Supplementary Information

Photoneutralization of Insulin in High Vacuum

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Cleavage conditions

All photocleavage experiments were performed with the frequency-quadrupled output of a short-pulse Nd:YAG laser whose beam was aligned antiparallel to the molecular ion beam. The laser had a pulse length of 10 ns, a wavelength of 266 nm in an essentially flat-top circular beam diameter of 2 mm at the location of the molecules. The laser was operated with a rate of 250 Hz and an energy of 200 µJ for tripeptides, at 500 Hz and 400 µJ for the nonapeptides, and at 1000 Hz and 400 µJ for insulin. The mass spectrometer readout was synchronized with the laser, to ensure that only those molecules were counted that had interacted with the laser pulse once. Data were recorded for 120 sec in negative ion mode and for 240 sec for positive ion mode.

Peptide spray

1 mg of peptide powder was dissolved in 1 ml of a 50:50 vol-%mixture of methanol and deionized water which was enriched by vol-1% of formic acid for experiments in positive mode and vol-1% of ammonia for the negative mode. The electrospray conditions were optimized for each species. Typical values an injection flow of 5 µl/min, a capillary voltage of +3.5 kV for positive and -3 kV for measurements in the negative mode. Nitrogen was used as sheath gas with a flow rate of 22 l/hr and as desolvation gas at 50 l/hr. The desolvation temperature was set to 140°C and the source temperature to 100°C.
Suppl. Figure 1. Photon induced cleavage for charge reduction.

Electrospray ionization volatilizes the peptides. The particles can be charge-reduced by collision with an ionized sheath gas before they are mass selected (m/z ≤ 30 kDa) in the quadrupole mass filter, top left. Cooling and/or collision induced dissociation can be realized in a buffer gas loaded hexapole ion guide. The ions in the interaction region are hit by a counter-propagating single UV laser pulse to trigger photodissociation of a photocleavable tag (PCT), bottom right. The surviving ions are detected by a time-of-flight mass spectrometer.

Charge reduction of labelled insulin (posIns) in positive ion-mode

Insulin (posIns) labelled with three photocleavable leaving groups (posLG) was charge reduced by photocleavage in positive ion mode from charge state +5 to +4. The spectra showed additional fragments in the low mass region (low mass in Suppl. Figure 2) compared to the charge reduction of negIns in negative ion mode (Fig.2 of the main text). Analysis of the mass signals of the leaving group posLG suggests that cleavage under H-transfer is somewhat favoured over homolysis in contrast to the observations with negIns where homolysis is favoured.
Suppl. Figure 2. Charge reduction of an ion beam of an insulin construct (posIns) equipped with three positively charge leaving groups (posLG) in positive ion mode. Black line: mass spectrum without laser irradiation; red line: mass spectrum with laser radiation at 266 nm.

Synthesis

Chemicals were purchased from Sigma Aldrich, Fluorochem, Novabiochem or Bachem and used as received unless otherwise noted. UPLC-MS analysis was performed with an Acquity UPLC-H Class Bio from Waters equipped with a PDA and a SQ detector 2 and the following column: ACQUITY UPLC, HSS T3 1.8 µm, 2.1 x 100 mm. Preparative HPLC separations were carried out with a Water Prep LC 4000 System and the following column: Agilent: XDB-C18, 21.2 x 150 mm, 5 µm. HRMS experiments were conducted with a Bruker maXis 4G. NMR experiments were performed at 25°C on Bruker Avance III NMR spectrometers operating at 600 or 500 MHz proton frequency. NMR spectrometers were equipped with inverse dual channel, broadband probe heads with z -gradients. $^{13}$C shifts were determined by 2D NMR experiments (HMBC and HMQC). $^{1}$H and $^{13}$C signals were assigned by 2D NMR (COSY, HMBC, HMQC). All $^{1}$H-NMR spectra below were recorded in DMSO-d$_6$ at 500 MHz and at room temperature (298 K).

Ultra-Performance Liquid Chromatography (UPLC): Column: ACQUITY UPLC, HSS T3 1.8 µm, 2.1 x 100 mm. Solvents were water and acetonitrile, respectively, each containing 0.1%
formic acid, later on referred to as (A) and (B). The flow rate was set to 0.61 mL/min and the
temperature to 40 °C. Method 1: 0 min – 90% A; 1 min – 90% A; 6 min – 100% B; 7.5 min –
100% B. Method 2: 0 min – 100% A; 1 min – 100% A; 6 min – 100% B; 7.5 min – 100% B.

Intermediate peptides 3 and 4 were synthesized by standard Fmoc-SPPS following a procedure
published previously.[23]

![Peptide Structure](image)

**Alkyne 5**

(3-Bromo-4-nitrophenyl)methanol (348 mg, 1.50 mmol, 1.00 eq.) was dissolved in triethylamine (15 mL). The solution was degassed by bubbling with nitrogen for 20 min before Cul (5.7 mg, 2 mol% and tetrakis-(triphenylphosphine)palladium (17.3 mg, 1 mol%) were
added and the reaction mixture degassed for another 10 min. Trimethylsilylacetylene (320 µl,
2.25 mmol, 1.50 eq.) was added and the reaction stirred under reflux overnight. After filtration
through Celite, tert-butyl methyl ether (100mL) was added and the solution was washed with
water (2 × 100 mL) and brine (100 mL). Volatiles were removed under reduced pressure and
the residue purified by column chromatography (cyclohexane /ethyl acetate, 10:1). 5 was
isolated as a slightly yellow solid (267 mg, 1.07 mmol, 71%).

![Alkyne 5 Structure](image)

$^1$H-NMR (500 MHz, MeOH-d$_4$, 298 K) δ 8.04 (s, 1 H, H-2), 7.83 (d, $^3$J$_{H,H}$ = 8.1 Hz, 1 H, H-5), 7.72 (d, $^3$J$_{H,H}$ = 8.1 Hz, 1 H, H-4), 4.93 (s, 2 H, H-7), 0.26 (s, 9 H, H-10); δ $^{13}$C (determined
by HMQC and HMBC experiments) δ 148.0 (C-1), 139.2 (C-6), 137.0 (C-4), 129.4 (C-5),
127.9 (C-2), 123.9 (C-3), 103.0 (C-8), 96.8 (C-9), 61.3 (C-7), -0.6 (C-10).

**Compound 6**

5 (249 mg, 1.00 mmol, 1.00 eq.), sodium 2,3,5,6-tetrafluoro-4-hydroxybenzenesulfonate (268
mg, 1.00 mmol, 1.00 eq.) and triphenylphosphine (315 mg, 1.20 mmol, 1.20 eq.) were
dissolved in dry DMF and cooled to 0°C. Diisopropyl-azodicarboxylate (294 µl, 1.50 mmol,
1.50 eq.) was added dropwise over 15 min. After completed addition the reaction was allowed
to warm to room temperature and stirring continued overnight. For work up volatiles were removed under reduced pressure and the remaining residue subjected to preparative HPLC. After lyophilization 6 was isolated as a brown solid (155 mg, 0.325 mmol, 33%).

UPLC-MS (ES-): 476.8 [100%, M - H+]. 1H-NMR (500 MHz, DMSO-d6, 298 K) δ 8.15 (d, 4J_H-H = 1.6 Hz, 1 H, H-2), 7.89 (dd, 3J_H-H = 8.1 Hz, 4J_H-H = 1.6 Hz, 1 H, H-4), 7.85 (d, 3J_H-H = 8.1 Hz, 1 H, H-5), 5.65 (s, 2 H, H-7), 0.26 (s, 9 H, H-14); δ 13C (determined by HMQC and HMBC experiments) δ 147.1 (C-1), 144.1 (C-9 or C-10), 142.1 (C-9 or C-10), 136.5 (C-4), 136.2 (C-8), 132.0 (C-6), 130.1 (C-5), 127.6 (C-2), 123.3 (C-3), 121.4 (C-11) 102.1 (C-12), 97.8 (C-13), 72.4 (C-7), -0.3 (C-14).

**Compound 7**

The trifluoroacetic acid salt of (2-aminoethyl)trimethylammonium trifluoroacetate was synthesized as described elsewhere [24].

The trifluoroacetic acid salt of (2-aminoethyl)trimethylammonium trifluoroacetate (1.51 g, 7.00 mmol, 1.00 eq.), 2,3,5,6-tetrafluoro-4-hydroxybenzoic acid (1.47 g, 7.00 mmol, 1.00 eq.), HBTU (2.92 g, 7.70 mmol, 1.10 eq.) and DIPEA (5.78 mL, 35.0 mmol, 5.00 eq.) were dissolved in DMF (200 mL) and stirred overnight at room temperature. Full conversion was confirmed by UPLC-MS. The solvent was removed under reduced pressure and the residue was directly used for the next step without further purification. A fraction was purified by preparative reversed phase HPLC for analytical purposes.

HRMS: calculated for: C13H15F4N2O2+: 295.1064; found 295.1066; 1H-NMR (500 MHz, DMSO-d6, 298 K) δ 9.06 (t, 3J_H-H = 5.7 Hz, 1 H, H-4), 3.70 (td, 3J_H-H = 6.8 Hz, 4J_H-H = 5.7 Hz, 2 H, H-3), 3.49 (t, 3J_H-H = 6.8 Hz, 2 H, H-2), 3.13 (s, 9 H, H-1); δ 13C (determined by HMQC and HMBC experiments) δ 158.2 (C-5), 63.0 (C-2), 52.3 (C-1), 33.2 (C-3), C-6, C-7, C-8 and C-9 not visible.
Compound 8

7 (crude, 1.60 g, 1.90 mmol, 1.00 eq.), 5 (474 mg, 1.90 mmol, 1.00 eq.) and PPh₃ (648 mg, 2.47 mmol, 1.30 eq.) were dissolved in dry DMF and the mixture cooled to 0 °C. DIAD (560 µl, 2.85 mmol, 1.50 eq.) was added slowly and the reaction was stirred at room temperature for 5 h. The solvent was removed under reduced pressure and the product was isolated from the residue by preparative reversed phase HPLC. 8 was isolated after lyophilisation as a brown solid (98.0 mg, 0.186 mmol, 10%).

HRMS: calculated for: C₂₄H₂₈F₄N₃O₄Si⁺: 526.1780; found 526.1780; ¹H-NMR (500 MHz, DMSO-d₆, 298 K) δ 9.21 (t, ³J_H-H = 5.8 Hz, 1 H, H-4), 8.17 (d, ⁴J_H-H = 1.7 Hz, 1 H, H-15), 7.93 (dd, ³J_H-H = 8.0 Hz, ⁴J_H-H = 1.7 Hz, 1 H, H-13), 7.87 (d, ³J_H-H = 8.0 Hz, 1 H, H-12), 5.72 (s, 2 H, H-10), 3.73 (td, ³J_H-H = 6.8 Hz, ⁴J_H-H = 5.8 Hz, 2 H, H-3), 3.49 (t, ³J_H-H = 6.8 Hz, 2 H, H-2), 3.14 (s, 9 H, H-1), 0.26 (s, 9 H, H-19); ¹³C (determined by HMQC and HMBC experiments) δ 157.7 (C-5), 147.3 (C-16), 136.5 (C-13), 131.6 (C-11), 130.0 (C-12), 127.6 (C-15), 123.4 (C-14), 101.8 (C-17), 97.8 (C-18), 72.4 (C-10), 63.0 (C-2), 52.4 (C-1), 33.5 (C-3), -0.5 (C-19), C-6, C-7, C-8 and C-9 not visible.

Photocleavable tripeptide 1-negLG

General procedure 1. To a suspension of 3 (20.0 mg, 56.2 µmol, 2.00 eq.) and 6 (13.9 mg, 28.1 µmol, 1.00 eq.) in water / tert-butanol (1 : 1. 5 mL) was added aq. sodium ascorbate (1 M, 200 µl, 7.12 eq.) and aq. CuSO₄ (1 M, 20 µl, 0.71 eq.) and the resulting mixture was stirred for 4 h at 55 °C. Subsequently volatiles were removed under reduced pressure and the resulting residue was purified by reversed phase HPLC. The product was obtained as a white powder after lyophilisation (6.4 mg, 8.4 µmol, 30%).

HRMS: calculated for: C₃₀H₃₅F₄N₇NaO₁₀S⁺: 784.1994; found 784.2001. ¹H-NMR (500 MHz, DMSO-d₆, 298 K) δ 8.83 (s, 1 H, H-14), 8.58 (d, ³J_H-H = 7.6 Hz, 1 H, H-7), 8.56 (d, ⁴J_H-H = 1.8
Hz, 1 H, H-21), 8.25 (dd, $^3J_{H-H}$=8.0 Hz, $^4J_{H-H}$ = 1.8 Hz, 1 H, H-17), 8.22 (d, $^3J_{H-H}$ = 8.1 Hz, 1 H, H-4), 8.04 (b, 3 H, H-27), 7.92 (d, $^3J_{H-H}$ = 8.1 Hz, 1 H, H-18), 5.68 (s, 2 H, H-22), 4.45-4.36 (m, 3 H, H-13 and H-6), 4.23 (ddd, $^3J_{H-H}$ =9.3 Hz, $^3J_{H-H}$ =8.0 Hz, $^3J_{H-H}$ = 5.8 Hz, 1 H, H-3), 3.75 (b, 1 H, H-9), 1.89 (p, $^3J_{H-H}$ = 7.1 Hz, 2 H, H-12), 1.75-1.69 (m, 2 H, H-10), 1.67-1.59 (m, 1 H, H-30), 1.57-1.47 (m, 2 H, H-29), 1.38-1.29 (m, 2 H, H-11), 1.25 (d, $^3J_{H-H}$ = 7.0 Hz, 3 H, H-28), 0.88 (d, $^3J_{H-H}$ = 6.6 Hz, 3 H, H-31 or H-32), 0.83 (d, $^3J_{H-H}$ = 6.5 Hz, 3 H, H-31 or H-32); $^{13}$C (determined by HMQC and HMBC experiments) $\delta$ 173.5 (C-2), 171.3 (C-5), 167.8 (C-8), 147.7 (C-20), 143.7 (C-15), 132.2 (C-16), 130.7 (C-18), 129.9 (C-19), 129.6 (C-17), 122.5 (C-14), 120.6 (C-21), 72.2 (C-22), 51.6 (C-9), 49.9 (C-3), 49.2 (C-13), 47.7 (C-6), 39.6 (C-29), 30.4 (C-10), 28.7 (C-12), 24.1 (C-30), 22.6 (C-31 or C-32), 21.0 (C-31 or C-32), 20.9 (C-11), 17.9 (C-28). C-23, 24, 25 and 26 are not visible).

**Photocleavable nonapeptide 2-negLG**

The synthesis followed the protocol for the synthesis of **1-negLG** (General procedure 1) starting with 4 (34.0 mg, 40.5 μmol, 2.00 eq.). The modified peptide was isolated as a white solid (7.0 mg, 5.5 μmol, 27%).

![Chemical structure of 2-negLG](image_url)

HRMS: calculated for: C$_{52}$H$_{73}$F$_{4}$N$_{13}$NaO$_{16}$S$^+$: 1266.4847; found 1266.4842; $^1$H-NMR (500 MHz, DMSO-d$_6$, 298 K) $\delta$ 8.83 (s, 1 H, H-1), 8.57 (d, $^3J_{H-H}$ = 7.6 Hz, 1 H, amid), 8.56 (d, $^4J_{H-H}$ = 1.8 Hz, 1 H, H-8) 8.25 (dd, $^3J_{H-H}$ = 8.0 Hz, $^4J_{H-H}$ = 1.8 Hz, 1 H, H-4), 8.14 - 8.00 (m, 7 H, 4 amide, 3 ammonium), 7.99 (d, $^3J_{H-H}$ = 7.8 Hz, 1 H, amid), 7.95 (d, $^3J_{H-H}$ = 7.4 Hz, 1 H, amid), 7.92 (d, $^3J_{H-H}$ = 8.1 Hz, 1 H, H-5), 7.84 (d, $^3J_{H-H}$ = 7.6 Hz, 1 H, amid), 5.68 (s, 2 H, H 9), 4.44-4.35 (m, 3 H, 2 H-C(α(lys)) and H-Cα), 4.35-4.16 (m, 5 H, H-Cα), 3.77-3.62 (m, 5 H, H-Cα), 1.89 (p, $^3J_{H-H}$ = 7.3 Hz, 2 H, H-C(α(lys)), 1.75-1.67 (m, 2 H, H-C(β(lys)), 1.66-1.57 (m, 3 H, H-C(γ(lys)), 1.56-1.43 (m, 6 H, H-C(β(ileu)), 1.38-1.31 (m, 2 H, H-C(γ(ileu)), 1.24 (d, $^3J_{H-H}$ = 7.1 Hz, 3 H, H-C(β(ala)), 1.20(d, $^2J_{H-H}$ = 7.2 Hz, 6 H, H-C(β(ala)), 0.91-0.85 (m, 9 H, H-C(α(ileu)), 0.85-0.80 (m, 9 H, H-C(β(ileu)).
Photocleavable tripeptide 1-posLG

The synthesis followed the protocol for the synthesis of 1-negLG (General procedure 1) starting with 3 (20.0 mg, 56.2 μmol, 2.00 eq.) and 8 (14.8 mg, 28.1 μmol, 1.00 eq.). The modified peptide was isolated as a white solid (4.5 mg, 4.5 μmol, 16%).

![Chemical structure of 1-posLG]

HRMS: calculated for: C_{36}H_{48}F_{4}N_{9}O_{8}+: 810.3556; found 810.3564; \(^1\)H-NMR (500 MHz, DMSO-d<sub>6</sub>, 298 K) \(\delta\) 9.25 (t, \(^3\)J<sub>H-H</sub> = 5.8 Hz, 1 H, H-28), 8.85 (s, 1 H, H-14), 8.60 (d, \(^3\)J<sub>H-H</sub> = 7.5 Hz, 1 H, H-7), 8.58 (d, \(^4\)J<sub>H-H</sub> = 1.8 Hz, 1 H, H-21), 8.29 (dd, \(^3\)J<sub>H-H</sub> = 8.1 Hz, \(^4\)J<sub>H-H</sub> = 1.8 Hz, 1 H, H-17), 8.23 (d, \(^3\)J<sub>H-H</sub> = 8.0 Hz, 1 H, H-4), 8.09 (b, 3 H, H-32), 7.96 (d, \(^3\)J<sub>H-H</sub> = 8.1 Hz, 1 H, H-18), 5.74 (s, 2 H, H-22), 4.46-4.36 (m, 3 H, H-13 and H-6), 4.22 (ddd, \(^3\)J<sub>H-H</sub> = 9.4 Hz, \(^3\)J<sub>H-H</sub> = 5.6 Hz, 1 H, H-3), 3.77 (b, 1 H, H-9), 3.73 (td, \(^3\)J<sub>H-H</sub> = 6.9 Hz , \(^3\)J<sub>H-H</sub> = 5.8 Hz, 2 H, H-29), 3.50 (t , \(^3\)J<sub>H-H</sub> = 6.9 Hz, 2 H, H-30), 3.14 (s, 9 H, H-31), 1.93-1.85 (m, 2 H, H-12), 1.77-1.70 (m, 2 H, H-10), 1.66-1.58 (m, 1 H, H-35), 1.55-1.49 (m, 2 H, H-34), 1.40-1.33 (m, 2 H, H-11), 1.25 (d, \(^3\)J<sub>H-H</sub> = 7.0 Hz, 3 H, H-33), 0.88 (d, \(^3\)J<sub>H-H</sub> = 6.6 Hz, 3 H, H-36), 0.82 (d, \(^3\)J<sub>H-H</sub> = 6.5 Hz, 3 H, H-36); \(^{13}\)C (determined by HMQC and HMBC experiments) \(\delta\) 173.7 (C-2), 171.4 (C-5), 167.7 (C-8), 157.6 (C-27), 147.7 (C-20), 143.6 (C-15), 137.6 (C-23), 132.3 (C-16), 130.6 (C-18), 129.8 (C-17 and C-19), 122.5 (C-14), 120.7 (C-21), 72.6 (C-22), 62.7 (C-30), 52.4 (C-31), 51.6 (C-9), 49.9 (C-3), 49.1 (C-13), 47.7 (C-6), 39.7 (C-34), 33.4 (C-29), 30.3 (C-10), 28.8 (C-12), 24.1 (C-35), 22.6 (C-36), 21.0 (C-36), 20.9 (C-11), 17.9 (C-33), C24, C-25 and C-26 not visible.

Photocleavable nonapeptide 2-posLG

The synthesis followed the protocol for the synthesis of 1-negLG (General procedure 1) starting with 4 (25.5 mg, 30.4 μmol, 2.00 eq.) and 8 (8.0 mg, 15.2 μmol, 1.00 eq.). The modified peptide was isolated as a white solid (5.7 mg, 3.8 μmol, 25%).

![Chemical structure of 2-posLG]
HRMS: calculated for: C_{58}H_{86}F_{4}N_{15}O_{14}+: 1292.6409; found 1292.6384; \!^{1}H-NMR (500 MHz, DMSO-d_{6}, 298 K) \!^{\delta} 9.23 (t, 1 H, H-14), 8.85 (s, 1 H, H-1), 8.59 (d, 3\!^{J_{H-H}} = 6.5 Hz, 1 H, amide), 8.58 (d, 4\!^{J_{H-H}} = 1.8 Hz, 1 H, H-8) 8.29 (dd, 5\!^{J_{H-H}} = 8.0 Hz, 6\!^{J_{H-H}} = 1.8 Hz, 1 H, H-4), 8.16-8.02 (m, 8 H, 4 amide, 3 ammonium), 8.00 (d, 2\!^{J_{H-H}} = 7.8 Hz, 1 H, amide), 7.98-7.93 (m, 2 H, H-5 and amide), 7.85 (d, 2\!^{J_{H-H}} = 7.5 Hz, 1 H, amide), 5.75 (s, 2 H, H-9), 4.46-4.37 (m, 3 H, 2 H-C\!_{\epsilon(Lys)} and H-C\!_{\alpha}), 4.35-4.16 (m, 5 H, H-C\!_{\alpha}), 3.77-3.62 (m, 7 H, H-C\!_{\alpha} and H-16), 3.50 (t, 2\!^{J_{H-H}} = 6.9 Hz), 3.14 (s, 9 H, H-17), 1.93-1.85 (m, 2 H, H-C\!_{\beta(Lys)}) 1.75-1.67 (m, 2 H, H-C\!_{\beta(Lys)}), 1.66-1.42 (m, 9 H, H-C\!_{\gamma(Leu)}, H-C\!_{\beta(Leu)}), 1.41-1.32 (m, 2 H, H-C\!_{\gamma(Lys)}), 1.25 (d, 2\!^{J_{H-H}} = 7.0 Hz, 3 H, H-C\!_{\beta(ala)}), 1.20(d, 2\!^{J_{H-H}} = 7.2 Hz, 6 H, H-C\!_{\beta(ala)}), 0.91-0.85 (m, 9 H, H-C\!_{\beta(Leu)}), 0.85-0.80 (m, 9 H, H-C\!_{\beta(Leu)}).

**Compound 9**

To a suspension of 2-azidoacetic acid (28.2 mg, 279 \(\mu\text{mol}, 1.50 \text{ eq.}\)) and 6 (88.8 mg, 186 \(\mu\text{mol}, 1.00 \text{ eq.}\)) in water / tert-butanol (1 : 1, 10 mL) was added aq. sodium ascorbate (1.00 M, 186 \(\mu\text{l}, 1.00 \text{ eq.}\)) and aq. CuSO\(_{4}\) (1.00 M, 37.0 \(\mu\text{l}, 0.20 \text{ eq.}\)) and the resulting mixture was stirred for 2 h at 55 °C. Subsequently volatiles were removed under reduced pressure and the resulting residue was purified by reversed phase HPLC. The product was obtained as a white powder after lyophilisation (26.0 mg, 49.2 \(\mu\text{mol} 27\%\)).

![Image](https://via.placeholder.com/150)

**UPLC-MS (ES-) m/z = 505.0 [100%, M - H\(^{+}\)], 251.9 [20%, M – 2 H\(^{+}\)]; \!^{1}H-NMR (500 MHz, D\(_2\)O, 298 K) \!^{\delta} 8.52 (d, 2\!^{J_{H-H}} = 1.8 Hz, 1 H, H-2), 8.48 (s, 1 H, H-13), 8.12 (dd, 3\!^{J_{H-H}} = 8.1 Hz, 4\!^{J_{H-H}} = 1.8 Hz, 1 H, H-4), 7.87 (d, 3\!^{J_{H-H}} = 8.1 Hz, 1 H, H-5), 5.71 (s, 2 H, H-7), 5.40 (s, 2 H, H-14); \!^{13}C (determined by HMQC and HMBC experiments) \!^{\delta} 171.4 (C-15), 148.0 (C-1), 145.7 (C-12), 139.1 (C-8), 132.0 (C-6), 131.9 (C-3), 131.6 (C-4), 131.5 (C-5), 125.1 (C-13), 122.7 (C-2), C-9, C-10 and C-11 are not visible.**

**Compound 10**

To a suspension of 2-azidoacetic acid (115 mg, 1.00 mmol, 2.00 eq.) and 8 (320 mg, 0.50 mmol, 1.00 eq.) in water / tert-butanol (1 : 1, 20 mL) was added aq. sodium ascorbate (1 M, 500 \(\mu\text{l}, 1.0 \text{ eq.}\)) and aq. CuSO\(_{4}\) (1 M, 100 \(\mu\text{l}, 0.2 \text{ eq.}\)) and the resulting mixture was stirred for
2 h at 55 °C. Subsequently volatiles were removed under reduced pressure and the resulting residue was purified by reversed phase HPLC. The product was obtained as a white powder after lyophilisation (280 mg, 429 µmol, 86%).

HRMS: calculated for: C_{23}H_{23}F_{4}N_{6}O_{6}+: 555.1610; found 555.1612; \(^1\)H-NMR (500 MHz, DMSO-d<sub>6</sub>, 298 K) δ 9.27 (t, \(^3\)J\textsubscript{H-H} = 5.7 Hz, 1 H, H-4), 8.82 (s, 1 H, H-18), 8.61 (d, \(^4\)J\textsubscript{H-H} = 1.8 Hz, 1 H, H-15), 8.32 (dd, \(^3\)J\textsubscript{H-H} = 8.0 Hz, \(^4\)J\textsubscript{H-H} = 1.8 Hz, 1 H, H-13), 7.95 (d, \(^3\)J\textsubscript{H-H} = 8.0 Hz, H-12), 5.75 (s, 2 H, H-10), 5.36 (s, 2 H, H-19), 3.73 (q, \(^3\)J\textsubscript{H-H} = 6.5 Hz, 2 H, H-3), 3.50 (t, \(^3\)J\textsubscript{H-H} = 6.8 Hz, 2 H, H-2), 3.14 (s, 9 H, H-1), \(^{13}\)C (determined by HMQC and HMBC experiments) δ 168.9 (C-20), 158.3 (C-5), 148.4 (C-16), 144.3 (C-17), 138.2 (C-9), 131.5 (C-11), 130.6 (C-12), 129.7 (C-13), 124.0 (C-18), 120.6 (C-15), 72.5 (C-10), 62.8 (C-2), 52.2 (C-1), 50.7 (C-19), 33.4 (C-3), C-6, C-7 and C-8 are not visible.

\textbf{negIns}

\section{negIns}

9 (10.0 mg, 18.9 µmol, 3.84 eq.), \(N,N,N',N''\)-tetramethyl-\(O\)-(N-succinimidyl)uronium tetrafluoroborate (TSTU, 5.90 mg, 19.7 µmol, 4.00 eq.) and \(N,N\)-diisopropylethylamine (DIPEA, 6.5 µl 39.4 µmol, 8.00 eq.), were dissolved in DMF (2 mL) and stirred for 1 h at room temperature. Then the solution was added to insulin (30.0 mg, 4.93 µmol, 1.00 eq.) dissolved in DMF (2 mL) and stirred for 2 h at room temperature afterwards the solvent was removed under reduced pressure and the crude was purified by preparative HPLC. \textbf{negIns} was isolated after lyophilisation (2.1 mg, 0.31 µmol, 18%).

UPLC-MS, Solvents were water and acetonitrile, respectively, each containing 0.1% formic acid, later on referred to as (A) and (B). The flow rate was set to 0.61 mL/min and the temperature to 40 °C. Method 1: 0 min – 90% A; 1 min – 90% A; 6 min – 100% B; 7.5 min – 100% B., \(T_R = 4.00\) min; m/z MS (ES+): 1455.7 [30%, M^{3+} + 8 H^{+}], 1819.0 [80%, M^{3+} + 7 H^{+}], 2425.2 [100%, M^{3+} + 6 H^{+}].
Suppl. Figure 3. Human insulin and its charged functionalization. Amino acid sequence of human insulin.

The three photocleavable tags (PCTs) are likely connected at the two N-termini and the side chain of the single lysine residue. The negative charge of $\text{negLG}^-$ is located on the $-\text{SO}_3^-$ group. The construct has a mass of 7271 Da.

**posIns**

10 (9.3 mg, 14.2 µmol, 2.54 eq.), TSTU (5.1 mg, 16.8 µmol, 3.00 eq.), DIPEA (2.8 µl, 16.8 µmol, 3.00 eq.), were dissolved in DMF (1 mL) and stirred for 1 h at room temperature. The solution was added to insulin (32.5 mg, 5.60 µmol, 1.00 eq.) dissolved in DMF (10 mL) and stirred for 2 h at room temperature. Subsequently the solvent was removed under reduced pressure and **posIns** isolated by preparative HPLC. The sample was finally lyophilised (3.4 mg, 0.46 µmol, 8%).

UPLC-MS method 1, $T_R = 4.00$ min; m/z MS (ES+): 928.2 [20%, $M^{3+} + 5\ \text{H}^+$], 1060.6 [60%, $M^{3+} + 4\ \text{H}^+$], 1237.1 [100%, $M^{3+} + 3\ \text{H}^+$], 1484.4 [60%, $M^{3+} + 2\ \text{H}^+$], 1855.5 [20%, $M^{3+} + \text{H}^+$].
Spectra and chromatography traces

Suppl. Figure 4: $^1$H-NMR (500 MHz, 298K, DMSO-d$_6$) of 1-negLG.

Suppl. Figure 5: UPLC of 1-negLG.
Suppl. Figure 6: $^1$H-NMR (500 MHz, 298K, DMSO-d$_6$) of 2-negLG.

Suppl. Figure 7: UPLC of the tripeptide 2-negLG.
Suppl. Figure 8: $^1$H-NMR (500 MHz, 298K, DMSO-d$_6$) of 1-posLG.

Suppl. Figure 9: UPLC of 1-posLG.
Suppl. Figure 10: $^1$H-NMR (500 MHz, 298K, DMSO-d$_6$) of 2-posLG.

Suppl. Figure 11: UPLC of 2-posLG.
Suppl. Figure 12: UPLC of negIns.

Suppl. Figure 13: UPLC of posIns.
Suppl. Figure 14: Photocleavage neutralization of negIns. The spectrum plotted in black shows mass-selected negIns\(^{-}\) before irradiation; the spectrum plotted in red was recorded under irradiation with 266 nm light.