}_3$), 1.40 (9H, s, $C(C\mathbf{H}_3)_3$), 1.41 (9H, s, $C(C\mathbf{H}_3)_3$), 1.42 (9H, s, $C(C\mathbf{H}_3)_3$), 1.42 (9H, s, $C(C\mathbf{H}_3)_3$), 1.42 (9H, s, $C(C\mathbf{H}_3)_3$), 1.51-1.68 (6H, m, **H**$^{e+c+i}$), 1.89-1.98 (2H, m, $C\mathbf{H}_2P$), 2.06-2.15 (4H, m, cyclen), 2.25-2.39 (4H, m, cyclen), 2.41-2.61 (4H, m, cyclen), 2.70-2.87 (5H, m, cyclen+**H**^{b}), 3.07-3.43 (10H, m, **H**$^{f+g+j+k}$), 3.65-3.86 (2H, m, **H**^{h}), 3.99-4.16 (4H, m, $(OC\mathbf{H}_2CH_3)_2$) ppm. ^{31}P NMR ($CDCl_3$, 283 MHz): δ 30.18 ppm. ^{13}C NMR ($CDCl_3$, 176 MHz): δ 16.51 (d, ^{3}J = 7, $POCH_2\mathbf{C}H_3$), 22.29 (d, ^{2}J = 5, $NCH_2\mathbf{C}H_2CH_2P$), 23.05 (d, ^{1}J = 143, $N(CH_2)_2\mathbf{C}H_2P$) 24.6 (**C**^{d}), 27.8 ($C(\mathbf{C}H_3)_3$), 27.8(**C**^{e}), 27.8 27.9, ($C(\mathbf{C}H_3)_3$), 28.1 (**C**^{c}), 29.2 ($C(\mathbf{C}H_3)_3$), 39.1 (**C**^{f}), 47.3, 48.1 (**C**^{cy}), 48.5 (**C**^{g}), 52.5, 52.6 (**C**^{cy}), 56.0 (d, ^{3}J = 17, $N\mathbf{C}H_2(CH_2)_2P$), 55.5, 55.7 ($\mathbf{C}H_2O$), 61.9 (**C**^{b}), 61.92 (d, ^{2}J = 7, $P(O\mathbf{C}H_2CH_3)_2$), 81.9, 82.0, 82.1, 82.7 ($\mathbf{C}(CH_3)_3$), 151.8, 151.9 ($C\mathbf{C}N$), 160.5, 160.7, 165.2, 172.8, 172.8, 172.9, 174.9 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{54}H_{95}DN_7O_{16}PNa$ requires 1153.661 $[M+Na]^+$; found 1153.657 $[M+Na]^+$.

[Gd.L^1]



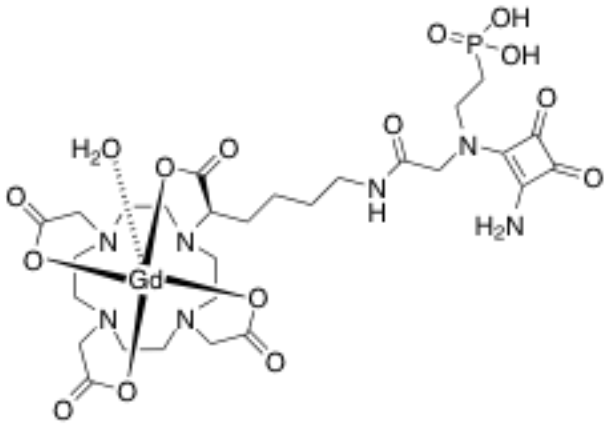
[Conjugate 1] (26 mg, 0.02 mmol) was dissolved in DCM (1 mL) with stirring. To this was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature overnight. Complete removal of the protecting groups was verified by ESI-MS at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed in *vacuo* to remove excess TFA. This process yielded **L^1** as a brown solid. MS (ES$^+$) *m/z* 685.7 $[M+H]^+$. **L^1** (13 mg, 0.02 mmol) was dissolved in purite H_2O (1 mL) and the pH adjusted to about 5.5 by the addition of NaOH (0.1 M). $GdCl_3.6H_2O$ (11.5 mg, 0.031 mmol) was added as a solution in H_2O (0.5 mL) and the reaction mixture stirred at 60 $^{\circ}$C overnight. The pH of the solution was periodically checked and adjusted to 6.5 with the addition of NaOH/HCl (0.1 M). Upon completion, excess gadolinium was removed by the addition of Chelex-100™ with stirring. The Chelex trap was filtered and the complex eluted with excess H_2O. Removal of the water by lyophilisation gave the complex as a light yellow solid. HR-MS (ES$^-$) $C_{28}H_{37}{}^{157}GdN_7O_{13}$ requires 837.1690 $[M-2H]^-$; found 837.1695. r_{1p} = 5.17 $mM^{-1}s^{-1}$ (60 MHz, 310K).

[Gd.L^2]



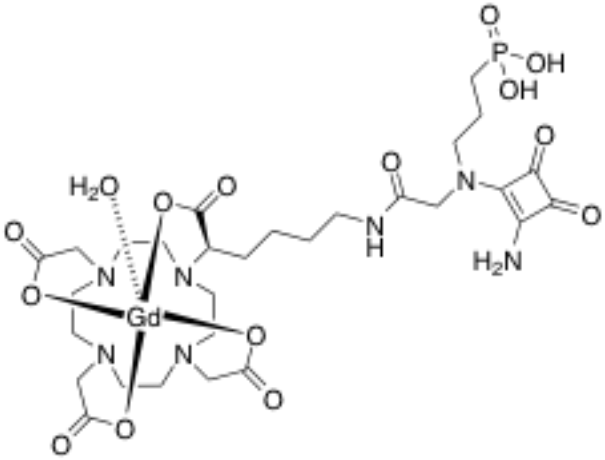
[Conjugate 2] (17 mg, 0.02 mmol) was dissolved in DCM (1 mL) with stirring. To this was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature overnight. Completion of the reaction was verified by ESI-MS at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed in *vacuo* to remove excess TFA. This process yielded the product as a light-brown solid. MS (ES$^+$) *m/z* 700.5 $[M+H]^+$. **L^2** (14 mg, 0.02 mmol) was dissolved in purite H_2O (1 mL) and the pH adjusted to 5.5 by the addition of NaOH (0.1 M). $GdCl_3.6H_2O$ (11.5 mg, 0.031 mmol) was added as a solution in H_2O (0.5 mL) and the reaction mixture stirred at 60 $^\circ$C overnight. The pH of the solution was periodically checked and adjusted to 6.5 with the addition of NaOH/HCl (0.1 M). Upon completion, excess gadolinium was removed by the addition of chelex 100™ with stirring. The chelex trap was filtered and the complex eluted with excess H_2O. Removal of the water by lyophilisation gave the complex as a light yellow solid. HR-MS (ES$^-$) $C_{29}H_{39}{}^{157}GdN_7O_{13}$ requires 851.1932 $[M-2H]^-$; found 851.1909. r_{1p} = 5.30 $mM^{-1}s^{-1}$ (60 MHz, 310K).

[Gd.L^3]



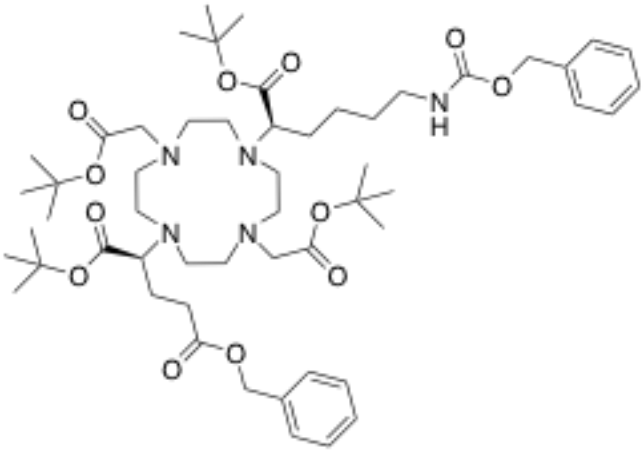
[Conjugate 3] (10 mg, 9 μmol) was dissolved in DCM (1 mL) with stirring. To this was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature for overnight. Loss of *tert*-butyl and BOC groups was verified by ESI-MS ($[M+H]^+$; m/z =791.5) at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed in *vacuo* to remove excess TFA. This process yielded the phosphonate ethyl ester as a light-brown solid. This residue was dissolved in DMF (1 mL) to which bromotrimethylsilane (20 μL, 0.15 mmol) was added dropwise. The resulting mixture was heated to 60 °C overnight until complete hydrolysis. The solvent was removed under reduced pressure, before the residue redissolved in H_2O, the pH adjusted to 6 and the aqueous washed with diethyl ether (x3). The solvent was removed by lyophilisation to give **L^3** as a light brown solid. MS (ES^+) *m/z* 735.0 $[M+H]^+$. **L^3** (7 mg, 9 μmol) was dissolved in purite H_2O (1 mL) and the pH adjusted to 5.5 by the addition of NaOH (0.1 M). $GdCl_3.6H_2O$ (4 mg, 10 μmol) was added as a solution in H_2O (0.5 mL) and the reaction mixture stirred at 60 °C overnight. The pH of the solution was periodically checked and adjusted to 6.5 with the addition of NaOH/HCl (0.1 M). Upon completion, excess gadolinium was removed by the addition of chelex 100™ with stirring. The chelex trap was filtered and the complex eluted with excess H_2O. Removal of the water by lyophilisation gave the complex as a light yellow solid. HR-MS (ES^-) $C_{28}H_{40}{}^{157}GdN_7O_{14}P$ requires 887.1697 $[M-2H]^-$; found 887.1686. r_{1p} = 4.68 $mM^{-1}s^{-1}$ (60 MHz, 310K).

[Gd.L^4]



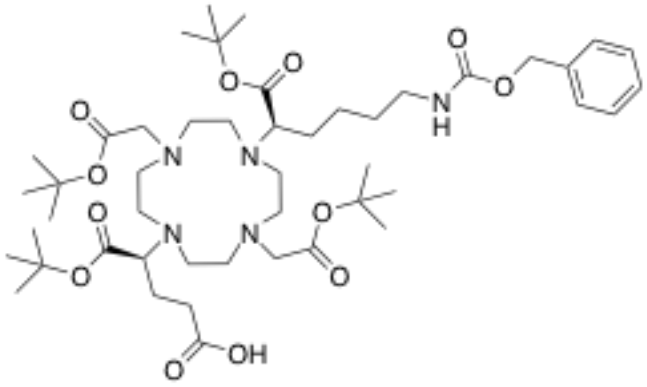
[Conjugate 4] (10 mg, 9 μmol) was dissolved in DCM (1 mL) with stirring. To this was added trifluoroacetic acid (1 mL) and the resulting solution stirred at room temperature for overnight. Loss of *tert*-butyl and BOC groups was verified by ESI-MS ([M+H]$^+$; m/z = 806.0) at which point the excess solvent was removed under reduced pressure. The residue was repeatedly re-dissolved in DCM (2 mL) and the solvent removed in *vacuo* to remove excess TFA. This process yielded the phosphonate ethyl ester as a light-brown solid. This residue was dissolved in DMF (1 mL) to which bromotrimethylsilane (20 μL, 0.15 mmol) was added dropwise. The resulting mixture was heated to 60 $^\circ$C overnight until complete hydrolysis. The solvent was removed under reduced pressure, before the residue redissolved in H_2O, the pH adjusted to 6 and the aqueous washed with diethyl ether (x3). The solvent was removed by lyophilisation to give **L^4** as a light brown solid. MS (ES$^+$) *m/z* 750.0 [M+H]$^+$. **L^4** (7 mg, 9 μmol) was dissolved in purite H_2O (1 mL) and the pH adjusted to 5.5 by the addition of NaOH (0.1 M). $GdCl_3.6H_2O$ (4 mg, 10 μmol) was added as a solution in H_2O (0.5 mL) and the reaction mixture stirred at 60 $^\circ$C overnight. The pH of the solution was periodically checked and adjusted to 6.5 with the addition of NaOH/HCl (0.1 M). Upon completion, excess gadolinium was removed by the addition of chelex 100™ with stirring. The chelex trap was filtered and the complex eluted with excess H_2O. Removal of the water by lyophilisation gave the complex as a light yellow solid. HR-MS (ES$^-$) $C_{28}H_{40}{}^{155}GdN_7O_{14}P$ requires 898.1754 [M-2H]$^-$; found 898.1788. r_{1p} = 4.80 mM^{-1}s^{-1} (60 MHz, 310K).

_(R)_-5-benzyl 1-_tert_-butyl 2-(7-(_(R)_-6(((benzyloxy)carbonyl)amino)-1-(_tert_-butoxy)-1-oxohexan-2-yl)-4,10-bis(2-(_tert_-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanedioate, 15



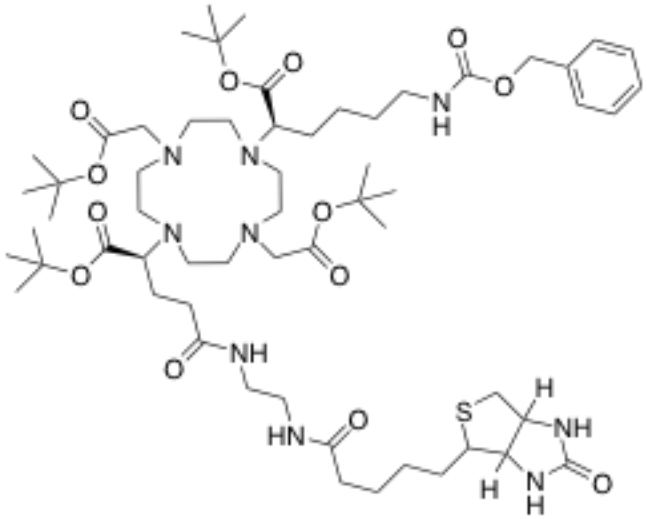
(R)-Di-*tert*-butyl 2,2'-(4-(6-(((benzyloxy)carbonyl)amino)-1-(*tert*-butoxy)-1-oxohexan-2-yl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate[4] (1.34 g, 1.87 mmol), K_2CO_3 (517 mg, 3.74 mmol) and *(S)*-*tert*-butyl 2-bromo-5-(2-phenylacetamido)pentanoate (801 mg, 2.24 mmol) were stirred as a solution in acetonitrile (20 mL) at 60 °C for 48 hours. The completion of the reaction was verified by TLC and the mixture cooled to room temperature, filtered and the filtrate concentrated under reduced pressure. The crude orange residue was then purified by column chromatography (100% DCM to 93:7 DCM MeOH utilizing 1% increments; R_f=0.4) to give the title compound as a light green oil (596 mg, 32%). ^{1}H NMR (700 MHz, $CDCl_3$) δ 1.22 – 1.36 (4H, m, C**H**$_2$), 1.39 (9H, s, C(C**H**$_3$)), 1.41 (18H, s, C(C**H**$_3$)), 1.44 (9H, s, C(C**H**$_3$)), 1.59 – 1.73 (2H, m, C**H**$_2$), 2.04 – 2.12 (1H, m, C**H**), 2.25 – 2.75 (12H, br, m, C**H**$_2$, C**H**$_2$ ring), 2.96 – 3.19 (8H, br, m, C**H**$_2$ ring), 3.26 – 3.31 (2H, m, C**H**$_2$), 3.34 – 3.35 (1H, m, C**H**), 3.46 (4H, s, C**H**$_2$), 5.04 (2H, s, C**H**$_2$), 5.06 (2H, s, C**H**$_2$), 5.15 (1H, s, N***H***), 7.25 – 7.35 (10H, m, **H**$_{Ar}$). ^{13}C NMR (176 MHz, $CDCl_3$) δ 24.4, 26.5, 27.7, 27.72 (C(**C**H$_3$)$_3$), 27.8, 28.1, 29.9, 32.6, 40.6 (**C**H$_2$), 55.6, 55.9, 56.4, 59.8 (**C**^{cy}), 61.2(**C**H$_2$CO$_2$^{t}Bu), 66.3, 67.6 (**C**HCO$_2$^{t}Bu), 68.4, 69.3 (Ar-**C**H$_2$CO), 81.7, 81.9, 82.4 (**C**(CH$_3$)$_3$), 127.9, 128.2, 128.3, 128.4, 128.6, 135.6, 136.5 (Ar-**C**), 156.4, 172.9, 173.4, 174.6, 175.1 (**C**O). HR-MS (ES$^+$) *m/z* $C_{54}H_{86}N_5O_{12}$ requires 996.6273 $[M+H]^+$; found 996.6293 $[M+H]^+$.

_(R)_-4-(7-((_R_)-6-(((benzyloxy)carbonyl)amino)-1-(_tert_-butoxy)-1-oxohexan-2-yl)-4,10-bis(2-(_tert_-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-5-(_tert_-butoxy)-5-oxopentanoic acid



15 (196 mg, 0.20 mmol) was dissolved in anhydrous THF (3 mL) and NaOH (17 mg, 0.40 mmol) was added as a solution in water (1 mL). The light yellow solution was stirred at room temperature and the progress of the reaction monitored by TLC. Upon complete conversion of the starting material, the solvent was removed under reduced pressure to give a light green solid which was dried fully on a high vacuum line before being used directly in the next step without further purification (181 mg, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 – 1.39 (2H, m, C**H**$_2$), 1.40 (9H, s, C(C**H**$_3$)), 1.41 (18H, s, C(C**H**$_3$)), 1.42 (9H, s, C(C**H**$_3$)), 1.69-1.78 (4H, m, C**H**$_2$,), 2.14 – 2.22 (1H, m, C**H**), 2.27 – 2.88 (12H, br, m, C**H**$_2$, C**H**$_2$ ring), 2.92 – 3.39 (8H, br, m, C**H**$_2$ ring), 3.42 – 3.51 (2H, m, C**H**$_2$), 3.49 – 3.55 (1H, m, C**H**), 3.67 (4H, s, C**H**$_2$), 4.63 (1H, s, COO**H**), 5.01 (2H, s, C**H**$_2$), 7.15 – 7.33 (5H, m, **H**$_{Ar}$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.5, 24.6, 26.9 (C(**C**H$_3$)$_3$), 27.2, 27.3, 30.2, 31.5, 39.6 (**C**H$_2$), 53.8, 54.9, 55.4, 58.3 (**C**^{cy}), 59.0 (**C**H$_2$CO$_2$^{t}Bu), 63.4, 66.9 (**C**HCO$_2$^{t}Bu), 67.9 (Ar**C**H$_2$O), 80.4, 80.8, 80.9 (**C**(CH$_3$)$_3$), 125.9, 127.2, 127.4, 141.0 (Ar-**C**), 155.6, 171.6, 174.9, 175.5, 179.1 (**C**O). HR-MS (ES$^+$) *m/z* $C_{47}H_{80}N_5O_{12}$ requires 906.5803 $[M+H]^+$; found 906.5763 $[M+H]^+$.

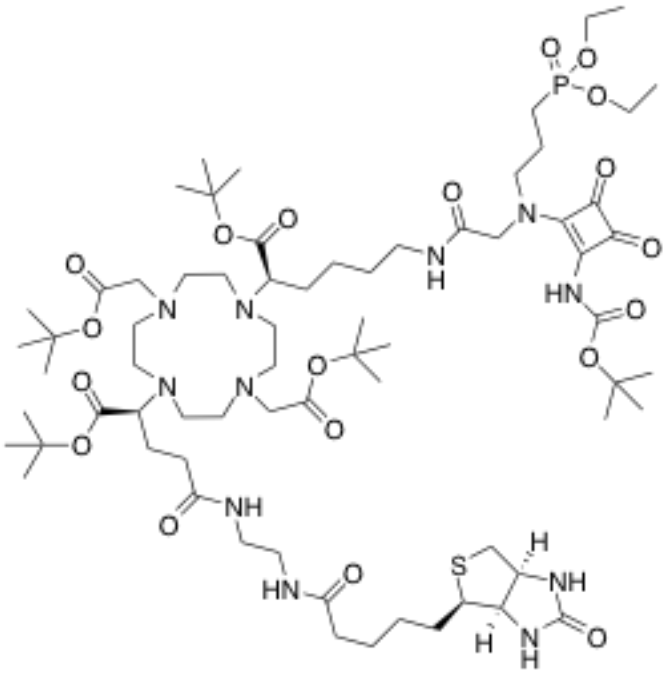
di-*tert*-butyl 2,2'-(4-((*R*)-6-(((benzyloxy)carbonyl)amino)-1-(*tert*-butoxy)-1-oxohexan-2-yl)-10-((*R*)-1-(*tert*-butoxy)-1,5-dioxo-5-((2-5-((3a*S*,4*S*,6a*R*)-2-oxohexhydro-1H-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethyl)amino)pentan-2-yl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate, 16



A solution of *(R)*-4-(7-(*(R)*-6-(((benzyloxy)carbonyl)amino)-1-(*tert*-butoxy)-1-oxohexan-2-yl)-4,10-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-5-(*tert*-butoxy)-5-oxopentanoic acid (181 mg, 0.2 mmol), EDC.HCl (46 mg, 0.24 mmol) and HOBt (32 mg, 0.24 mmol) in anhydrous DMF (1 mL) were stirred under argon at room temperature for 15 minutes. To the mixture was added a solution of the Biotin ethylenediamine[5] (57.5 mg, 0.2 mmol) and NMM (44 μL, 0.4 mmol) in anhydrous DMF (0.7 mL) dropwise over 5 minutes. The resulting mixture was stirred at room temperature overnight, before a second addition of the coupling reagents (EDC.HCl 46 mg, 0.24 mmol; HOBt 32 mg, 0.24 mmol). The reaction mixture was again stirred overnight at room temperature. Upon no further reaction by ESI-MS, the DMF was removed under reduced pressure and the residue taken up into EtOAc (30 mL). The organic phase was washed with saturated $NaHCO_3$ (30 mL) and the aqueous repeatedly extracted with EtOAc (3 x 30 mL). The combined organics were washed with brine (40 mL), dried over $MgSO_4$, filtered and evaporated. The crude residue was then purified by column chromatography (100% DCM to 80:18:2 DCM/MeOH/NH_4OH; R_f=0.13) to give the title compound as a viscous brown oil (70 mg, 30 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.32-1.56 (41H, m, C(C**H**$_3$)$_3$,

C**H**$_2$), 1.57-1.69 (5H, m), 1.70-1.90 (3H, m), 2.09-2.25 (4H, m), 2.49-2.67 (6H, m), 2.68-2.77 (6H, m), 2.78-2.92 (6H, m), 3.04-3.23 (9H, m), 3.23-3.41 (5H, m), 4.27 (1H, dd, ^{3}J = 7.8, 4.8, NHC**H**CHS), 4.45 (1H, dd, ^{3}J = 7.8, 4.8, NHC**H**CH$_2$S), 5.06 (2H, s, OC**H**$_2$Ph), 5.89 (1H, br, s, N**H**), 6.51 (1H, br, s, N**H**), 7.28-7.35 (5H, m, Ar-**H**). ^{13}C NMR (151 MHz, $CDCl_3$) δ 23.6, 25.6, 27.9, 28.0 (**C**H/**C**H$_2$), 28.2, 28.3, 28.4 (C(**C**H$_3$)$_3$), 29.7, 29.9, 33.3, 35.9, 39.4, 40.6, 41.0, 49.5, 50.0, 50.7 (**C**H/**C**H$_2$), 53.0, 53.2, 55.7, 56.7 (**C**^{cy}), 60.3, 61.8 (**C**H$_2$CO$_2$^{t}Bu), 63.5, 64.5 (**C**HCO$_2$^{t}Bu), 66.6 (Ar**C**H$_2$O), 80.9, 81.0, 81.1 (**C**(CH$_3$)$_3$), 128.1, 128.2, 128.5, 136.8 (**C**-Ar), 156.6, 164.1, 171.3, 172.5, 173.0, 173.9, 175.4 (**C**O) ppm. HR-MS (ES$^+$) *m/z* $C_{59}H_{100}N_9O_{13}S$ requires 1174.716 $[M+H]^+$; found 1174.719 $[M+H]^+$.

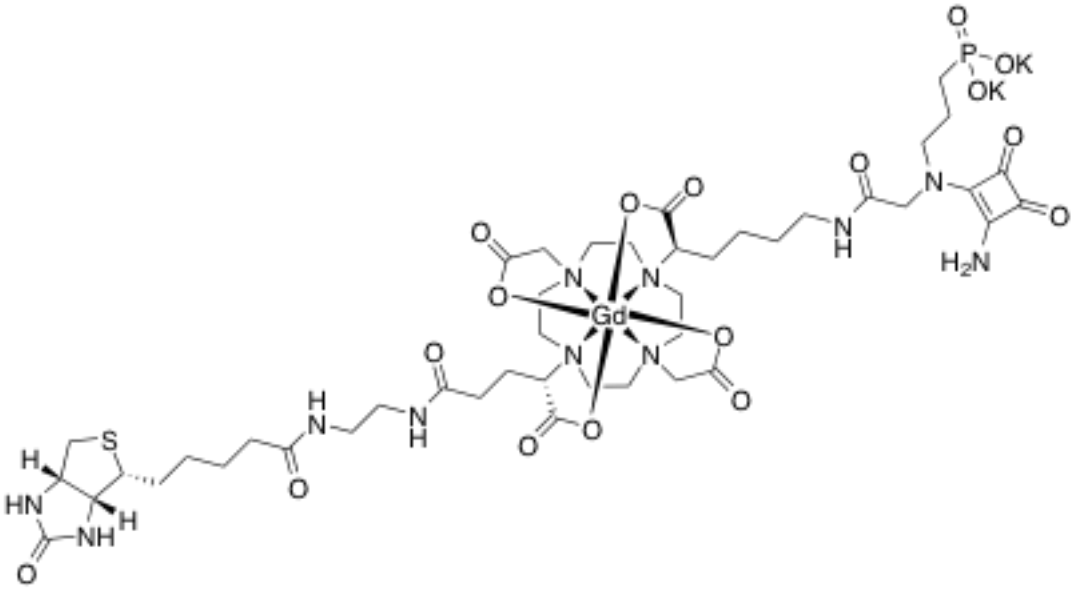
[Conjugate 5], 17



16 (70 mg, 0.06 mmol) was dissolved in MeOH (10 mL) to which $Pd(OH)_2$ (20 mg) was added. The vessel was then loaded onto a Parr hydrogenator (P_{H2}=40 bar) and the reaction mixture agitated over 72 hours. After removal of the CBz protecting group (MS (ES$^+$) *m/z* 520.9 $[M+2H]^{2+}$), the mixture was filtered to remove excess palladium and washed excessively with MeOH. The amine residue was then evaporated to dryness. A solution of the amine (62 mg, 0.06 mmol) and NMM (13 μL, 0.12 mmol) in anhydrous DMF (1 mL) were then added dropwise over 5 minutes to a prestirred solution of the acid, **13** (28 mg, 0.06 mmol),

EDC.HCl (14 mg, 0.07 mmol) and HOBt (10 mg, 0.07 mmol) in anhydrous DMF (1 mL). The resulting mixture was stirred at room temperature overnight, before a second addition of the coupling reagents (EDC.HCl 14 mg, 0.07 mmol; HOBt 10 mg, 0.07 mmol). The reaction mixture was again stirred overnight at room temperature. Upon no further reaction by ESI-MS, the DMF was removed under reduced pressure and the residue taken up into EtOAc (20 mL). The organic phase was washed with saturated $NaHCO_3$ (20 mL) and the aqueous repeatedly extracted with EtOAc (3 x 20 mL). The combined organics were washed with brine (30 mL), dried over $MgSO_4$, filtered and evaporated. The crude residue was then purified by RF-HPLC to give the desired compound as a brown film (5 mg, 6%). ^{1}H NMR (700 MHz, $CDCl_3$) δ 1.22-1.54 (53H, br, m), 1.62-2.48 (35H, br, m), 2.70-3.51 (22H, br, m), 4.10 (4H, br, m). ^{31}P NMR (283 MHz, CDCl3) δ 30.49 ppm. HR-MS (ES^+) *m/z* $C_{69}H_{121}N_{11}O_{19}SP$ requires 736.422 $[M+2H]^{2+}$; found 736.420 $[M+2H]^{2+}$.

[Gd.L^5]



To a solution of **[conjugate 5]** (5 mg, 0.003 mmol) in anhydrous THF (1 mL), was added a solution of KOH (1 mg, 0.02 mmol) in H_2O (200 µL) and the resulting mixture heated to 60 °C for 4 hours. After this period, the solvent was removed under reduced pressure to give the crude residue. Removal of the phosphonate ethyl esters was verified by ESI-MS ($[M+2K]^{2+}$; m/z = 745.5). The free phosphonic acid was then partially dissolved in DCM (1 mL) to which TFA (1

mL) was added. The resulting yellow solution was stirred at room temperature overnight before removal of the solvent under reduced pressure. The crude product was redissolved in DCM and concentrated under reduced pressure. This process was repeated 3 times to yield the deprotected ligand ($[M+2H]^{2+}$ m/z = 545.1). The residue was then dissolved in H_2O (1 mL) and the pH adjusted to 5.5 by the addition of NaOH (0.1M). $GdCl_3.6H_2O$ (2 mg, 5.4 µmol) was added as a solution in H_2O (0.2 mL) and the reaction mixture stirred at 60 °C overnight. The pH of the solution was periodically checked and adjusted to 5.5 with the addition of NaOH/HCl (0.1 M). Upon completion, excess gadolinium was removed by the addition of chelex 100 with stirring. The Chelex trap was filtered and the complex eluted with excess H_2O. Removal of the water by lyophilisation gave the complex as a light yellow solid. r_{1p} = 7.24 $mM^{-1}s^{-1}$ (60 MHz, 310K). HR-MS (ES^+) $C_{44}H_{69}{}^{157}GdN_{11}O_{17}PSK_2$ requires 440.672 $[M+H+2K]^{3+}$; found 440.671 $[M+H+2K]^{3+}$.

Cell culture

Cells were cultured according to a previously reported protocol.[6] In short, the NSC-34 cells were cultured in a 1 : 1 mixture of Dulbecco's modified Eagle's (DMEM) and F12 medium supplemented with fetal bovine serum (FBS, 10% v:v), non essential amino acids (0.5%) and Penicillin/Streptomycin (0.1%). At approximately 90% confluence after 3 healthy passages, cells were sub-cultured into a different growth medium, which contained a mixture of DMEM/Ham's F12 (1:1), FBS (1%), non-essential amino acids (1%) and Penicillin/Streptomycin (0.1%). Cells were allowed to proliferate over a period of several days to allow for the growth of functional NMDA receptors.

NIH-3T3 cells were cultured as previously reported.[7]

Immunofluorescence measurements

NSC-34 cells grown and differentiated for 1 week on surface-modified glass chamber slides (Thermo Scientific Nunc, Denmark) were fixed for 10 min at room temperature with 4% paraformaldehyde (Roti®-Histofix 4%, Roth,

Germany). Non-specific antibody binding sites were blocked by incubating for 30 min with 10% (v/v) goat-serum in PBST. Fixed cells were then incubated for 2 hours with the primary antibodies for the NMDA receptor subunits NMDAR-1 or NMDAR-2B (diluted in 1% BSA (Roth, Germany) in PBST) in a humidified chamber at RT. After washing, cells were incubated with secondary antibodies in 1% BSA in PBST in the humidified chamber in the dark. Afterwards cellular DNA was stained with 16.2µM Hoechst 33342 (Merck, Germany) for 15 min and slides were mounted overnight with Mowiol 4-88 (Roth, Germany) containing 50 mg/mL of the anti-bleaching reagent 1,4-diaza-bicyclo[2.2.2]octane (Roth, Germany). Microscopic images were taken on a Zeiss Axiovert 200 M microscope (Germany) using appropriate fluorescence filters and a Zeiss Plan APOCHROMAT 63x/1.4 oil DIC objective. Volocity Acquisition and Visualization software 5.2.0 (Perkin Elmer, USA, MA) was used for image capture and analysis. The used antibodies were as follows and all antibodies were obtained from Abcam (UK, Cambridge). Primary antibodies, rabbit polyclonal to NMDAR2B or NMDAR1; secondary antibody, goat polyclonal to rabbit IgG – H&L conjugated to Cy3.

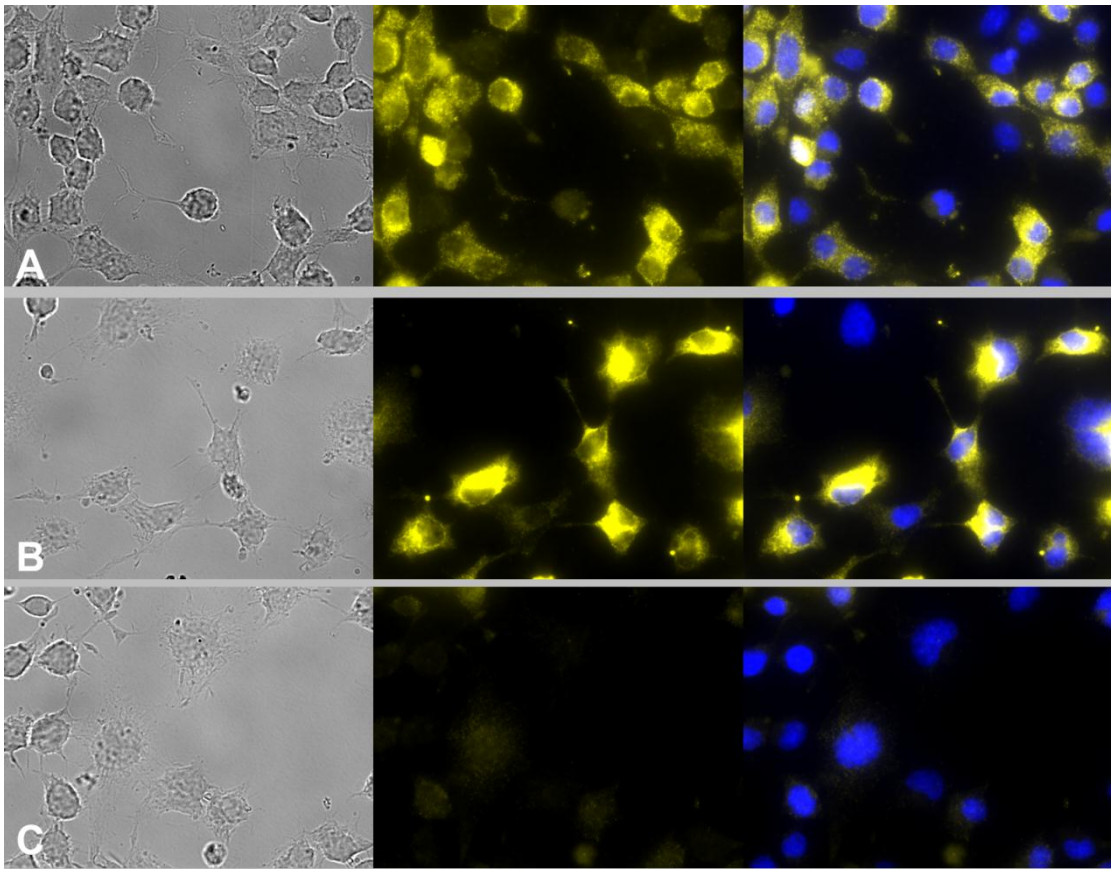


Figure S1: Immunostaining of differentiated NSC-34 cells for NMDAR subunit 2B and NMDAR-1. NSC-34 cells were differentiated for 1 week in surface-modified glass chamber slides. (A) From left to right: Differential interference contrast image showing cell morphology, NMDAR 2B (yellow, Cy 3™) visualized after staining with specific antibodies and overlay with counterstained cell nuclei (blue, Hoechst 33342). (B) as (A) but with specific antibodies for the NMDAR1 subunit on different cells. (C) Control staining for non-specific binding of the 2nd antibody (goat anti-rabbit-Cy 3™). The middle image was normalized to the brightness and contrast of the Cy 3™ image in (A), demonstrating very low non-specific binding of the 2nd antibody. The intensity is equally low when the normalization was done to the Cy 3™ image in B (data not shown). [Carried out at MPI, Germany]

Cytotoxicity

IC_{50} values were determined using the MTT assay, as described elsewhere[8] which makes use of the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) to a purple formazan product by the mitochondrial dehydrogenase of viable cells. This insoluble formazan was quantified spectrophotometrically upon dissolution in DMSO. Approximately 5×10^3 differentiated NSC-34 cells in 100 μL culture medium were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. Complex solutions were added to triplicate wells to give final concentrations over a 2-log range. Following 24 h incubation, MTT (1.0 mM) was added to each well, and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plates were shaken for 20 seconds and the absorbance measured immediately at 540 nm in a microplate reader. IC_{50} values were determined as the drug concentration required to reduce the absorbance to 50% of that in the untreated, control wells, and represent the mean for data from at least three independent experiments

Table S1: Observed absorbance at 540 nm of **[Gd.L$^{1\text{-}4}$]** at varying concentrations giving IC_{50} > 200 μM. (control blank wells and untreated cells (7 well each) gave an averaged absorbance value of 0.258 and 1.689 respectively)

Conc/μM	[Gd.L^1]	[Gd.L^2]	[Gd.L^3]	[Gd.L^4]
5	1.746	1.605	1.634	1.824
10	1.593	1.801	1.762	1.554
30	1.501	1.425	1.489	1.822
50	1.432	1.467	1.456	1.675
100	1.488	1.756	1.782	1.430
150	1.816	1.768	1.554	1.478
200	1.653	1.478	1.853	1.724

HSA-relaxivity titration

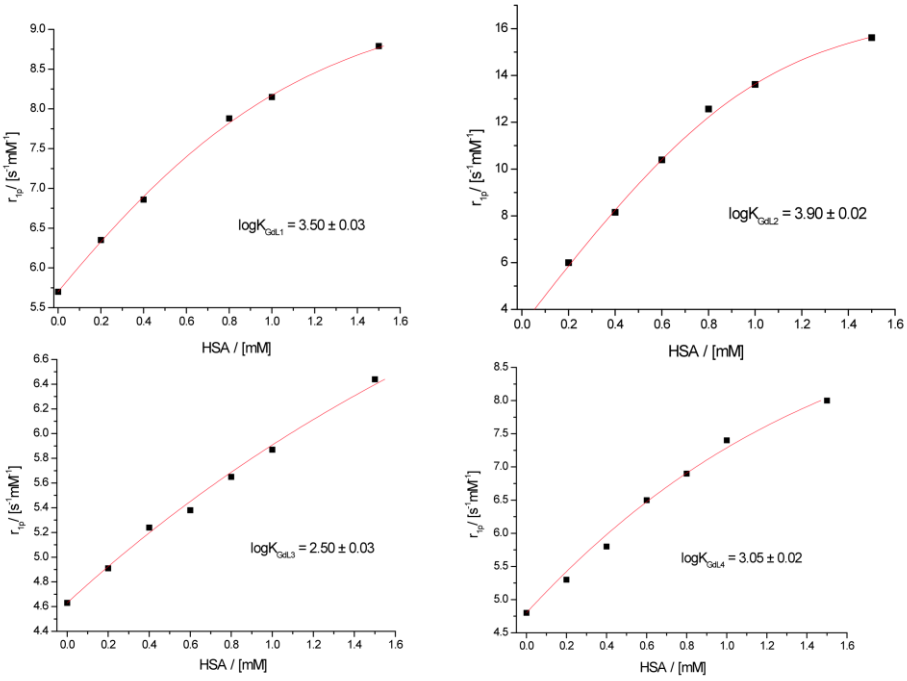


Figure S2: Binding constant determination of **[Gd.L$^{1\text{-}4}$ vs HSA]** showing the fit(line) to the data derived by iterative least squares fitting assuming a 1:1 binding interaction. Errors quoted are statistical errors in the fitting (1.4 T, pH 7, 310 K).

Measurement of cellular relaxation rates at 3T

Differentiated NSC-34 cells were grown to confluency and trypsinised with Trypsin/EDTA 0.05/0.02% (w/v) (Biochrom AG, Germany), centrifuged and re-suspended to 1 x 10^7 cells/ 500 µL HBSS in 750 µL Eppendorf tubes. A maximum of 20 µL of highly concentrated stock solutions of the compounds were added to give 200 µM solutions; cells were incubated for 45 min. at 37°C, 10% CO_2. Afterwards, cells were centrifuged (300×g, 5 min., RT) and the supernatant was kept for MR- measurements. Cells were washed once with HBSS, centrifuged again (300×g, 5 min., RT) and re-suspended carefully in 500 µL of HBSS. In some experiments the wash-solution was also kept for MR-measurements. Cells were allowed to settle down before making MR-measurements. MR imaging of the cell pellets and supernatants, data analysis and determination of the cellular

relaxation rates $R_{1,cell}$ were assessed from the measured T_1 values, according to the literature.

Briefly, MR measurements were performed on a clinical 3T (128 MHz, 21°C) human MR scanner (MAGNETOM Tim Trio, Siemens Healthcare, Germany). Longitudinal relaxation times (T_1) were measured using an inversion recovery sequence to obtain images from a 1 mm thick slice through the samples. The inversion time (T_i) was varied from 23 ms to 3000 ms in about 12

17 steps. Images were read out with a turbo spin echo technique, acquiring 5 echoes per scan. The repetition time (T_R) was 10.000 ms to ensure complete relaxation. A matrix of 256 x 256 voxels was used over a field-of-view of 110 x 110 mm^2. Six averages per T_i were possible within 18 min.

Fitting to relaxivity curves was done using self-written routines in MATLAB 6.5 R13 (The Mathworks Inc., USA). T_1 relaxation data with varying $t = T_R$ were fitted to $S=S_0(1-a \times \exp(- t/T_1))$. Nonlinear least-squares fitting of three parameters S_0 (initial signal at t=0), T_1 and a was done for each voxel with the Gauss-Newton method (MATLAB function nlinfit). For each fitted parameter, the 95% confidence intervals were calculated (MATLAB functions nlparci, nlpreci) and used as an error estimate of the fitted relaxation times T_1 and S_0. The fit procedure resulted in parameter maps of T_1, S_0 and corresponding error maps σT_1, σS_0.

Circular image-regions in the tubes were defined as Regions Of Interest (ROIs), and the means and distribution width of the relaxation times of voxels in these regions were calculated. An iterative Gaussian fit was used to determine mean and standard deviation (SD) of a distribution with outliers' correction. For this purpose, a distribution histogram was first fitted to a Gaussian to estimate the mean and SD. The tails of the distribution were then discarded by using a threshold of three SDs. A repeated fit proved to be robust and converged to the 'true' Gaussian mean and width of the distribution barring the outliers, observed as a result of the non-linear fit of noisy voxels. The processing of the relaxation data thus resulted in specific $R_1 = 1/T_1$ values for each tube sample, including the standard deviation in the selected ROI ensemble. The ensemble error matched closely the errors of a single-voxel fit, which showed that no further systematic errors were introduced by the image encoding.

Confocal Microscopy and Cell Spectral Imaging

Cell images and co-localization experiments were obtained using a Leica SP5 II microscope. A HeNe laser was used to visualize SA-AF488 fluorescence. The microscope was equipped with a triple channel imaging detector, comprising two conventional PMT systems and a HyD hybrid avalanche photodiode detector. The latter part of the detection system, when operated in the BrightRed mode, is capable of improving imaging sensitivity above 550 nm by 25%, reducing signal to noise by a factor of 5. The pinhole was always determined by the Airy disc size, calculated from the objective in use (HCX PL APO 63x/1.40 NA LbdBlue), using the lowest excitation wavelength (488 nm). Scanning speed was adjusted to 400 Hz in a bidirectional mode, to ensure both sufficient light exposure and enough time to collect the emitted light from the optical probes (1024 x 1024 frame size, a pixel size of 120 x 120 nm and depth of 0.89 μm). The 3D reconstruction was achieved using a novel saturation elimination algorithm update of the existing ImageJ 1.46r 3D plug-in using, LSCM images recorded on the above detailed Leica SP5 II microscope. In these z-stack images a deliberate 20% overlap in the applied axial resolution was introduced, determined by the optics used and the experimental parameters detailed above.

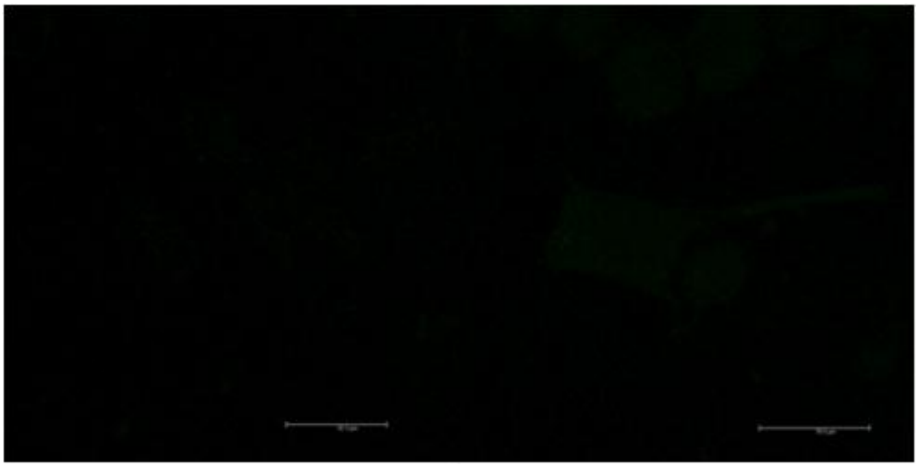


Figure S3: Live cell LSCM image of (*left*) untreated differentiated NSC-34 cells and (*right*) incubation of AvidinAlexaFluor® 488 conjugate (2.5 μM, 10 mins), with differentiated NSC-34 cells, showing no auto fluorescence.

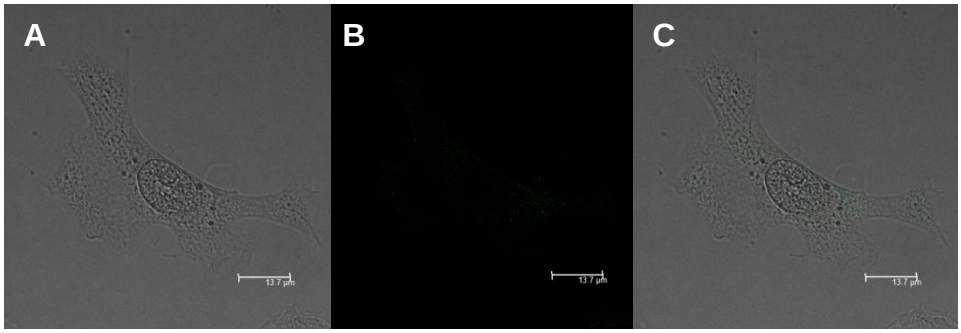


Figure S4: Live cell LSCM images of simultaneous loading of **[Gd.L^5]** (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 10 mins), with NIH-3T3 cells. (A and B) visualization of AlexaFluor®488 using our previously established imaging parameters and simultaneously recorded transmission image respectively; (C) Merged image showing no cellular staining.

Glutamate/Aspartate wash experiment

The average image intensity of each phase was assessed as triplicates using 3 cells (excluding the axon) within each image. In the case of the simultaneous experiment (Fig. 2A, main manuscript), the maximum Contrast Transfer Function (CTF), displaying an even Gaussian distribution profile was found the be 173 (out of 256, 0-255). Keeping all experimental parameters constant throughout these experiments (Leica x63, Oil, 1.40 NA LmdBlue, 400 Hz bi-

directional scan, voxel size (120 x 120 x 790 nm, λ_{ex} = 488 nm (0.5 mW), λ_{em} = 505 -555 nm (HyD 50% gain), for the aspartate wash, the images were less bright giving rise to an evenly distributed CTF profile with a highest value of 66, which equates to 38% of original intensity from original experimental parameters.

For the glutamate wash, an evenly distributed CTF was found with the highest value only to be 17 which gives 10.2% of the original intensity from the first experiment. Although this gives rise to a large background noise of 10. This value was found to be of similar portion (35) when the gain has been increased to 100% showing a linear correlation (note if laser power would have been increased it would show a 2 exponential increase).

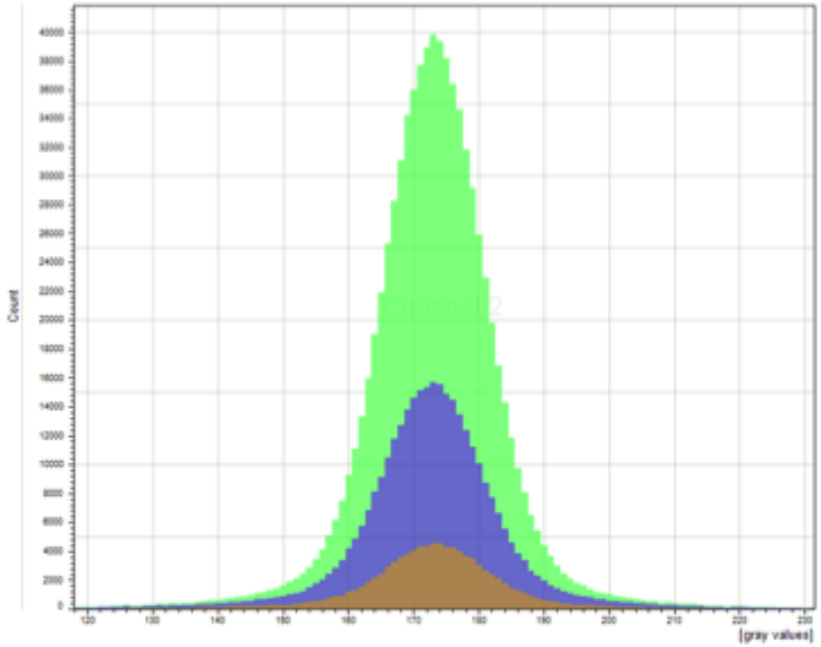


Figure S5: Gaussian distribution plot of recorded intensity count of a unified stained cell standardising the average contrast transfer function (CTF) intensities to the maximum brightness of the original simultaneous loading experiment (173). Original simultaneous loading experiment of **[Gd.L^5]** (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 10 mins) (green 40000 counts) and comparing it to the post-glutamate wash (orange 4080 counts, 10.2%) and post-aspartate wash (purple 15200 counts, 38 %)).

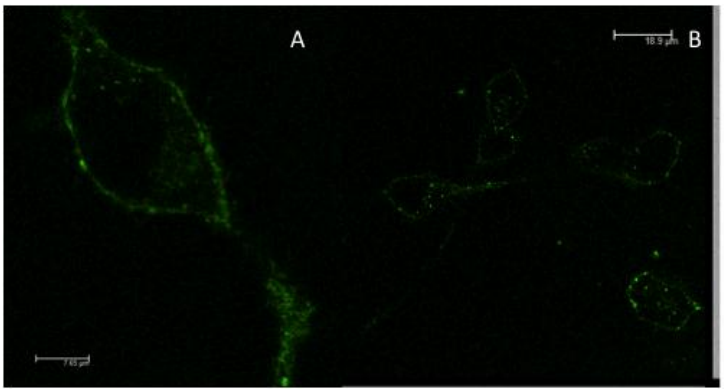


Figure S6: Live cell LSCM images of differentiated NSC-34 cells and visualization of AvidinAlexaFluor® 488 conjugate $\lambda_{ex}/\lambda_{em}$ = 488/505-555 nm. (A) Original simultaneous loading of **[Gd.L^5]** (10 μM), and AvidinAlexaFluor® 488 conjugate (2.5 μM, 10 mins), showing cell surface localization; (B) Simultaneous incubation with a solution of **[Gd.L^5]** (10 μM) and AvidinAlexaFluor® 488 conjugate (2.5 μM) for 10 minutes. Then washing the cells with 5 volumetric aliquots of an aspartate rich (1 mM) culture media, showing only 38% original fluorescence intensity from the original cell staining experiment.

References

1. C.-W. Lee, H. Cao, K. Ichiyama and T. M. Rana, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 4243-4246.
2. P. Bakó, T. Novák, K. Ludányi, B. Pete, T. László and G. Keglevich *, *Tetrahedron: Asymmetry*, 1999, **10**, 2373-2380.
3. A. M. Nyström and K. L. Wooley, *Tetrahedron*, 2008, **64**, 8543-8552.
4. S. M. Vibhute, J. Engelmann, T. Verbic, M. E. Maier, N. K. Logothetis and G. Angelovski, *Org. Biomol. Chem.*, 2013, **11**, 1294-1305.
5. A. Eisenführ, P. S. Arora, G. Sengle, L. R. Takaoka, J. S. Nowick and M. Famulok, *Bioorg. Med. Chem.*, 2003, **11**, 235-249.
6. C. J. Eggett, S. Crosier, P. Manning, M. R. Cookson, F. M. Menzies, C. J. McNeil and P. J. Shaw, *J. Neurochem.*, 2000, **74**, 1895-1902.
7. J. Yu, D. Parker, R. Pal, R. A. Poole and M. J. Cann, *J. Am. Chem. Soc.*, 2006, **128**, 2294-2299.
8. E. J. New, A. Congreve and D. Parker, *Chem. Sci.*, 2010, **1**, 111-118.