Supplementary Figure 1. Neurotransmitter and amino acid structures

(**a**) Neurotransmitters to be used as ligands in neuronal receptor PTLs contain amine and/or hydroxyl moieties susceptible of reacting with an electrophilic reactive group. (**b**) Structure of nucleophilic amino acids (protein side chains) targeted by electrophilic reactive groups.

Supplementary Figure 2. Optimization of the Click Reactions conditions

Conditions A (Cu2O and NaAsc): to a solution of azide (compounds **1** or **2**, 1 mg, 1 equiv), NaAsc (4 equiv), $Cu₂O$ (2.4 equiv) in water (30 μ L) was added a solution of 4pentynoic acid (1.1 equiv) in THF (4 μL) and the reaction was stirred at RT. The conversion of the reaction was determined by HPLC.

Conditions B (Cu₂O): To a solution of azide (compounds 1 or 2, 1 mg, 1 equiv), Cu₂O (2.4 equiv) in water (30 μL) was added a solution of 4-pentynoic acid (1.1 equiv) in THF (4 μL) and the reaction was stirred at rt. The conversion of the reaction was determined by HPLC.

Conditions C (CuSO⁴ and NaAsc): To a solution of azide (compounds **1** or **2**, 1 mg, 1 equiv), NaAsc (4 equiv), $CuSO₄$ (2.4 equiv) in water (30 µL) was added a solution of 4pentynoic acid (1.1 equiv) in THF (4 μL) and the reaction was stirred at rt. The conversion of the reaction was determined by HPLC.

HPLC analyses were performed on an Alliance 2695 system with UV detection at 360 nm using a ZORBAX Eclipse Plus C18 (4.6 mm \times 75 mm, 3.5 µm) column under the following chromatography conditions: mobile phase A, H_2O with 0.2% HCO₂H; mobile phase B, CH₃CN with 0.2% HCO₂H; flow rate, 1.0 mL min⁻¹; injection volume, 5 µL; elution gradient, 0.0−5.0 min, 5−90% B; 5.0−7.0min, 90% B; 7.0−8.0 min, 90−100% B; 8.0−10.0 min, 100% B; 10.0−11.0 min, 100−5% B; 11.0−15.0 min, 5% B.

Progression of click reaction monitored by HPLC (see text above section for details). **(a)** Scheme of the reaction between azide **1** and 4-pentynoic acid. **(b)** Percentage of azide **1** conversion versus reaction time under different reaction conditions. **(c)** Scheme of the reaction between azide **2** and 4-pentynoic acid. **(d)** Percentage of azide **2** conversion versus reaction time under different reaction conditions.

Supplementary Figure 3. Structures of Compounds Obtained by Click Reaction with azide 1

Supplementary Figure 4. Structures of Compounds Obtained by Click Reaction with azide 2

Supplementary Figure 5. Generation of compound 9 and side product characterization by UPLC-MS analysis

1: HRMS calculated for C₂₅H₂₉N₈O₆: 537.2210 [M-H]⁻. Found: 537.2216. I: HRMS calculated for $C_{31}H_{37}N_8O_9$: 665.2684 [M-H]⁻. Found: 665.2668. II: HRMS calculated for $C_{37}H_{49}N_{10}O_{10}$: 793.3633 [M-H]⁻. Found: 793.3613. Illa/IIIb: HRMS calculated for C₃₁H₃₅N₈O₈: 647.2578 [M-H]⁻. Found: 647.2548.

Scheme of the reaction between azide **1** and NHS-alkyne **6**

Supplementary Figure 6. Generation of compound 9 and side product characterization by UPLC-MS analysis

UPLC-MS analysis of the reaction mixture from **Supplementary Figure 5** at 60 min after the addition of lysine

Supplementary Figure 7. Generation of compound 10 and side product characterization by UPLC-MS analysis

Scheme of the reaction between azide **2** and NHS-alkyne **8**.

Supplementary Figure 8. Generation of compound 10 and side product characterization by UPLC-MS analysis

UPLC-MS analysis of the reaction mixture from **Supplementary Figure 7** at 60 min after the addition of lysine

Supplementary Figure 9. Generation of compound 11 and HPLC analysis.

(**a**) Scheme of the reaction between azide **1** and epoxide-alkyne **3** to yield **11**. (**b**) HPLC chromatograms of azide **1** and reaction mixture at 30 min

(**a**) Scheme of the reaction between azide **1** and epoxide-alkyne **4** to yield **12**. (**b**) UPLC-MS analysis of the reaction mixture at 30 min

Supplementary Figure 11. UV-Visible absorption spectra and photochromism of compounds 9 and 10.

(**a-b** and **d-e**) Absorption spectrum of non-illuminated compounds (black), after 5 min of illumination at 380 nm (violet) and after 5 min at 500 nm (green). (**a**) Absorption spectra of compound **9** (15 µM) in 1% DMSO and 99% PBS and (**b**) in 100% DMSO. (**c**) Time course of the absorption at 350 nm of compound **9** (15 µM, 25 ºC) in the dark after 5 min of illumination with 380 nm light. The calculated relaxation lifetime is 84.07 min. (**d**) Absorption spectra of compound **10** (15 µM) in 1% DMSO and 99% PBS and (**e**) in 100% DMSO. (**f**) Time course of the absorption at 350 nm of compound **10** (15 µM, 25 ºC) in the dark after 5 min of illumination with 380 nm light. The relaxation lifetime of compound **10** (15 µM) is 74.82 min. For absorbance measurements, we used compounds **9** and **10** previously prepared and immediately stored in 100% DMSO at - 20 ºC.

(**a**) Structure of compound **11** bearing an epoxide moiety as a reactive group (in red) (**b**). Whole-cell current from HEK293 cells expressing GluK1 and incubated with **11**. Green bar indicates 500 nm illumination, and violet 380 nm. Red bars indicate when the bath solution perfused contained 300 µM glutamate and yellow 1 mM DNQX. (**c**) Quantification of the variation of photocurrent for cells incubated with compound **11**, and compound **9** as a positive control for light activation. Photocurrent is normalized by current induced by perfusion of glutamate. Bars are mean ± s.e.m, n=5 for compound **9** and n=6 for compound **11**.

Supplementary Figure 13. Basal *(trans)* **photocurrents.**

Current induced by the *trans* conformation of the compounds versus its length, in number of bonds from the reactive carbonyl group to the *C*-4 of glutamate (see **Supplementary Table 1**). Compounds **9** and **10** have a length of 28 and 35 bonds, respectively. Data points are indicated as mean ± s.e.m, equal n's as in **Figure 3c**.

Supplementary Figure 14. Action spectra traces

(**a-d**) Whole cell recordings after HEK239 cells expressing GluK1 were incubated with compounds **9** or **10**. (**a** and **c**) Representative activation spectra of compound **9** (**a**) and **10** (**c**) assayed by measuring photocurrent in response to light pulses ranged from 350 to 470 nm, while resting wavelength was set to 500 nm (maximal deactivation). (**b** and **d**) Representative deactivation spectra of compound **9** (**b**) and **10** (**d**) assayed by measuring photocurrent in response to light pulses ranged from 380 to 600 nm, while resting wavelength was set to 380 nm (maximal activation). Quantification is shown in **Figure 3h**.

Supplementary Figure 15. PTL is a full agonist.

Whole cell current recording from a HEK293 cell expressing GluK1 and incubated with compound **9**. We compared binding of a full agonist (red bar, 300 µM glutamate) and binding of the PTL by photoswitching between the activating and deactivating configurations (violet and green bars, respectively). The binding of the PTL under violet light leads to higher activation of the receptor than free glutamate, indicating that the compound acts as full agonist $⁶$.</sup>

Photosensitivity of compound 9 was determined from the dependence of τ_{on} of the photocurrent with light intensity. In HEK293 cells expressing GluK1 and incubated with compound **9**, photocurrents were elicited at different intensities of violet light (purple trace, right axis). To calculate τ_{on} $^{-1}$ (black trace, left axis.), the photocurrent onset was fitted to an exponential function. Dots are mean \pm s.e.m, n=1 photocurrent for 0% intensity, n=2 photocurrents for 3% intensity and n=3 photocurrents for 10 to 100% intensity.

Supplementary Figure 17. Mass spectrometry characterization of purified intact S1S2 GluK1 domain protein.

Mass spectrometry characterization of purified intact S1S2 GluK1 domain protein (**a**, **b**, **c**) and after conjugation with compound **9** (**d**, **e**, **f**) and compound **10** (**g**, **h**, **i**). Total ion current chromatograms of intact protein (**a**) and after conjugation to compound **9** (**d**) and compound **10** (**g**). Mass spectra of intact protein (**b**) and after conjugation to compound **9** (**e**) and compound **10** (**h**). The deconvoluted monoisotopic mass spectra of the protein prior to conjugation (**c**) displays a single peak corresponding to the intact protein (theoretical mass 29196.8892 Da, mass error 3.35 ppm). The deconvoluted monoisotopic mass spectra of the protein conjugated to compound **9** (**f**) displays two peaks corresponding to the unconjugated (mass 29196.9590) and conjugated protein (mass 29845.2603). The deconvoluted monoisotopic mass spectra of the protein conjugated to compound **10** (**i**) displays a single peak that demonstrates almost complete conjugation (theoretical mass 29936.2069 Da, mass error 1.96 ppm). Results in (**f**) and (**i**) indicate that the stoichiometric ratio of S1S2 GluK1 conjugation to compounds **9** and **10** is 1:1.

Supplementary Figure 18. PTL conjugation to S1S2 GluK1 domain protein and effect of illumination.

Relative quantification by targeted MS/MS analysis of compound **9** lysine conjugation in S1S2 GluK1 protein domain under 380nm illumination. For each conjugated lysine, ratio peak areas of the extracted ion chromatograms (XIC) of conjugated peptides vs non-conjugated peptides were represented for dark and violet illumination. Significance was calculated by a linear mixed effects model, $*$ p<0.05. N = 3 conjugation experiments, bars are mean ± standard deviation.

Supplementary Figure 19. Photoswitchable activity of free, hydrolyzed PTL compounds.

(**a**) Whole-cell current recording in HEK239 cells expressing GluK1 perfused with compound **10** at 1 μM (light blue bar) and 10 μM (dark blue bar). Light pulses are indicated in green (500 nm) and violet (380 nm), absence of light is colored in dark. (**b**) Quantification of the photoresponse obtained in *cis* and *trans* conformations when compound **10** was perfused at 0.1 μM (n=2), 1 μM (n=3), 10 μM (n=4) and 30 μM ($n=3$). Points are mean \pm s.e.m.

Supplementary Figure 20. Photoresponses of GluK1 Lys734Ala-Lys497Ala mutant

Whole cell current recording from a HEK cell expressing GluK1-Lys734Ala-Lys497Ala.

We mutated to alanine (which does not bear nucleophilic reactive groups) the main lysine involved in conjugation (Lys734Ala, see **Fig. 4** and **Supplementary Figs. 17** and **18**) as well as another residue located near the glutamate binding site that was identified as a partial conjugation site in preliminary assays (Lys497Ala). The double mutant Lys734Ala-Lys497Ala expresses at a similar level to the wildtype, but after conjugation to compound **9** photoresponses are not abolished in the absence of these two lysines. This result suggests that one or more of the 18 remaining lysines exposed on the surface of the GluK1 LBD (**Fig. 4f**) can act as surrogate conjugation sites of compound **9** in the absence of Lys497 and Lys734. Red and orange marks indicate glutamate and DNQX perfusion respectively; green, violet and black marks correspond to illumination at 500 nm, 380 nm and absence of illumination.

Supplementary Figure 21. Photocurrents in DRG neurons

Quantification of current responses induced by glutamate (black dots, left axis) and light (violet dots, right axis) in DRG neurons incubated with 0, 1, 3, 6 or 24 µM of compound **9** and Con A, previous to the experiment. Incubation with increasing concentration of compound **9** reduces response to glutamate and increases photoresponses. We determined an optimal concentration window (3 to 6 uM of compound **9**) in which photoresponses are maximal and glutamate response is still present. Dots are mean \pm s.e.m, for glutamate responses n ranges from 6 to 21, for photoresponses: n=7 for incubation with 3 µM of compound **9** and n=5 for incubation with 6 µM of compound **9**.

Supplementary Figure 22. Retinal photoresponses are stable in time

Raster plot and integrated time course of the firing rate of MEA from flat-mounted degenerated retinas from *rd10* mice incubated with compound **9**. On top, photoresponse of a retina right after the incubation with the TCP. On bottom, retinal photoresponses are still observed after 5 h of the TCP treatment.

Supplementary Figure 23. GluK1 ligand binding domain sequence alignment

GluK1 Ligand Binding Domain Segment 1

Sequence alignment using ClustalW2 of the GluK1 ligand binding domain segments from several species frequently used as animal models for retina⁷. Segment 1 comprises amioacids 446 to 559, and segment 2 aminoacids 682 to 821 of the *R. norvegicus* gene, access number NP_001104587.1. The alignment shows that GluK1 ligand binding domain amino acid sequence is highly conserved across species, including lysines were the TCP conjugates. Lysines and arginines are highlighted in red.

NMR spectra

Supplementary Figure 24. ¹H NMR (500 MHz, CD3OD) and ¹³C NMR (125 MHz, CD3OD) spectra of 21

Supplementary Figure 25. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 26

Supplementary Figure 26. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 27

Supplementary Figure 27. ¹H NMR (400 MHz, DMSO-d6) and ¹³C NMR (100 MHz, DMSO-d6) spectra of 22

Supplementary Figure 28. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 23

Supplementary Figure 29. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 24

Supplementary Figure 30. ¹H NMR (400 MHz, CD3OD) and ¹³C NMR (100 MHz, CD3OD) spectra of 28

Supplementary Figure 31. ¹H NMR (400 MHz, CD3OD) and ¹³C NMR (100 MHz, CD3OD) spectra of 29

Supplementary Figure 32. ¹H NMR (400 MHz, CD3OD) and ¹³C NMR (100 MHz, CD3OD) spectra of 1

Supplementary Figure 33. ¹H NMR (400 MHz, CD3OD) and ¹³C NMR (100 MHz, CD3OD) spectra of 2

Supplementary Figure 34. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 4

Supplementary Figure 35. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 31
Supplementary Figure 36. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 32

,OH Ω \int_{0}^{∞} 33 \mathbb{F}^5 -99 3.5 f1 (ppm) 2.5 4.0 3.0 $\rm _{1.92}^ 2.04$ $E+2$ $F_{60.1}$ $\frac{1}{3.5}$ 6.5 5.5 5.0
f1 (ppm) $\frac{1}{2.5}$ 9.0 8.5 $\overline{8.0}$ 7.5 7.0 6.0 4.5 4.0 3.0 2.0 $\begin{array}{c|c} -\text{79.11} \\ -\text{75.13} \end{array}$ -64.97 -58.52 -34.72 -177.4 ,OH $\frac{1}{\circ}$ 33 $\frac{110}{110}$ $\frac{100}{100}$ $\frac{90}{f1}$ $\frac{80}{f1}$ 120 180 170 160 150 140 130 70 60 50 40 30 $\overline{20}$ 10 $\overline{0}$

Supplementary Figure 37. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 33

Supplementary Figure 38. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 34

Supplementary Figure 39. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 5

Supplementary Figure 40. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 6

Supplementary Figure 41. ¹H NMR (400 MHz, CDCl3) and ¹³C NMR (100 MHz, CDCl3) spectra of 7

HPLC Chromatograms of compounds 1 and 2

The purity of compounds **1** and **2** was >95% as determined by HPLC on an Alliance 2695 system with UV detection at 360 nm using a ZORBAX Eclipse Plus C18 (4.6 mm × 75 mm, 3.5 μm) column under the following chromatography conditions: mobile phase A, H₂O with 0.2% HCO₂H; mobile phase B, CH₃CN with 0.2% HCO₂H; flow rate, 1.0 mL min−1 ; injection volume, 5 μL; elution gradient, 0.0−5.0 min, 5−90% B; 5.0−7.0min, 90% B; 7.0−8.0 min, 90−100% B; 8.0−10.0 min, 100% B; 10.0−11.0 min, 100−5% B; 11.0−15.0 min, 5% B.

Supplementary Figure 42. HPLC chromatograms of compounds 1 and 2

Supplementary Table 1. Summary of the resulting length of each PTL prepared.

Length is calulated in number of bonds from the reactive carbonyl group to the *C*-4 of glutamate, see the bonds highlighted in red for compounds **10** and **9** (upper and lower structures respectively) in the schemes below the table.

Supplementary Table 2. Theoretical ion fragment tables

Theoretical ion fragment tables of peptides QQSALVK(**9**)NSDEGIQR,b and y ion fragments found in the MS/MS spectra (**Fig. 4b**) are showed in red and blue, respectively.

QQSALVK(9)NSDEGIQR

Charge: +3, Monoisotopic m/z: 774.38201 Da (+1.51 mmu/+1.95 ppm), MH+: 2321.13149 Da

Neutral losses

Precursor ions

Supplementary Table 3. Theoretical ion fragment tables

Theoretical ion fragment tables of peptides QQSALVK(**10**)NSDEGIQR, b and y ion fragments found in the MS/MS spectra (**Fig. 4b**) are showed in red and blue, respectively.

QQSALVK(10)NSDEGIQR

Charge: +4, Monoisotopic m/z: 603.80098 Da (+0.75 mmu/+1.25 ppm), MH+: 2412.18208 Da

Neutral losses

Precursor ions

Supplementary table 4. Identified peptides

Supplementary table 5. Selected ions for targeted analysis

Supplementary table 6. Integrated peak area

Integrated peak areas was based on extracted ion chromatograms (XICs) of up to 3 highest ranked MS/MS fragment ions masses

Supplementary Notes 1. Chemical Synthesis

General Methods.

Solvents were dried prior to use with alumina in a solvent purification system or distilled and dried by standard methods. FT-IR spectra are reported in cm^{-1} . ¹H and ¹³C NMR spectra were obtained in CDCl₃, CD₃OD or DMSO-d₆ solutions at 500 MHz (for ¹H) and 100 MHz (for ¹³C). Chemical shifts (*δ*) are reported in ppm relative to the singlet at 7.26 ppm of CDCI₃ for ¹H and in ppm relative to the center line of a triplet at 77.16 ppm of CDC I_3 for ¹³C. Optical rotations were measured with a Jasco P-1030 polarimeter, and specific rotations are reported in 10⁻¹ deg cm² g⁻¹. The HRMS spectra were recorded on a Waters LCT Premier Mass spectrometer with electrospray ionization (ESI). Melting points were measured on a Mettler Toledo MP70 melting point apparatus. UV-Vis analyses were performed in a UV-1700 PharmaSpec SHIMADZU UV-Vis spectrometer on a 50 μ M solution of the corresponding compound in HPLC-quality solvent. All the starting materials for the synthesis of the azobenzenes and Alkyne-NHS Esters were obtained from commercial suppliers and were used without further purifications. 4- Azidoaniline **25** was prepared from 4-iodoaniline according to the procedure of Wei *et al*. 1 (Scheme S2). Alkyne-NHS Ester **8** and epoxide-NHS Ester **3** were purchased from Alfa Aesar and Sigma-Aldrich, respectively.

Synthesis of azobenzenes 9 – 20

The preparation of the final compounds **9** – **20** is shown in Scheme S1. The azides **1** and **2** were synthesized from azobenzenes **21** and **22**, respectively. Azobenzene **21** was prepared from commercially available 4,4'-azodianiline and azobenzene **22** was obtained using classical diazo-coupling chemistry (Scheme S2).

Amide coupling of 21 and 22 with a pyroglutamate acid derivative^{2, 3} gave the advanced intermediates **23** and **24** in 78% and 57% yield, respectively. Then, these compounds were transformed into the final azides **1** and **2** after hydrolysis of the pyroglutamate with concomitant saponification of the ethyl ester and acidic removal of the *tert*butoxycarbonyl (Boc) protecting group (Scheme S1 step b) in 73% and 43% yield, respectively. Triazole compounds **9**–**20** were obtained by click chemistry using the Cu(I) catalyzed azide-alkyne cycloaddition reaction⁴ by mixing in H_2O/THF (7.5:1), 4 equivalents of sodium ascorbate (NaAsc), 2.4 equivalents of the solid $Cu₂O$, and 1 equivalent of azide **1** or **2**. After stirring for 5 min at room temperature, 1.1 equivalents of the alkyne compounds **3**–**8** (Scheme S1 squared figure) in THF were added. The reaction was stirred for 30 min at room temperature (step c).

Scheme S1. Synthesis of azobenzenes **9** – **20**. a

^aReagent and conditions: (a) HATU, DIPEA, EtOAc; (b) 1. LiOH, THF/H₂O. 2. HCl, EtOAc; (c) $Cu₂O$, NaAsc, THF/H₂O.

Synthesis of azobenzenes 21 and 22

The preparation of azobenzenes **21** and **22** is shown in Scheme S2. To outline the strategy of the non-commercially available azo block compounds, we used two different routes. Azo-compound **21** was synthesized by monoacylation of the commercially available 4,4'-diaminoazobenzene with 4-bromobutyryl chloride, followed by nucleophilic substitution of the bromide with sodium azide in the presence of TBAI. The azo-compound **21** was obtained in 79% yield.

Azo compound **22** was obtained from 4-iodoaniline, which was converted into 4 azidoaniline **25** by the substitution of the iodide with the azido group (Scheme S2 step b) in a nearly quantitative yield. Then, compound **25** was coupled with nitroso compound **26** (Scheme S2 step d), which was obtained from commercially available *tert*-butyl (4-aminophenyl)carbamate (step c, 47% yield), in acetic acid media to give the resulting azo compound **27** in 91% yield. Further acidic deprotection of *tert*butoxycarbonyl (Boc) group afforded in 90% yield the desired azo compound **22**.

Scheme S2. Synthesis of azobenzenes **21** and **22**. a

^aReagent and conditions: (a) 1. 4-bromobutyryl chloride, DIPEA, THF, rt. 2. NaN₃, TBAI, DMF, 70 $°C$; (b) NaN₃, 10 mol% CuI, 20 mol% L-proline, 20 mol% NaOH, DMSO, 60 °C; (c) oxone, CH_2Cl_2 , rt; (d) AcOH, rt; (e) HCl, EtOAc.

(*E***)-***N***-(4-((4-Aminophenyl)diazenyl)phenyl)-4-azidobutanamide (21).**

To a stirred solution of 4,4'-diaminoazobenzene (1.0 g, 4.71 mmol) and DIPEA (0.49 mL, 2.83 mmol) in THF (40 mL) was added a solution of 4-bromobutyryl chloride (0.27 mL, 2.36 mmol) in THF (20 mL) at 0 °C. The reaction mixture was stirred at room temperature for 20 h, followed by removal of solvent *in vacuo*. The residue was suspended in H_2O and extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with brine, dried over $MqSO₄$, filtered and concentrated. The resulting crude was directly used in the next step without further purification.

To a solution of the above crude in DMF (50 mL) were added tetrabutylammonium iodide (TBAI) (0.43 g, 1.18 mmol) and N_3 (0.61 g, 9.41 mmol). The reaction mixture was stirred at 70 °C for 24 h. The reaction mixture was cooled to room temperature and the solvent was removed under vacuum. The residue was taken up in EtOAc (60 mL) and washed with water (30 mL). The organic layer was washed brine (3×30 mL), dried over MgSO4, filtered and the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography (95:5 to 10:1 $CH_2Cl_2/EtOAc$ gradient) to afford 0.60 g (1.86 mmol, 79%) of **21**. mp: 155–157 ºC R^f : 0.5 $(CH_2Cl_2/EtOAC 90:10)$. IR (film): $v = 3429, 3293, 3202, 2097, 1659, 1595, 1580, 1536,$ 1403, 1356, 1243, 848, 832, 639 cm⁻¹. ¹H NMR (δ, 500 MHz, CD₃OD): 7.76–7.66 (m, 6H), 6.74–6.70 (m, 2H), 3.40 (t, *J* = 6.6 Hz, 2H), 2.49 (t, *J* = 7.2 Hz, 2H), 2.02–1.93 (m, 2H). ¹³C NMR (δ, 125 MHz, CD₃OD): 173.41, 153.29, 150.57, 145.75, 141.28, 125.96, 123.78, 121.19, 115.21, 51.95, 34.80, 25.94. HRMS calculated for $C_{16}H_{18}N_7O$: 324.1567 [M+H]⁺. Found: 324.1570.

*tert***-Butyl (4-nitrosophenyl)carbamate (26).**

To a flask containing a solution of *tert*-butyl (4-aminophenyl)carbamate (100 mg, 0.48 mmol) in CH_2Cl_2 (4 mL) was added an aqueous solution of oxone (0.169 M, 0.72 mmol). After stirring for 3 h at room temperature, the color of the solution changes from white to brown. The mixture was diluted with CH_2Cl_2 (25 mL), washed first with 1 M HCl solution (3 x 50 mL), then with a saturated solution of NaHCO₃ (3 x 50 mL) and brine (3 x 50 mL). The organic layer was dried over $Na₂SO₄$, filtered and concentrated. The resulting residue was purified by column chromatography (CH_2Cl_2) to afford 50.3 mg $(0.23 \text{ mmol}, 47\%)$ of 26 as a green olive solid. mp: 111–113 °C. R_f: 0.6 (100% CH₂Cl₂). IR (film): $υ = 3280$, 2984, 1733, 1718, 1528, 1100 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃) 7.88 (d, *J =* 8.1 Hz, 2H), 7.58 (d, *J =* 8.8, 2H), 6.87 (s, 1H), 1.54 (s, 9H). ¹³C NMR (*δ*, 100 MHz, CDCl₃): 164.12, 151.92, 145.67, 123.59, 117.37, 82.15, 28.33. HRMS calculated for $C_{11}H_{13}N_2O_3$: 221.0926 [M-H]. Found: 221.0915.

(*E***)-***tert***-Butyl (4-((4-azidophenyl)diazenyl)phenyl)carbamate (27).**

To a solution of **26** (373 mg 1.68 mmol) in 10 mL of AcOH was added 4-azidoaniline **25** (188 mg 1.40 mmol) and the reaction mixture was stirred at room temperature for 5 days. After neutralization of the acidic medium with a saturated solution of NaHCO₃ (140 mL), the mixture was diluted with $CH₂Cl₂$ (100 mL). The aqueous layer was

extracted with CH_2Cl_2 (2 x 100 mL) and the combined organic layers were washed with H₂O (3 x 100 mL) and brine (3 x 100 mL), then dried over MgSO₄, filtered and concentrated *in vacuo*. The crude was purified by column chromatography (9:1 Hexane/EtOAc) to afford **27** (430 mg, 91%) as a bright orange solid. mp: 161–164 ºC. R_f : 0.8 (Hexane/EtOAc 4:1). IR (film): $v = 3373$, 2978, 2931, 2400, 2249, 2111, 1698, 1603, 1575, 1149 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 7.93–7.86 (m, 4H), 7.51 (d, J = 8.9 Hz, 2H), 7.16–7.12 (m, 2H), 6.67 (brs, 1H), 1.54 (s, 9H). ¹³C NMR (*δ*, 100 MHz, CDCl3): 152.44, 150.03, 148.30, 142.24, 141.24, 124.50, 124.23, 119.68, 118.41, 81.28, 28.45. HRMS calculated for $C_{17}H_{19}N_6O_2$: 339.1569 [M+H]⁺. Found: 339.1557.

(*E***)-4-((4-Azidophenyl)diazenyl)aniline hydrochloride (22).**

To a flask containing **27** (0.38 g, 1.12 mmol) was added a freshly prepared solution of HCl in EtOAc (20 mL) *(see General procedure for HCl in EtOAc preparation)*. After stirring for 2 h at room temperature, an argon flow was passed through the solution to remove all HCl and the solvent was removed under reduced pressure. The resulting purple solid was triturated with Et_2O (3×30 mL) and filtered to give 22 (0.28 g, 1.01 mmol, 90%). mp: >350 °C. IR (film): $v = 2847$, 2547, 2114, 1595, 1556, 1488, 1279 cm ¹. ¹H NMR (δ, 400 MHz, DMSO-d₆): 7.89–7.84 (m, 2H), 7.83–7.77 (m, 2H), 7.30–7.25 (m, 2H), 7.10–7.03 (m, 2H). ¹³C NMR (*δ*, 100 MHz, DMSO-d6): 155.62, 149.18, 141.23, 138.19, 124.76, 123.81, 120.01, 118.02. HRMS calculated for $C_{12}H_{11}N_6$: 239.1045 [M+H]⁺. Found: 239.1019.

Synthesis of azides 1 and 2

(2*S***,4***R***)-1-(***tert***-Butyl)-2-ethyl 4-(4-((4-((***E***)-(4-(4-azidobutanamido) phenyl) diazenyl) phenyl) amino)-4-oxobutyl)-5-oxopyrrolidine-1,2-dicarboxylate (23).**

To a solution of pyroglutamate derivative^{2, 3} (1.07 g, 3.09 mmol) in EtOAc (40 mL) containing DIPEA (1.10 mL, 6.18 mmol), HATU (2.35 g, 6.18 mmol) was added and the mixture was stirred at room temperature for 15 min. Consequently, a solution of **21** (1.0

g, 3.09 mmol) in EtOAc (10 mL) was added, and the reaction was stirred at 40 ºC for 16 h. The mixture was diluted with EtOAc (100 mL) and washed with H_2O (2 \times 150 mL) and brine (2 \times 150 mL). The organic layer was dried over MgSO₄, filtered and concentrated. The reaction crude was purified by column chromatography (4:1 to 2:1 Hexane/EtOAc gradient) to give **23** (1.56 g, 2.41 mmol, 78%) as an orange solid. mp: 166–169 °C. R_f: 0.2 (Hexane/EtOAc 2:1). [α]_D: n.d. IR (film): $v = 3309$, 2981, 2934, 2096, 1788, 1742, 1657, 1590, 1528, 1296, 1244, 844 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl3): 7.78–7.74 (m, 4H), 7.67–7.48 (m, 4H), 4.50 (dd, *J* = 9.6, 1.5 Hz, 1H), 4.16 (q, *J* = 7.2 Hz, 2H), 3.36 (t, *J* = 6.4 Hz, 2H), 2.64–2.56 (m, 1H), 2.45 (t, *J* = 7.2 Hz, 2H), 2.37 (td, *J* = 7.2, 2.5 Hz, 2H), 2.19 (ddd, *J* = 13.2, 8.5, 1.5 Hz, 1H), 2.00–1.89 (m, 3H), 1.88– 1.79 (m, 1H), 1.77–1.69 (m, 3H), 1.41 (s, 9H), 1.22 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (*δ*, 100 MHz, CDCl₃): 175.97, 171.37, 171.34, 170.53, 149.46, 149.19, 149.02, 140.60, 140.21, 123.92, 123.89, 120.10, 84.02, 77.36, 61.97, 57.41, 50.85, 41.64, 37.26, 34.29, 29.70, 28.46, 28.00, 24.70, 22.79, 14.34. HRMS calculated for $C_{32}H_{40}N_8O_7Na$: 671.2912 [M+Na]⁺. Found: 671.2939

(2*S***,4***R***)-1-***tert***-Butyl-2-ethyl-4-(4-((4-((***E***)-(4-azidophenyl)diazenyl)phenyl)amino)4 oxbutyl) -5-oxopyrrolidine-1,2-dicarboxylate (24).**

To a solution of pyroglutamate derivative^{2, 3} (0.57 g, 1.65 mmol) in EtOAc (20 mL) containing DIPEA (1.16 mL, 6.62 mmol), HATU (1.26 g, 3.31 mmol) was added and the mixture was stirred at room temperature for 15 min. Consequently, a solution of **22** (0.50 g, 1.82 mmol) in EtOAc (10 mL) was added, and the reaction was stirred at 55 \degree C for 18 h. The mixture was diluted with EtOAc (250 mL) and washed with NaHCO₃ solution (3 \times 150 mL) and brine (3 \times 150 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated. The reaction crude was purified by column chromatography (CH₂Cl₂/EtOAc 9:1) to give **24** (0.53 g, 0.94 mmol, 57%) as an orange solid. mp: 69–73 °C. R_f: 0.32 (CH₂Cl₂/EtOAc 4:1). [α]_D: +79.94 (*c* = 0.87, CHCl₃). IR (film): $v = 3336$, 2977, 2933, 2111, 1781, 1741, 1592, 1531, 1297, 1144 cm⁻¹. ¹H NMR (*δ*, 400 MHz, CDCl3): 8.40–6.89 (m, 8H), 4.60–4.54 (m, 1H), 4.17 (q, *J* = 7.1 Hz, 2H), 2.73–2.60 (m, 1H), 2.52–2.22 (m, 3H), 2.10–1.97 (m, 1H), 1.97–1.87 (m, 1H), 1.87– 1.76 (m, 2H), 1.48 (9H, s), 1.42 (1H, s), 1.29 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (*δ*, 100 MHz, CDCl₃): 175.79, 171.34, 149.98, 149.37, 148.81, 142.29, 141.11, 125.62, 124.52, 123.96, 119.97, 119.65, 61.93, 57.40, 41.68, 37.24, 30.43, 29.79, 28.51, 28.00, 22.83, 14.33. HRMS calculated for C₂₈H₃₂N₇O₆: 562.2414 [M-H]⁻. Found: 562.2429.

General procedure for the ring-opening hydrolysis reaction. Synthesis of 29 as a representative example.

To a solution of **24** (67.0 mg, 0.119 mmol) in THF (1.2 mL) at 0 ºC was added 1.0 M aqueous solution of LiOH (1.2 mL). After stirring for 2 h, the mixture was acidified to pH 2 with 1.0 M HCl solution and partitioned between EtOAc (20 mL) and $H₂O$ (20 mL). The layers were separated and the aqueous phase was extracted with EtOAc (2 x 20 mL). The combined organic layers were dried over $Na₂SO₄$, filtered and concentrated *in vacuo*. The residue obtained was purified by silica gel chromatography.

(2*R***,4***S***)-2-(4-((4-((***E***)-(4-(4-Azidobutanamido) phenyl) diazenyl) phenyl) amino)-4 oxobutyl)-4-((***tert***-butoxycarbonyl) amino) pentanedioic acid (28).**

Following the general hydrolysis procedure, 1.2 g (1.87 mmol, 81% yield) of **28** as an orange solid were obtained from 1.5 g (2.31 mmol) of **23**. The compound was purified by flash chromatography (98:2 to 90:10 $CH_2Cl_2/MeOH$ gradient with 1% AcOH). mp: 198–199 °C. R_f: 0.2 (MeOH/CH₂Cl₂ 5:95 + 1 % AcOH). [α]_D: +8.6 (*c* = 0.25, MeOH). IR (film): $v = 3303$, 2931, 2097, 1656, 1530, 1408, 1367, 1252, 1152, 845 cm⁻¹. ¹H NMR (*δ*, 400 MHz, CD3OD): 7.85–7.83 (m, 4H), 7.74–7.72 (m, 4H), 4.21–4.06 (m, 1H), 3.39 (t, *J* = 6.7 Hz, 2H), 2.65–2.54 (m, 1H), 2.50 (t, *J* = 7.3 Hz, 2H), 2.46–2.38 (m, 2H), 2.28–2.14 (m, 1H), 1.98–1.93 (m, 2H), 1.82–1.56 (m, 5H), 1.43 (s, 9H). ¹³C NMR (*δ*, 100 MHz, CD3OD): 178.65, 175.96, 175.20, 174.19, 173.46, 158.11, 150.09, 142.52, 142.49, 124.52, 121.09, 121.06, 80.51, 53.48, 51.91, 43.11, 37.73, 34.80, 33.39, 28.72, 28.52, 25.85, 24.35. HRMS calculated for $C_{30}H_{37}N_8O_8$: 637.2734 [M-H]. Found: 637.2740.

(2*R***,4***S***)-2-(4-((4-((***E***)-(4-Azidophenyl)diazenyl)phenyl)amino)-4-oxobutyl)-4-((***tert***butoxycarbonyl)amino)pentanedioic acid (29).**

Following the general hydrolysis procedure, 43 mg (0.077 mmol, 65% yield) of **29** as an orange solid were obtained from 67 mg (0.119 mmol) of **24**. The compound was purified by flash chromatography (CH₂Cl₂/MeOH 9:1 with 1% AcOH). mp: 98–99 °C. R_f: 0.45 (CH₂Cl₂/MeOH 9:1 with 1% AcOH). $\lbrack \alpha \rbrack_{D}$: -31.56 (*c* = 1.08, MeOH). IR (film): $v =$ 3317, 2927, 2407, 2113, 1703, 1595, 1530, 1153 cm⁻¹. ¹H NMR (δ, 400 MHz, CD₃OD): 7.78 (d, *J* = 8.5 Hz, 2H), 7.74 (d, *J* = 8.8 Hz, 2H) 7.64 (d, *J* = 8.8 Hz, 2H), 7.07 (d, *J* = 8.5 Hz, 2H), 4.14–3.91 (m, 1H), 2.58–2.40 (m, 1H), 2.39–2.23 (m, 2H), 2.20–2.03 (m, 1H), 1.78–1.45 (m, 5H), 1.34 (s, 9H). ¹³C NMR (δ, 100 MHz, CD₃OD): 178.62, 175.90, 175.18, 174.19, 158.10, 151.15, 149.92, 143.83, 142.82, 125.41, 124.68, 121.04, 120.66, 43.10, 37.77, 34.81, 33.39, 28.73, 24.33, 20.75. HRMS calculated for C₂₆H₃₀N₇O₇: 552.2207 [M-H]. Found: 552.2219.

General procedure for the NH-Boc deprotection reaction. Synthesis of 1 as a representative example.

To a flask containing **28** (1.2 g, 1.87 mmol) was added a freshly prepared solution of HCI in EtOAc (50 mL) *(see General procedure for HCI in EtOAc preparation)*. The color of the solution changes from orange to dark purple. After stirring for 2 h at room temperature, an argon flow passed through the solution to remove all HCl and the solvent was removed under reduced pressure. The resulting purple solid was triturated with $Et₂O$ (3 \times 30 mL) and filtered.

(2*S***,4***R***)-2-Amino-4-(4-((4-((E)-(4-(4-**

azidobutanamido)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)pentanedioic acid hydrochloride (1).

Following the general -NH-Boc deprotection procedure, 0.98 g (1.70 mmol, 91% yield) of 1 were obtained from 1.2 g (1.87 mmol) of 28. mp: 181–183 °C. $[\alpha]_D$: +29.9 ($c =$ 0.125, MeOH). IR (film): $v = 3320, 2927, 2866, 2095, 1711, 1665, 1590, 1518, 1494,$ 1240, 1153, 844 cm⁻¹. ¹H NMR (δ, 400 MHz, CD₃OD): 7.88–7.84 (m, 4H), 7.79–7.73 (m, 4H), 4.03 (dd, *J* = 8.0, 6.0 Hz, 1H), 3.41 (t, *J* = 6.7 Hz, 2H), 2.76–2.67 (m, 1H), 2.56–2.45 (m, 4H), 2.42–2.33 (m, 1H), 2.01–1.92 (m, 3H), 1.86–1.68 (m, 4H). ¹³C NMR (δ, 100 MHz, CD3OD): 176.32, 172.81, 172.21, 170.16, 148.82, 148.79, 141.27, 141.19, 123.18, 119.79, 119.76, 51.18, 50.59, 41.02, 36.16, 33.48, 31.84, 31.27, 24.54, 22.49. HRMS calculated for $C_{25}H_{29}N_8O_6$: 537.2210 [M-H]. Found: 537.2200.

(2*S***,4***R***)-2-Amino-4-(4-((4-((***E***)-(4-azidophenyl)diazenyl)phenyl)amino)-4 oxobutyl)pentanedioic acid hydrochloride (2).**

Following the general -NH-Boc deprotection procedure, 19 mg (39.02 µmol, 72% yield) of **2** were obtained from 30 mg (54.20 μmol) of **29**. mp: 188 °C. [α]_D: + 17.24 (*c* = 0.89, MeOH). IR (film): $v =$, 2924, 2111, 1708, 1664, 1533, 1492, 1405, 1281, 1209, 1153 cm⁻¹. ¹H NMR (δ, 400 MHz, DMSO-d₆): 7.94–7.81 (m, 6H), 7.38–7.25 (m, 2H), 3.79 (t, *J* = 7.0 Hz, 1H), 2.71–2.61 (m, 1H), 2.19–1.74 (m, 3H), 1.73–1.42 (m, 5H). ¹H NMR (δ, 400 MHz, CD3OD): 7.95–7.91 (m, 2H), 7.90–7.85 (m, 2H), 7.81–7.74 (m, 2H), 7.20– 7.25 (m, 2H), 4.02 (dt, *J* = 12.9, 6.5 Hz, 1H), 2.75–2.67 (m, 1H), 2.52–2.44 (m, 2H), 2.38 (ddd, *J* = 14.9, 9.2, 5.9 Hz, 1H), 1.96 (ddd, *J* = 14.5, 8.0, 4.9 Hz, 1H),1.65–1.87 (m, 4H). ¹³C NMR (δ, 100 MHz, CD₃OD): 177.63, 174.16, 171.49, 151.24, 150.04, 143.99, 142.84, 125.44, 124.69, 121.10, 120.74, 52.53, 42.36, 37.51, 33.19, 32.63, 23.82. HRMS calculated for $C_{21}H_{24}N_7O_5$: 454.1839 [M+H]⁺. Found: 454.1856.

General procedure for HCl in EtOAc preparation

To prepare a 0.5 L solution of HCl in EtOAc (2 M) for the -NH-Boc deprotection we need a closed addition system, previously dried. On the round bottom flask, acetyl chloride (71.11 mL, 1 eq.) and EtOAc (401.78 mL) was introduced and maintained at 0 ºC with an ice bath. EtOH (58.40 mL, 1 eq.) was introduced in the addition funnel and dropwise addition was kept during 45 min. Once complete, the content could be stored in the fridge for long periods. EtOAc and EtOH were previously dried with 4 \AA molecular sieves.

Synthesis of alkynes 4–7

The preparation of the alkynes **4** – **7** is shown in Schemes S3 and S4. Epoxide **4** was obtained by *O*-alkylation of alcohol **30**⁵ with (±)-epichlorohydrin (Scheme S3). Alkyne-NHS esters **5** – **7** were obtained by treating acids pent-4-ynoic acid, **33** or **34** with DCC and *N*-hydroxysuccinimide (Scheme S4 step c). Acids **33** and **34** were synthesized by *O*-alkylation of *t*-butyl 3-hydroxypropanoate, followed by deprotection of Boc group (Scheme S4 steps a and b).

Scheme S3. Synthesis of epoxide 4.^a

*^a*Reagent and conditions: (a) NaOH, TBAHS, rt.

2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethanol (30). Compound **30** was prepared from bis(2 hydroxyethyl)ether according to the procedure reported by Gerland *et al.*⁵

2-((2-(2-(Prop-2-yn-1-yloxy)ethoxy)ethoxy)methyl)oxirane (4).

A mixture of 2-(2-(prop-2-yn-1-yloxy)ethoxy)ethanol **30** (370 mg, 2.57 mmol), tetrabutylammonium hydrogensulfate (TBAHS, 87 mg, 0.26 mmol) and 50% NaOH aq solution (2 mL) was vigorously stirred at room temperature and then cooled to 0° C. To this mixture was added (\pm) -epichlorohydrin (474 mg, 5.13 mmol). The resulting suspension was stirred for 18 h at room temperature. The reaction mixture was diluted with $H₂O$ (20 mL) and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with brine, dried over $MqSO₄$, filtered and concentrated. The resulting clear oil was purified by flash chromatography (4:1 to 1:2 hexane/EtOAc gradient) to give 4 (363 mg, 1.81 mmol, 71%). IR (film): $v = 2873$, 1351, 1251, 1091, 1032, 911, 840 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 4.20 (d, J = 2.4 Hz, 2H), 3.78 (dd, *J* = 11.7, 3.0 Hz, 1H), 3.63–3.72 (m, 8H), 3.43 (dd, *J* = 11.7, 5.9 Hz, 1H), 3.15 (ddt, *J* = 5.9, 4.2, 2.9 Hz, 1H), 2.78 (t, *J* = 4.6 Hz, 1H), 2.60 (dd, *J* = 5.0, 2.7 Hz, 1H), 2.42 (t, *J* = 2.4 Hz, 1H). ¹³C NMR (*δ*, 100 MHz, CDCl3): 79.77, 74.63, 72.11, 70.86, 70.75, 70.58, 69.24, 58.53, 50.94, 44.40. HRMS calculated for $C_{10}H_{16}O_4$ Na: 223.0946 [M+Na]⁺. Found: 223.0936.

*^a*Reagent and conditions: (a) TBAHS, NaOH/toluene, 5-iodopent-1-yne or 3 bromoprop-1-yne, rt, 64–74%; (b) TFA, CH₂Cl₂, rt, 99%; (c) DCC, Nhydroxysuccinimide, THF, 61–94%.

General procedure for the alkylation of *t***-butyl 3-hydroxypropanoate. Synthesis of 32 as a representative example.**

To a solution of *t*-butyl 3-hydroxypropanoate (0.61 ml, 4.12 mmol) and 5-iodopent-1 yne (0.4 ml, 2.06 mmol) in toluene (5 mL) was added a solution of NaOH (1.48 g, 37.1 mmol) in H2O (3 mL) and tetrabutylammonium hydrogensulfate (TBAHS*,* 0.70 g, 2.06 mmol) at room temperature. After stirring for 4 h at room temperature, the solvents were removed under reduced pressure. The resulting residue was dissolved in EtOAc (30 mL) and washed with $H₂O$ (15 mL). The aqueous phase was extracted with EtOAc $(3 \times 30 \text{ mL})$. The combined organic layers were washed with brine, dried over MgSO₄, and evaporated to dryness to give a residue, which was purified by flash chromatography (hexane/EtOAc 20:1) to give the desired products.

*t-***Butyl 3-(prop-2-yn-1-yloxy)propanoate (31).**

Following the general procedure, 317 mg (1.72 mmol, 64% yield) of **31** were obtained from 0.3 mL (2.71 mmol) of 3-bromoprop-1-yne. IR (film): $v = 2960$, 2924, 2855, 1731, 1466, 1368, 1260, 1159 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 4.13–4.18 (m, 2H), 3.71– 3.80 (m, 2H), 2.49–2.55 (m, 2H), 2.42 (t, *J* = 2.4 Hz, 1H), 1.45 (s, 9H). ¹³C NMR (*δ*, 100 MHz, CDCl₃): 170.78, 80.85, 79.73, 74.56, 65.76, 58.35, 36.22, 28.24. HRMS calculated for $C_{10}H_{16}O_3$ Na: 207.0997 [M+Na]⁺. Found: 207.1010.

*t-***Butyl 3-(pent-4-yn-1-yloxy)propanoate (32).**

Following the general procedure, 324 mg (1.53 mmol, 74% yield) of **32** were obtained from 0.4 mL (2.06 mmol) of 5-iodopent-1-yne. IR (film): $v = 2955$, 2922, 2852, 1732, 1463, 1376, 1274, 1159 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 3.66 (t, *J* = 6.4 Hz, 2H), 3.52 (t, *J* = 6.1 Hz, 2H), 2.47 (t, *J* = 6.4 Hz, 2H), 2.27 (td, *J* = 7.1, 2.7 Hz, 2H), 1.93 (t, *J* = 2.7 Hz, 1H), 1.73–1.80 (m, 2H), 1.45 (s, 9H). ¹³C NMR (δ, 100 MHz, CDCl₃): 171.10, 84.12, 80.65, 69.33, 68.53, 66.64, 36.52, 28.69, 28.24, 15.31. HRMS calculated for $C_{12}H_{20}O_3$ Na: 235.1310 [M+Na]⁺. Found: 235.1318.

General procedure for the hydrolysis of esters 31–32. Synthesis of 34 as a representative example.

To a stirred solution of *t-*butyl 3-(pent-4-yn-1-yloxy)propanoate **32** (0.32 g, 1.53 mmol) in CH_2Cl_2 (4 mL) was added TFA (1 mL). The resulting mixture was stirred at room temperature for 4 h. Once the reaction was finished, the solvents were removed under reduced pressure. The resulting residue was dissolved in EtOAc (30 mL) and washed with HCl 1 N (20 mL). The aqueous layer was extracted with EtOAc (2 x 20 mL). The combined organic layers were dried over MgSO₄, and evaporated to dryness to give a residue, which was used in the next reaction without further purification.

3-(Prop-2-yn-1-yloxy)propanoic acid (33).

Following the general procedure, 215 mg (1.68 mmol, 99% yield) of **33** were obtained from 310 mg (1.68 mmol) of *t*-butyl 3-(prop-2-yn-1-yloxy)propanoate 31. IR (film): $v =$ 3284, 2924, 2854, 1717, 1440, 1190, 1102, 1067 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 4.19 (d, *J* = 2.4 Hz, 2H), 3.84 (t, *J* = 6.2 Hz, 2H), 2.70 (t, *J* = 6.2 Hz, 2H), 2.46 (t, *J* = 2.4 Hz, 1H). ¹³C NMR (*δ*, 100 MHz, CDCl3): 177.44, 79.11, 75.13, 64.97, 58.52, 34.72. HRMS calculated for $C_6H_7O_3$: 127.0395 [M-H]⁻. Found: 127.0387.

3-(Pent-4-yn-1-yloxy)propanoic acid (34).

Following the general procedure, 235 mg (1.50 mmol, 99% yield) of **34** were obtained from 324 mg (1.53 mmol) of *t*-butyl 3-(pent-4-yn-1-yloxy)propanoate 32. IR (film): $v =$ 3293, 2926, 2874, 1713, 1433, 1186, 1112, 1060 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 3.73 (t, *J* = 6.2 Hz, 2H), 3.58 (t, *J* = 6.2 Hz, 2H), 2.64 (t, *J* = 6.2 Hz, 2H), 2.28 (td, *J* = 7.0, 2.7 Hz, 2H), 1.95 (t, *J* = 2.7 Hz, 1H), 1.76–1.80 (m, 2H). ¹³C NMR (*δ*, 100 MHz, CDCl3): 175.43, 83.84, 69.64, 68.76, 66.00, 34.77, 28.48, 15.29. HRMS calculated for $C_8H_{11}O_3$: 155.0708 [M-H]⁻. Found: 155.0681.

General procedure for the synthesis of NHS 5–7. Synthesis of 7 as a representative example.

To a solution of 3-(pent-4-yn-1-yloxy)propanoic acid (0.2 g, 1.28 mmol) in anhydrous THF (5 mL) were added DCC (0.26 g, 1.28 mmol) and *N*-hydroxysuccinimide (0.15 g, 1.28 mmol) at 0 °C. The suspension was stirred at room temperature for 4 h. Crystalline dicyclohexylurea (DCU) was filtered off and washed with cold, dry THF. The filtrate was concentrated under reduced pressure, dissolved in dry THF (3 mL) and
cooled to 0 ºC for 30 minutes. The insoluble DCU was again filtered off and the process was repeated 2 more times. Filtration and evaporation afforded crude compounds, which were purified as indicated below.

2,5-Dioxopyrrolidin-1-yl pent-4-ynoate (5).

Following the general procedure, 160 mg (0.71 mmol, 61% yield) of **5** were obtained from 150 mg (1.17 mmol) of pent-4-ynoic acid. IR (film): $v = 2929$, 2845, 1814, 1783, 1732, 1205, 1068 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 2.79-2.92 (m, 6H), 2.58-2.66 (m, 2H), 2.05 (t, *J* = 2.7 Hz, 1H). ¹³C NMR (*δ*, 100 MHz, CDCl3): 168.84, 166.96, 80.79, 69.99, 30.27, 25.53, 14.06. HRMS calculated for $C_9H_9NO_4Na$: 218.0429 [M+Na]⁺. Found: 218.0435.

2,5-Dioxopyrrolidin-1-yl 3-(prop-2-yn-1-yloxy)propanoate (6).

Following the general procedure, 350 mg (1.79 mmol, 88% yield) of **6** were obtained from 0.2 g (2.04 mmol) of 3-(prop-2-yn-1-yloxy)propanoic acid (**33**). The compound was purified by flash chromatography (7:2 to 7:3 hexane/EtOAc gradient). IR (film): $v =$ 2929, 2873, 1814, 1782, 1729, 1201, 1062 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 4.21 (d, *J* = 2.4 Hz, 2H), 3.90 (t, *J* = 6.4 Hz, 2H), 2.93 (t, *J* = 6.4 Hz, 2H), 2.84 (brs, 4H), 2.46 (t, *J* = 2.4 Hz, 1H). ¹³C NMR (δ, 100 MHz, CDCl₃): 169.09, 169.07, 166.62, 79.19, 75.09, 64.30, 58.51, 31.99, 25.68. HRMS calculated for $C_{10}H_{11}NO_5Na$: 248.0535 [M+Na]⁺. Found: 248.0515.

2,5-Dioxopyrrolidin-1-yl 3-(pent-4-yn-1-yloxy)propanoate (7).

Following the general procedure, 305 mg (1.2 mmol, 94% yield) of **7** were obtained from 0.2 g (1.28 mmol) of 3-(pent-4-yn-1-yloxy)propanoic acid **34**. The compound was purified by flash chromatography (7:3 to 6:4 hexane/EtOAc gradient). IR (film): $v =$ 2929, 2873, 1815, 1782, 1736, 1205, 1066 cm⁻¹. ¹H NMR (δ, 400 MHz, CDCl₃): 3.80 (t, *J* = 6.4 Hz, 2H), 3.57 (t, *J* = 6.1 Hz, 2H), 2.88 (t, *J* = 6.4 Hz, 2H), 2.81–2.87 (m, 4H), 2.29 (td, *J* = 7.1, 2.6 Hz, 2H), 1.94 (t, *J* = 2.7 Hz, 1H), 1.75–1.84 (m, 2H).¹³C NMR (*δ*, 100 MHz, CDCl3): 169.06, 166.85, 84.08, 69.60, 68.59, 65.39, 32.29, 28.54, 25.72, 15.22. HRMS calculated for C₁₂H₁₅NO₅Na: 276.0848 [M+Na]⁺. Found: 276.0843.

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