Supplementary Information for:

Optical Control of Metabotropic Glutamate Receptors

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Supplementary Figure 1

**Figure S1.** Design of photoswitches for control of mGluR2: Apparent access channel and tether model screening. (a) Homology model of mGluR2 ligand binding domain in the closed, glutamate-bound, conformation (based on structure of mGluR3, PBD: 2E4U) reveals an access channel through which glutamate (red) is seen from the surface of the protein. The 4’ D hydrogen of glutamate (yellow) is visible through the access channel, but the L hydrogen (green) is not. (b, c) Stick representation of glutamate shows the directions in which 4’D and L substituents may project. The protein is transparent to illustrate that the orientation is the same as in A. While the 4’D position points away from the protein surface, the 4’L position points into the protein suggesting accessibility only of the former. (d, e) D-Tether models of differing lengths have distinct effects on mGluR2 activation of GIRK1 channels in HEK293 cells. (e) 1 mM D-Tether-0 activates mGluR2 (i.e. functions as an agonist), evoking GIRK1 current that is smaller (26 ± 5%, n=6) than that evoked by 1 mM glutamate. (e) 1 mM D-Tether-1 does not activate mGluR2, but co-application with 1 mM glutamate reduces the response (45 ± 1%, n=4) compared to glutamate alone, indicating antagonistic activity.
**Supplementary Figure 2**

**Figure S2.** Photo-antagonism of mGluR2. (a) Representative trace of currents in mGluR2-L300C after labeling with D-MAG-1. Similarly to LimGluR2-block (S302C) 380 nm light (violet bar) does not induce current in the absence of glutamate, but after application of 1 mM glutamate (black bar) induces a decrease in current amplitude. (b) Glutamate-concentration dependence of photoantagonism by LimGluR-block (n=6 cells). (c) Representative trace showing extent of photoblock by LimGluR2-block over a range of glutamate concentrations. After application of 10 mM Glutamate, photo-antagonism is reduced, indicating a competitive mechanism of antagonism.
Supplementary Figure 3

**Figure S3.** Photo-agonism of mGluR2. (a) Representative trace of currents induced by either 380 nm illumination or application of 1 mM glutamate in LimGluR2 during voltage ramps from -80 to +20 mV. Ramp currents were subtracted from baseline ramps taken in absence of illumination or glutamate. Currents show inward rectification typical of GIRK current. Inset shows close overlay of normalized traces. (b) When glutamate is applied after 380 nm
illumination (violet bar), a further increase in inward current is seen, indicating that D-MAG-0 functions as a full agonist and does not occlude glutamate activation. Green bars indicate 500 nm illumination. (c) Application of glutamate at a range of concentrations followed by photoswitching indicates that D-MAG-0 never functions as an antagonist. At sub-saturating concentrations, MAG increases inward current further indicating that it functions as a full agonist. (d) Glutamate titration curves for mGluR2 and LimGluR2 indicate a minor decrease in glutamate affinity for LimGluR2. Titration curves for individual cells were fit and EC$_{50}$ and $n_h$ values were averaged. (n=6 cells each). (e) LimGluR2 maintains sensitivity to L-CCG-1, a commonly used group II mGluR agonist. (f) LimGluR2 maintains sensitivity to LY341495, a commonly used group II mGluR competitive antagonist.
Figure S4. Characterization of LimGluR2 bistability and the kinetics of its activation and deactivation of GIRK. (a) Representative trace showing that activation of LimGluR2 using a 250 ms pulse of illumination at 380 nm (15 mw/mm²) and deactivation by a 1 s pulse at 500 nm (20 mw/mm²) produce GIRK currents of similar amplitude and kinetics (of activation desensitization and deactivation) as does switching illumination between the wavelengths for extended times. (b) Summary comparison of GIRK activation kinetics evoked by short pulses of illumination (250 ms at 380 nm) versus long exposures (≥ 5 s at 380 nm). (c) Summary comparison of GIRK deactivation kinetics evoked by short pulses of illumination (1 s at 500 nm) versus long exposures (≥ 10 s at 500 nm). (b, c) Lines show values for individual cells and bars show averages.
Supplementary Figure 5

Figure S5. Comparison between the activation of GIRK1 channels by optical activation of rat rhodopsin (RO4) and LimGluR2 in HEK293 cells. (a, b) Representative currents in response to light pulses for RO4 (a) and LimGluR2 (b) in cells co-expressing GIRK1. (b) Summary of on and off kinetics for GIRK1 currents evoked by optical activation of RO4 and LimGluR2. The time to 90% off was significantly longer in RO4 (unpaired, 1-tailed t test, p=4x10⁻⁵). (d, e) Representative currents in response to repetitive optical activation (bouts of 10 s activation, followed by 90 s deactivation) of RO4 (d) and LimGluR2 (e). (f) Summary of peak current amplitudes elicited by repeated optical activation of RO4 and LimGluR2. A significant rundown of photo-current amplitude was seen for RO4 but not LimGluR2 (* indicates p=0.016 for paired, 1-tailed t test)
Supplementary Figure 6

**Figure S6.** LimGluR2 mediated control of neuronal excitability. (a) Representative trace of photo-current induced by illumination at 380 nm (violet bar) and extinguished by illumination at 500 nm (green bar) in a whole cell voltage-clamped neuron in 60 mM [K⁺]o extracellular solution. (b) Representative trace showing many rounds of repetitive suppression of spiking activity by photo-activation of LimGluR2.
Supplementary Figure 7

**Figure S7.** LimGluR2 inhibition of autaptic synaptic transmission. (A)-(C), EPSC kinetics (A: decay time; B: time to peak, C: normalized overlay) are constant despite decrease in amplitude induced by optical activation of LimGluR2. (A) EPSC decay time constant unchanged in 500 nm vs. 380 nm illumination (paired, 2-tailed t-test, p=0.91; n=15 sweeps/condition). (B) Time to peak (time from peak of pre-synaptic spike to peak of EPSC) and jitter (S.E.M. of time to peak) are unchanged in 500 nm vs. 380 nm illumination (paired, 2-tailed t-test, p=0.74; n=15 sweeps/condition). (C) Overlay of normalized average of 15 sweeps for both 380 nm and 500 nm illumination indicates no significant change in timing or shape of EPSC. Note, EPSC amplitude was reduced by 40% in this cell under 380 nm illumination. (d) Representative behavior of autapse during 25 Hz stimulation under illumination with either 500 nm light (green bar) or 380 nm light (violet bar). Each trace is an average of 8 trains. (e, f) Average EPSC amplitude (e) or normalized amplitude (f) during a 25 Hz train under 380 or 500 nm illumination. (g) Summary of bistable inhibition in n=4 cells under the same protocol as (d): 2 minutes at 500 nm followed by 1 second of illumination at 380 nm (violet arrow) and 3 minutes in the dark before returning to 500 nm illumination for 2 more minutes.
Supplementary Figure 8

Figure S8. LimGluR2-block mediated modulation of excitability and transmission. (a) Representative trace showing photoswitching of LimGluR2-block in a current-clamped neuron. Blockade of mGluR2 under 380 nm light increased firing frequency and was reversed by 500 nm illumination. (b) Summary of firing frequency modulation in a representative cell. Firing frequency was determined for each round of photoswitching (5-20 s per photoswitch) over a 10 minute recording. (paired, 2-tailed t-test, p=0.028). (c) Representative EPSCs in response to 380 nm (violet trace) or 500 nm illumination (green trace) in autaptic neurons expressing LimGluR2-block. Each trace is an average of 12 sweeps for each illumination condition. (d) Summary of LimGluR2-block enhancement of EPSC amplitude. Each line represents a single cell and the violet point indicates the amplitude in 380 nm light and the green point indicates the amplitude in 500 nm light. All values were normalized to the amplitude in 500 nm. (e) Representative IPSCs in response to 380 nm (violet trace) or 500 nm illumination (green trace) in autaptic neurons expressing LimGluR2-block. Each trace is an average of 12 sweeps for each illumination condition. (f) Summary of LimGluR2-block enhancement of IPSC amplitude. IPSCs were unaffected by LimGluR2-block.
**Supplementary Figure 9**

**Figure S9.** Hippocampal slice controls indicate that LimGluR2 does not harm cell health and is orthogonal. (a) Average resting potential is not altered in cells expressing LimGluR2 and labeled with D-MAG-0 relative to cells with D-MAG-0 but without mGluR2-300C or unlabeled mGluR2-300C expressing cells. (b) No photoswitching was seen in tdTomato-transfected cells labeled with D-MAG-0 or (c) cells expressing LimGluR2 (mGluR2-300C) but not labeled with D-MAG-0. (d) tdTomato-expressing cells labeled with D-MAG-0 show no change in spike firing in response to current injection when illuminated with 380 nm or 500 nm.
Figure S10. Pan-neuronal expression of LimGluR2 and labeling with D-MAG-0 do not modify basal activity levels and escape response threshold. (a) Schematic of system used for measurement of swimming behavior and escape response in 48 well plates for different genetic lines with or without D-MAG-0 labeling (see Online Methods for details). (b)
Representative image of individual zebrafish in wells of a 48-well plate during tracking of swimming. (c,d) Representative tracks of single zebrafish larvae with either GAL4 pan-neuronal driver driving Kaede fluorescent protein alone (elavl3:gal4; UAS:kaede control) (a) or also containing LimGluR2 (elavl3:gal4; UAS:kaede; UAS:LimGluR2) (b). (Tracking performed with an in-house written Matlab script.) (e) Total distance travelled at 5-6 dpf by elavl3:gal4; UAS:kaede control (n = 24) or elavl3:gal4; UAS:kaede; UAS:LimGluR2 (n = 24). Larvae were imaged for 25 minutes under infrared illumination at 30fps in 48-well microplates. Total distance swum was measured by the tracking of the centroid of each fish for the duration of the recording. (f) Sound stimulus intensity-probability of escape curve is not affected by LimGluR2 expression. The escape threshold of 5-6 dpf zebrafish larvae was determined by administering a randomized sequence of 120 sound stimuli at 10 different voltage levels, with a 30 second inter-stimulus interval (ISI) for elavl3:gal4; UAS:kaede control and elavl3:gal4; UAS:kaede; UAS:LimGluR2. Stimuli duration and frequency were 20ms and 900Hz, respectively. Experiment performed in a 48-well microplate, under infrared illumination and recorded at 30fps. Prior to testing larvae were acclimated for 30 minutes. Escapes were automatically determined by subtraction and thresholding of the first two frames after the stimulus. Accuracy of the detection method was verified by visual inspection of movies. (g) Sound stimulus intensity-probability of escape curve is not affected by D-MAG-0 treatment. 1181:gal4; UAS:kaede 5-6dpf zebrafish larvae were treated with D-MAG-0 or control medium. Curve was determined as described above for (f). (h) Labeling of elav3:gal4; UAS:kaede; UAS:LimGluR2 fish with D-MAG-0 does not modify ASR threshold before receptor activation with 380 nm light. Individual head-mounted fish, D-MAG-0-labeled or controls, were exposed to stimuli of increasing amplitude stimuli with an ISI of 10 seconds. The threshold was defined as the lowest sound able to initiate an escape response in >50% of trials. Graph shows the initial threshold of individual fish in the two groups.
Supplementary Table 1

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<td>x</td>
<td>x</td>
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<tr>
<td>Q42C</td>
<td>x</td>
<td>Antagonist (9±4%; n=4)</td>
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Table S1. Cysteine screen of mGluR2. Results of photoswitching of D-MAG-0 and D-MAG-1 attached at each of the 7 positions tested. “x” indicates to photoresponse. Agonistic and antagonistic effects are quantified (mean ± s.e.m) relative to 1mM glutamate. Data from ≥2 different coverslips for all conditions tested.
**Chemical Synthesis of D-MAG-0 and D-MAG-1**

Unless stated otherwise, all reactions were performed in oven- or flame-dried glassware under a positive pressure of nitrogen or argon using dry solvents. Commercial reagents and dry solvents were used as received with the following exceptions: Tetrahydrofuran (THF) was distilled from benzophenone/sodium prior to use. Diisopropylethylamine (DIPEA) was distilled over calcium hydride immediately before use. Solvents for chromatography were obtained as technical grade and distilled prior to use under reduced pressure. Reactions were magnetically stirred and monitored by NMR spectroscopy or analytical thin-layer chromatography (TLC) using E. Merck 0.25 mm silica gel 60 F254 precoated glass plates. TLC plates were visualized by exposure to ultraviolet light (UV, 254 nm) and/or exposure to an aqueous solution of ceric ammoniummolybdate (CAM), an aqueous solution of potassium permanganate (KMnO$_4$), an acidic solution of vanillin or a solution of ninhydrin in ethanol followed by heating with a heat gun. Flash column chromatography was performed using silica gel (60 Å, 40-63 μm, Merck) and a forced flow of eluant at 1.3–1.5 bar pressure. Yields refer to chromatographically and spectroscopically ($^1$H and $^{13}$C NMR) pure material.

**Instrumentation**

Nuclear magnetic resonance (NMR) spectra were recorded on Varian VNMRS 300, VNMRS 400, INOVA 400 or VNMRS 600 spectrometers. Proton chemical shifts ($^1$H) are expressed in parts per million (δ scale) and are calibrated using the residual undeuterated solvent as an internal reference (CHCl$_3$: δ 7.26, DMSO-$d_6$: δ 2.50). Data for $^1$H NMR spectra are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant, integration). Multiplicities are reported as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, app = apparent, or combinations thereof. Carbon chemical shifts ($^{13}$C) are expressed in parts per million (δ scale) and are referenced to the carbon resonances of the solvent (CDCl$_3$: δ 77.16, DMSO-$d_6$: δ 39.52). Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum BX II...
(FTIR System) and intensities are denoted as follows: w = weak, m = medium, s = strong, br = broad. IR data is reported in frequency of absorption (cm\(^{-1}\)). Mass spectroscopy (MS) experiments were performed on a Thermo Finnigan LTQ FT (ESI) instrument. Optical rotation at the sodium D line (589 nm) was determined on a Krüss polarimeter P8000T at 25 °C and is reported as \(10^{-1} \cdot \text{deg} \cdot \text{cm}^2 \cdot \text{g}^{-1}\). UV spectra (UV) were recorded on a Varian Cary 50 Scan UV spectrometer. Melting points were determined with a Stanford Research Systems MPA120 apparatus and are uncorrected.
Figure 1. Stereoselective synthesis of DMAG0 starting from L-glutamic acid (S1).
Experimental Procedures and Product Characterization

(S)-dimethyl 2-(tert-butoxycarbonylamino) pentanedioate (S2)

\[
\text{MeOOC} - \text{COOMe} \quad \text{NHBoc} \\
\text{S2}
\]

(S)-dimethyl 2-(tert-butoxycarbonylamino)pentanedioate (S2) was synthesized according to [1].

(2S,4S)-dimethyl 2-allyl-4-(tert-butoxycarbonylamino) pentanedioate (S3)

\[
\text{MeOOC} - \text{COOMe} \quad \text{NHBoc} \\
\text{S3}
\]

(2S,4S)-dimethyl 2-allyl-4-(tert-butoxycarbonylamino)pentanedioate (S3) was synthesized according to [2].

(5S,7S)-7-(tert-butoxycarbonylamino)-8-methoxy-5-(methoxycarbonyl)-8-oxo-octanoic acid (S4)

\[
\text{HOOC} - \text{COOMe} \quad \text{NHBoc} \\
\text{S4}
\]

To a solution of (2S,4S)-dimethyl 2-allyl-4-(tert-butoxycarbonylamino) pentanedioate (1.50 g, 4.76 mmol) and freshly distilled acrylic acid (0.46 mL, 6.66 mmol) in degassed CH2Cl2 (25 mL) was added solid Grubbs II catalyst (238 mg, 0.238 mmol) and the mixture was heated under reflux for 4 h. The reaction was allowed to cool to room temperature and was passed over a plug of celite eluting with ethyl acetate. After removal of the solvent under
reduced pressure, the tan residue was dissolved in ethyl acetate (100 mL) and Pd/C (10% Pd; 507 mg, 0.476 mmol) was added. The vessel was purged with H2 and the reaction was stirred overnight at room temperature. The mixture was filtered over a plug of celite eluting with ethyl acetate and the solvent was removed under reduced pressure. Normal phase column chromatography (SiO2; CH2Cl2:ethyl acetate:AcOH = 8:2:1% to 6:4:1%) yielded 1.40 g (81%, 2 steps) of (5S,7S)-7-(tert-butoxycarbonylamino)-8-methoxy-5-(methoxycarbonyl)-8-oxo-octanoic acid (S4) as a tan oil. [α]D = +8.8 (c 1.0, CH2Cl2); IR (ATR): ν = 2954 (w), 1708 (s), 1515 (m), 1436 (m), 1367 (m), 1212 (m), 1157 (s), 1052 (m), 1023 (w) cm⁻¹; 1H NMR (CDCl3, 300 MHz) δ 1.43 (s, 9H), 1.62 (br m, 4H), 1.99 (m, 2H), 2.34 (t, 3J = 6.7 Hz, 2H), 2.49 (m, 1H), 3.68 (s, 3H), 3.72 (s, 3H), 4.34 (m, 1H), 4.99 (d, 3J = 8.6 Hz, 1H) ppm; 13C NMR (CDCl3, 75.5 MHz) δ 178.4, 175.9, 172.9, 155.5, 80.3, 52.6, 52.3, 52.0, 42.0, 34.6, 33.7, 31.7, 28.4, 22.2 ppm; HRMS (ESI) – Calc. for C16H27NNaO₈ [M+Na]+: 384.1634. Found: 384.1626.

(2S,4S)-dimethyl 2-(4-(4-((E)-(4-aminophenyl)diazenyl)phenylamino)-4-oxobutyl)-4-(tert-butoxycarbonylamino)pentanedioate (S5)

![Chemical Structure](image)

To a solution of (5S,7S)-7-(tert-butoxycarbonylamino)-8-methoxy-5-(methoxy carbonyl)-8-oxo-octanoic acid (1.20 g, 3.32 mmol) in DMF (150 mL) was added 4,4′-azo dianiline (1.41 g, 6.64 mmol), DIPEA (2.30 mL, 13.3 mmol) and HBTU (1.39 g, 3.65 mmol) and the mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate (500 mL) and a saturated aqueous
solution of NaHCO$_3$ (200 mL). The organic phase was washed with saturated aqueous NaHCO$_3$ (200 mL), water (200 mL) and brine (200 mL), dried over MgSO$_4$, filtered and concentrated. Normal phase column chromatography (SiO$_2$, CH$_2$Cl$_2$:ethyl acetate = 4:1 to 3:2) afforded 1.23 g (67%) of (2S,4S)-dimethyl 2-(4-(4-((E)-(4-aminophenyl)diazenyl)phenylamino)-4-oxobutyl)-4-(tert-butoxycarbonylamino)pentanedioate (S5) as an orange solid. mp (ethyl acetate) = 82.8-88.5 °C; $[\alpha]_D = +32.0$ (c 0.1, CH$_2$Cl$_2$); UV: $\lambda_{\text{max}} = 384$ nm; IR (ATR): $\tilde{\nu} = 3356$ (w), 2950 (w), 1686 (m), 1625 (m), 1594 (s), 1527 (s), 1505 (s), 1424 (m), 1392 (m), 1366 (m), 1297 (m), 1241 (s), 1150 (s), 1139 (s) cm$^{-1}$; $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ (trans isomer) 1.46 (s, 9H), 1.67-1.84 (br m, 4H), 1.97 (m, 2H), 2.36 (m, 2H), 2.54 (m, 1H), 3.69 (s, 3H), 3.72 (s, 3H), 4.08 (br s, 2H), 4.35 (m, 1H), 5.12 (d, $^3 J = 9.0$ Hz, 1H), 6.73 (d, $^3 J = 8.9$ Hz, 2H), 7.70 (br d, $^3 J = 8.8$ Hz, 2H), 7.78 (d, $^3 J = 8.8$ Hz, 2H), 7.82 (d, $^3 J = 8.9$ Hz, 2H), 8.14 (br. m, 1H) ppm; $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ (trans isomer) 176.0, 172.7, 171.2, 155.9, 149.5, 149.3, 145.7, 139.9, 125.1, 123.4, 119.9, 114.8, 80.6, 52.7, 52.1, 52.0, 41.3, 36.7, 35.5, 30.9, 28.5, 23.1 ppm; HRMS (ESI) – Calc. for C$_{28}$H$_{37}$N$_5$NaO$_7$ [M+Na]$^+$: 578.2591. Found: 578.2584.

(2S,4S)-dimethyl 2-(4-(4-((E)-(4-aminacetamido)phenyl)diazenyl)phenylamino)oxobutyl)-4-(tert-butoxycarbonylamino)pentanedioate (S6)

To a solution of Fmoc glycine (1.31 g, 4.39 mmol) in THF (20 mL) was added oxalyl chloride (2.31 mL; 4.61 mmol; 1M in CH$_2$Cl$_2$) and one drop of DMF and the mixture was stirred for 45 min at 0 °C. The solvent was distilled off with a short distillation bridge under reduced pressure. The residue was dissolved in THF (20 mL) and added to a solution of (2S,4S)-dimethyl 2-(4-(4-((E)-(4-aminophenyl)diazenyl)phenylamino)-4-oxobutyl)-4-(tert-
butoxycarbonylamino)pentanedioate (1.22 g, 2.20 mmol), DIPEA (1.52 mL, 8.78 mmol) and DMAP (26.8 mg, 0.220 mmol) in THF at 0 °C. The mixture was stirred for 1 h at 0 °C and then for 1 h at room temperature. The reaction was diluted with ethyl acetate (500 mL) and washed successively with a saturated aqueous solution of NH₄Cl (200 mL), water (200 mL) and brine (200 mL), dried over MgSO₄, filtered and concentrated. The residue was dissolved in DMF (200 mL) and piperidine (1.30 mL, 13.2 mmol) was added. The mixture was stirred overnight at room temperature and then concentrated under reduced pressure. The residue was partitioned between a saturated aqueous solution of NaHCO₃ (300 mL) and ethyl acetate. The organic phase was washed with saturated aqueous NaHCO₃ (2 x 100 mL), water (100 mL) and brine (100 mL), dried over Na₂SO₄, filtered and concentrated. The product was purified using normal phase column chromatography (SiO₂, CH₂Cl₂:MeOH:acetic acid:water = 90:10:1%:1% to 80:20:1%:1%). The product containing fractions were washed with a saturated aqueous solution of NaHCO₃ (200 mL), dried over Na₂SO₄, filtered and concentrated to yield 803 mg (60%, 2 steps) of (2S,4S)-dimethyl 2-(4-(4-(((E)-(4-(2-aminoacetamido)phenyl)diazenyl)phenyl amino)-4-oxobutyl)-4-(tert-butoxycarbonylamino)pentanedioate (S6) as a red solid. mp (CH₂Cl₂/MeOH) = 146-156 °C; [α]D = +30.0 (c 0.1, CH₂Cl₂); UV: λmax = 368 nm; IR (ATR): ν = 3282 (w), 2920 (m), 2850 (m), 1682 (m), 1591 (m), 1528 (s), 1500 (m), 1434 (m), 1409 (m), 1366 (m), 1299 (m), 1249 (s), 1153 (s) cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ (trans isomer) 1.46 (s, 9H), 1.61-1.80 (br m, 6H), 1.92-2.01 (m, 2H), 2.35-2.41 (m, 2H), 2.50-2.57 (m, 1H), 3.51 (s, 2H), 3.69 (s, 3H), 3.72 (s, 3H), 4.36 (ddd, ²J = 14.0 Hz, ³J = 9.0 Hz, ⁴J = 4.8 Hz, 1H), 5.13 (d, ³J = 9.0 Hz, 1H), 7.70-7.78 (m, 4H), 7.85-7.97 (m, 4H), 8.27 (s, 1H), 9.64 (s, 1H) ppm; ¹³C NMR (CDCl₃, 100 MHz) δ (trans isomer) 176.0, 172.7, 171.3, 171.1, 155.9, 149.2, 149.0, 140.0, 129.3, 124.0, 123.9, 119.8, 119.6, 80.6, 52.7, 52.1, 52.0, 45.3, 41.3, 36.7, 35.5, 30.9, 28.5, 23.1 ppm; HRMS (ESI) – Calc. for C₃₀H₄₁N₆O₈ [M+H]⁺: 613.2986. Found: 613.2975.
(2S,4S)-2-(4-(4-((E)-(4-(2-aminoacetamido)phenyl)diazenyl)phenylamino)-4-oxo butyl)-4-(tert-butoxycarbonylamino)pentanedioic acid (S7)

To a solution of (2S,4S)-dimethyl 2-(4-(4-((E)-(4-(2-aminoacetamido)phenyl)diazenyl)phenylamino)-4-oxobutyl)-4-(tert-butoxycarbonylamino)pentanedioate (760 mg, 1.24 mmol) in THF/water (100 mL; 2:1) was added LiOH (743 mg, 31.0 mmol) at 0 ºC and the reaction was stirred for 1 h. The mixture was neutralized by addition of formic acid (1.17 mL, 31.0 mmol) and THF was removed under reduced pressure. The residual aqueous solution was added on top of a RP18 column, washed with water/formic acid (100 mL, 0.1% formic acid) and the crude product was purified by reversed phase column chromatography (C18; water:MeCN:formic acid = 99.9:0.1 to 74.9:25:0.1) to yield 529 mg (73 %) of (2S,4S)-2-(4-(4-((E)-(4-(2-aminoacetamido)phenyl)diazenyl)phenyl amino)-4-oxo-butyl)-4-(tert-butoxycarbonyl amino)pentanedioic acid (S7) as an orange solid. mp (water/MeCN) > 250 ºC (decomp. over 180 ºC indicated by loss of color); [α]D = -14.8 (c 0.1, acetonitrile:water = 9:1); UV: λmax = 369 nm; IR (ATR): ν = 2929 (w), 2362 (m), 2337 (m), 1662 (m), 1592 (m), 1497 (m), 1301 (m), 1248 (m), 1153 (m) cm⁻¹; ¹H NMR (DMSO-d₆, 400 MHz) δ (trans isomer) 1.36 (s, 9H), 1.49-1.65 (br m, 4H), 1.81-1.66 (br m, 2H), 2.30-2.43 (m, 3H), 3.80 (s, 2H), 3.91 (dd, 3J = 8.0 Hz, 3J = 8.00 Hz, 1H), 6.63 (d, 3J = 7.9 Hz, 1H), 6.63-7.93 (br m, 7H), 7.78-7.84 (m, 2H), 8.27 (br s, 3H), 10.28 (s, 1H) ppm; ¹³C
NMR (DMSO-\(d_6\), 100 MHz) \(\delta\) (trans isomer) 176.4, 174.3, 171.5, 164.2, 155.1, 148.0, 147.5, 142.2, 140.8, 123.5, 123.4, 119.5, 119.3, 77.9, 52.2, 41.7, 36.6, 34.5, 33.5, 30.9, 28.2, 23.0 ppm; HRMS (ESI) – Calc. for C\(_{28}\)H\(_{37}\)N\(_6\)O\(_8\) [M+H]\(^+\): 585.2673. Found: 585.2665.

\((2S,4S)-2\text{-amino-4-}(4\text{-}(4\text{-}(E)\text{-}(4\text{-}(2,5\text{-dioxo-2,5-dihydro-1H-pyrrol-1-yl})acetamido)phenyl)diazenyl)phenylamino)-4-oxobutyl)pentanedioic acid hydrochloride (DMAG0)

\[
\begin{align*}
\text{HOOC} & \quad \text{COOH} \\
\text{NH}_2 & \cdot \text{HCl}
\end{align*}
\]

\text{DMAG0}

To a solution of \((2S,4S)-2\text{-}(4\text{-}(4\text{-}(E)\text{-}(4\text{-}(2\text{-aminoacetamido)phenyl})diazenyl)phenylamino)-4-oxo butyl)\text{-}4\text{-}(tert-butoxycarbonylamino)pentanedioic acid (50.0 mg, 42.8 \mu\text{mol}) in saturated aqueous \text{NaHCO}_3 (7 mL) was added finely ground \text{N}-\text{methoxycarbonylmaleimide} (26.5 mg, 117 \mu\text{mol}) under vigorous stirring at 0 °C. The mixture was stirred for 30 min and then diluted with \text{THF} (7 mL). The ice bath was removed for 10 min. The solution was re-cooled to 0 °C, acidified to pH 1-2 with an aqueous solution of 1.0 M \text{H}_2\text{SO}_4 and extracted with ethyl acetate (2 x 25 mL). The combined organic extratcs were dried over \text{Na}_2\text{SO}_4, filtered and concentrated. Normal phase column chromatography (SiO\(_2\), \text{CH}_2\text{Cl}_2:MeOH:acetic acid:water = 90:10:0.6%:0.6%) yielded the maleimide adduct as an orange solid which was directly treated with a saturated solution of \text{HCl} in ethyl acetate (20 mL). After stirring for 2 h at room temperature, the resulting purple suspension was diluted with diethyl ether (30 mL) and the solid was collected by sedimentation using a centrifuge. The product was resuspended in diethyl ether (20 mL) and sedimented again. Removal of the solvent afforded 40.0 mg
(70%, 2 steps) of (2S,4S)-2-amino-4-(4-(4-((E)-(4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido) phenyl)diazenyl) phenyl-amino)-4-oxobutyl)pentanedioic acid hydrochloride (DMAG0) as a purple solid. mp (diethyl ether) > 250 °C (decomp. over 180 °C indicated by loss of color); [α]D = +81.8 (c 0.1, aqueous phosphate buffer pH = 7.4, 0.1% DMSO); UV: λmax = 362 nm; IR (ATR): ν = 2916 (w), 1710 (s), 1592 (s), 1535 (s), 1499 (m), 1428 (m), 1412 (m), 1300 (m), 1250 (s), 1151 (s) cm⁻¹; ¹H NMR (DMSO-d₆, 600 MHz) δ 1.55-1.65 (br m, 4H), 1.86 (ddd, ²J = 14.3 Hz, ³J = 7.9 Hz, ³J = 6.1 Hz, 1H), 2.07 (ddd, ²J = 14.3 Hz, ³J = 8.5 Hz, ³J = 6.9 Hz, 1H), 2.40 (br m, 2H), 2.61 (m, 1H), 3.80 (br s, 1H), 4.34 (s, 2H), 7.16 (s, 2H), 7.72-7.80 (m, 2H), 7.80-7.92 (m, 6H), 8.44 (br s, 3H), 10.42 (s, 1H), 10.83 (s, 1H) ppm; ¹³C NMR (DMSO-d₆, 100 MHz) δ 175.5, 171.4, 170.7, 170.6, 165.3, 147.8, 147.5, 142.1, 141.1, 135.0, 123.4 (2 C), 119.4, 119.2, 50.3, 40.5, 40.3, 36.2, 31.7, 31.1, 22.2 ppm; HRMS (ESI) – Calc. for C₂₇H₂₉N₆O₈ [M+H]⁺: 565.2047. Found: 565.2039.

(2S,4S)-dimethyl 2-(tert-butoxycarbonylamino)-4-(4-oxo-4-(phenylamino)butyl)
pentanedioate (S8)

To a solution of (5S,7S)-7-(tert-butoxycarbonylamino)-8-methoxy-5-(methoxy carbonyl)-8-oxo-octanoic acid (50 mg, 0.138 mmol) in DMF (5 mL) was added HBTU (56 mg, 152 µmol), DIPEA (48 µL, 277 µmol) and aniline (16 mg, 166 µmol). The solution was stirred overnight at room temperature. Ethyl acetate (20 mL) and saturated aqueous NaHCO₃ solution (30 mL) were added and the mixture was extracted with ethyl acetate (4 x 20 mL). The combined organic extracts were washed with 10% aqueous NaCl solution (2 x 20 mL)
and brine. The organic phase was dried over MgSO₄, filtered and concentrated. Normal phase column chromatography (SiO₂, CH₂Cl₂, 3% MeOH) yielded 42 mg (97%) of (2S,4S)-dimethyl 2-(tert-butoxycarbonylamino)-4-(4-oxo-4-(phenylamino)butyl) pentanedioate (S8) as a colorless oil. [α]D = -16.6 (c 1, CH₂Cl₂); IR (ATR): ν = 3318 (w), 2951 (w), 1731 (s), 1715 (s), 1690 (s), 1668 (s), 1599 (s), 1538 (s), 1499 (s), 1441 (s), 1366 (m), 1159 (s) cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 1.45 (s, 9H), 1.58-1.83 (br m, 4H), 1.96 (m, 2H), 2.33 (m, 2H), 2.52 (m, 1H), 3.68 (s, 3H), 3.71 (s, 3H), 4.34 (m, 1H), 5.08 (d, ²J = 8.8 Hz, 1H), 7.08 (t, ³J = 7.4 Hz, 1H), 7.30 (app t, ³J = 7.9 Hz, 2H), 7.40 (d, ³J = 7.9 Hz, 2H), 7.91 (br s, 1H) ppm; ¹³C NMR (CDCl₃, 100 MHz) δ 176.0, 172.8, 171.1, 155.8, 136.3, 129.1, 124.2, 120.0, 80.5, 52.6, 52.1, 41.4, 38.8, 36.7, 35.4, 31.1, 28.5, 23.1 ppm; HRMS (ESI) – Calc. for C₂₂H₃₂N₂NaO₇ [M+Na]⁺: 459.2107. Found: 459.2100.

(2S,4S)-2-amino-4-(4-oxo-4-(phenylamino)butyl)pentanedioic acid hydro-chloride (DTM0)

To a solution of (2S,4S)-dimethyl 2-(tert-butoxycarbonylamino)-4-(4-oxo-4-(phenylamino)butyl) pentanedioate (30 mg, 69 µmol) in THF/water (1:1, 6 mL) was added LiOH (66 mg, 2.7 mmol) at 0 ºC and the mixture was stirred for 1 h. The mixture was neutralized by addition of formic acid (104 µL, 2.7 mmol) and THF was removed under reduced pressure. The crude product was placed on a RP18 column and washed with H₂O/0.1% formic acid. Reversed phase column chromatography (C18; H₂O:MeOH:formic acid = 90:10:0.1% to 60:40:0.1%) yielded Boc-protected DTM0 as a colorless solid, which
was used directly in the next step. The obtained diacid was treated with HCl-saturated ethyl acetate (10 mL) and stirred for 2 h at room temperature. The suspension was treated with diethyl ether (50 mL) and the product was collected by sedimentation using a centrifuge. The residual solvent was removed under reduced pressure to yield 11 mg (46 %, 2 steps) of (2S,4S)-2-amino-4-(4-oxo-4-(phenylamino)butyl)pentanedioic acid hydrochloride (DTM0) as a colorless, hygroscopic solid. mp (diethyl ether) > 250 °C (decomp. over 200 °C); \( [\alpha]_D = +68.0 \) (c 0.1, aqueous phosphate buffer pH = 7.4); IR (ATR): \( \tilde{\nu} = 2924 \) (w), 1701 (m), 1656 (m), 1595 (s), 1539 (s), 1498 (s), 1442 (s), 1311 (w), 1199 (s), 1154 (m) cm\(^{-1} \); \(^1\)H NMR (DMSO-\( d_6 \), 600 MHz) \( \delta \) 1.48-1.62 (br s, 4H), 1.83 (m, 1H), 2.00 (m, 1H), 2.32 (t, \( ^3J = 6.9 \) Hz, 2H), 2.60 (m, 1H), 2.73 (app t, \( J = 7.5 \) Hz, 1H), 7.01 (m, 1H), 7.27 (m, 2H), 7.59 (m, 2H), 8.34 (br s, 2H), 9.97 (s, 1H) ppm; \(^{13}\)C NMR (DMSO-\( d_6 \), 150 MHz) \( \delta \) 175.5, 170.9, 170.6, 139.3, 128.6, 122.9, 119.0, 50.5, 40.5, 36.1, 32.0, 31.1, 22.4 ppm; HRMS (ESI) – Calc. for C\(_{15}\)H\(_{20}\)N\(_2\)NaO\(_5\) [M+Na]\(^+\): 331.1270. Found: 331.1264.

\( (2S,4S)\)-dimethyl 2-(\( tert \)-butoxycarbonylamino)-4-(4-oxo-4-(2-oxo-2-(phenylamino)ethylamino)butyl)pentanedioate (S9)

![Image of the molecule](image)

To a solution of (5S,7S)-7-(\( tert \)-butoxycarbonylamino)-8-methoxy-5-(methoxy carbonyl)-8-oxo-octanoic acid (50 mg, 138 µmol) in DMF (5 mL) was added HBTU (58 mg, 152 µmol), DIPEA (48 µL, 277 µmol) and 2-amino-N-phenylacetamide hydrochloride (28 mg, 152 µmol). The solution was stirred overnight at room temperature. Saturated aqueous NaHCO\(_3\) solution (30 mL) was added and the mixture extracted with ethyl acetate (3 x 20 mL). The
combined organic extracts were washed with a saturated aqueous NH₄Cl solution (1 x 30 mL), 10% aqueous NaCl solution (2 x 20 mL) and brine (20 mL). The organic phase was dried over MgSO₄, filtered and concentrated. Normal phase column chromatography (SiO₂, CH₂Cl₂, 3% MeOH) yielded 68 mg (99 %) of (2S,4S)-dimethyl 2-(tert-butoxycarbonylamino)-4-(4-oxo-4-(2-oxo-2-(phenylamino)ethylamino)butyl)pentanedioate (S9) as a colorless oil. [α]D = +3.2 (c 1, CH₂Cl₂); IR (ATR): \( \tilde{\nu} = 3296 \text{ (w)}, 2952 \text{ (w)}, 1731 \text{ (s)}, 1697 \text{ (s)}, 1652 \text{ (s)}, 1600 \text{ (m)}, 1526 \text{ (s)}, 1499 \text{ (s)}, 1443 \text{ (s)}, 1366 \text{ (m)}, 1249 \text{ (m)}, 1160 \text{ (s)} \text{ cm}^{-1} \);

\(^1\)H NMR (DMSO-\(d_6\), 600 MHz) \( \delta 1.42 \text{ (s, 9H)}, 1.55-1.74 \text{ (br m, 4H)}, 1.86 \text{ (m, 1H)}, 1.95 \text{ (m, 1H)}, 2.29 \text{ (t, }^{3}J = 6.7 \text{ Hz, 2H)}, 2.49 \text{ (m, 1H)}, 3.64 \text{ (s, 3H)}, 3.70 \text{ (s, 3H)}, 4.13 \text{ (m, 2H)}, 4.33 \text{ (m, 1H)}, 5.05 \text{ (d, }^{2}J = 8.9 \text{ Hz, 1H)}, 6.92 \text{ (br s, 1H)}, 7.09 \text{ (t, }^{3}J = 7.4 \text{ Hz, 1H)}, 7.30 \text{ (app t, } J = 7.9 \text{ Hz, 2H)}, 7.53 \text{ (d, }^{3}J = 7.5 \text{ Hz, 2H}) \text{ ppm}; \ ^{13}\text{C NMR (DMSO-}d_6\text{, 150 MHz)} \delta 175.9, 173.8, 172.8, 167.4, 155.7, 137.9, 129.1, 124.5, 120.0, 80.4, 52.6, 52.2, 52.0, 44.7, 41.8, 35.7, 34.9, 31.5, 28.4, 23.1 \text{ ppm}; \text{ HRMS (ESI) – Calc. for C}_{24}\text{H}_{35}\text{N}_{3}\text{NaO}_8 [\text{M+Na}]^+: 516.2322. \text{ Found: 516.2312.} \\

(2S,4S)-2-amino-4-(4-oxo-4-(2-oxo-2-(phenylamino)ethylamino)butyl)pentane-dioic acid hydrochloride (DTM1)

![DTM1 structure](image)

To a solution of (2S,4S)-dimethyl 2-(tert-butoxycarbonylamino)-4-(4-oxo-4-(2-oxo-2-(phenylamino)ethylamino)butyl)pentanedioate (40 mg, 81 µmol) in THF/water (1:1, 6 mL) was added LiOH (78 mg, 3.24 mmol) at 0 °C and the mixture was stirred for 1 h. The solution
was neutralized by addition of formic acid (122 µL, 3.24 mmol) and THF was removed under reduced pressure. The crude product was placed on a RP18 column and washed with H₂O/0.1% formic acid. Reversed phase column chromatography (C18; water:MeOH:formic acid = 90:10:0.1% to 60:40:0.1%) yielded Boc-protected DTM1 as a colorless solid which was used directly in the next step. The obtained diacid was treated with HCl-saturated ethyl acetate (10 mL) and stirred for 2 h at room temperature. The suspension was treated with diethyl ether (50 mL) and the product was collected by sedimentation using a centrifuge. Removal of the solvent afforded 24 mg (74 %, 2 steps) of (2S,4S)-2-amino-4-(4-oxo-4-(2-oxo-2-(phenylamino)ethylamino)butyl)pentane-dioic acid hydrochloride (DTM1) as a colorless, hygroscopic solid. mp (diethyl ether) > 250 ºC (decomp. over 210 ºC); [α]D = +62.4 (c 0.1, aqueous phosphate buffer pH = 7.4); IR (ATR): ν = 2929 (w), 1597 (m), 1542 (s), 1498 (s), 1445 (m), 1411 (m), 1312 (m), 1202 (s) cm⁻¹; ¹H NMR (DMSO-d₆, 600 MHz) δ 1.40-1.59 (br m, 4H), 1.82 (dt, J = 13.9 Hz, J = 6.9 Hz, 1H), 2.04 (app dt, J = 14.4 Hz, J = 7.1 Hz, 1H), 2.17 (br m, 2H), 2.59 (br m, 1H), 3.79 (app t, J = 7.1 Hz), 3.87 (d, J = 5.9 Hz, 2H), 7.02 (t, J = 7.4 Hz, 1H), 7.28 (app t, J = 7.9 Hz, 2H), 7.60 (d, J = 7.6 Hz, 2H), 8.25 (t, J = 5.8 Hz, 1H), 8.49 (br s, 3H), 10.15 (s, 1H) ppm; ¹³C NMR (DMSO-d₆, 100 MHz) δ 175.6, 172.4, 170.7, 168.0, 139.0, 123.2, 119.1, 50.3, 42.7, 40.3, 35.0, 31.8, 31.1, 22.5 ppm; HRMS (ESI) – Calc. for C₁₇H₂₄N₃O₆ [M+H]+: 366.1665. Found: 366.1659.
NMR Spectra of D-MAG-0
Supplementary References
