Supporting information

A Synthetic Approach to 'Click' *Neo*glycoprotein Analogues of EPO Employing One-Pot Native Chemical Ligation and CuAAC Chemistry

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Table of contents

Experimental

Reagents

All solvents and reagents were used as supplied. Dicyclohexylcarbodiimide (DCC), Fmoc-amino acids, S-Trityl-*β*-mercaptopropionic acid and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from GL Biochem (Shanghai, China). Sugar azides (**4**-**7**)and *N*-Boc-L-propargylglycine (**26**) were prepared as reported in our previous work [1-4]. *p*-Cresol*, N*,*N*'-diisopropylethylamine (DIPEA), ethanedithiol (EDT), methoxylamine hydrochloride, 4- mercaptophenylacetic acid (MPAA), piperidine, and triisopropylsilane (TIS) were purchased from Sigma Aldrich (St Louis, MO). *N*-methylpyrrolidine (NMP) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl) were purchased from Fluka (Buchs, Switzerland). Trifluoroacetic acid (TFA) was obtained from Halocarbon (River Edge, NJ). Boc-L-thiazolidine- 4-carboxylic acid was purchased from NovaBiochem (San Diego, CA). HF was purchased from Matheson Tri-Gas (Basking Rigde, NJ). VA-044 was purchased from Wako Chemicals (Osaka, Japan). Boc amino acids, Boc-Ala-PAM linker and Fmoc-Asp (OtBu)-HMPP linker were purchased from Polypeptides (Strasbourg, France). Amino methyl polystyrene resin (AM-PS) was made as previously described [5].

Peptide Analysis and Purification

Crude peptide products were analysed for purity by analytical RP-HPLC (Dionex P680 equipped with a 4 channel UV detector) at 210 and 254 nm using a Phenomenex Gemini C_{18} column (110 Å, 2.0 mm x 50 mm; 5 µm) at 0.2 ml/min and a linear gradient of 5-65% solvent B over 30 min. The binary solvent system consisted of: 0.1% TFA (*v/v*) in H2O (A) and 0.1% TFA (*v/v*) in MeCN (B). Peptide identity was confirmed by LC-MS (Dionex Ultimate 3000 equipped with a Thermo Finnegan MSQ mass spectrometer) using ESI positive mode. Purification of crude peptides was performed by semi-prep RP-HPLC (Dionex P680 equipped with a 4 channel UV detector) at 210 and 254 nm using a Phenomenex Gemini C18 column (110 Å, 10 mm x 250 mm; 5 µm)at 5.0 ml/min using a shallow gradient of increasing concentrations of solvent B as required for individual peptides. The binary solvent system consisted of: 0.1% TFA (*v/v*) in H2O (A) and 0.1% TFA (*v/v*) in MeCN (B). Fractions containing the pure target peptide were identified by analytical RP-HPLC, then combined and lyophilised. In some instances, for the larger ligated peptides, peptide analysis made use of a Phenomenex

Jupiter C4 column (300 Å, 50 mm x 2.0 mm; 5 µm) with a 0.2 ml/min flowrate, as specified in the respective figure legends. A Phenomenex Jupiter C4 column (300 Å, 250 mm x 10 mm; 5 µm) semi-pep column was employed for SPE and RP-HPLC purification for larger peptides in specified instances, with a flow rate of 5.0 ml/min. High resolution electrospray ionization mass spectrometry (ESI-MS) of purified peptides presented in figures were recorded on recorded on a Bruker micrOTOFQ mass spectrometer.

Synthesis of EPO1 - EPO4 Peptides (**8-11**)

Peptides were prepared manually by following a literature procedure; "*in situ* neutralization" Boc-chemistry stepwise solid phase peptide synthesis [6], employing HBTU / DIPEA for sequential amino acid couplings. Peptides were synthesised at 0.2 mmol scale on Boc-Ala-Pam-AM-PS resin to which the thioester generating linker *S*-trityl mercaptopropionic acid was installed. Side-chain protection for amino acids was as follows: Arg(Tos, Tos = tosyl), Asp(OcHex, cHex = Cyclohexyl), Asn(Xan, Xan = 9-xanthenyl), Cys(4-MeBzl, Bzl = benzyl), Cys(Acm, Acm = acetomidomethyl), Gln(Xan), Glu(OcHex), His(Tos), Lys(2-Cl-Z, Z = carboxybenzyl), Ser(Bzl), Thr(Bzl), Trp(CHO), Tyr(2-Br-Z). Boc-L-thiazolidine-4-carboxylic acid (Thz, Boc-L-thiaproline) was introduced to protect the N-terminal Cys of EPO3 and EPO4 peptides. The resulting peptides were then side-chain deprotected (excluding Trp(CHO) residues) and simultaneously cleaved from the resin support by treatment with anhydrous HF containing 10% (*v/v*) *p*-cresol for 1 h at 0 ^{o}C. After evaporation of the HF under reduced pressure, the crude products were precipitated and triturated with cold diethyl ether. The peptide products were then dissolved in 50% aqueous acetonitrile and lyophilised to obtain the crude product before purification by RP-HPLC. After purification by RP-HPLC and lyophilisation, EPO1 – EPO4 (**8** - **11**) peptides were obtained as a fluffy white powders. EPO1 (**8**) (55 mg, 6.1% yield). EPO2 (**9**) (38 mg, 4.1% yield). EPO3 (**10**) (40 mg, 5.7% yield). EPO4 (**11**) (80 mg, 12.6% yield). For characterisation, see **Figure S1 – S4**.

Synthesis of EPO5 (**12**)

Solid phase peptide synthesis was performed using a Liberty Microwave Peptide Synthesiser (CEM Corporation, Mathews, NC) using the Fmoc/tBu strategy. The peptide was synthesised on Fmoc-L-Asp(O*t*Bu)-HMPP-AM-PS resin at 0.2 mmol scale. Side chain protection of amino

acids was as follows: Arg(Pbf, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Asn(Trt, Trt = trityl), Asp(tBu), Cys(Trt), Gln(Trt), Glu(tBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Tyr(tBu) and Trp(Boc). The Fmoc group was deprotected with 20% *v/v* piperidine in DMF for 30 seconds followed by a second deprotection for 3 min using a microwave power of 60 W for both deprotections. The maximum temperature for both deprotections was set to 75°C. The coupling step was performed with 5 equivalents of the Fmoc protected amino acid in DMF (0.2 M), 4.5 equivalents of HBTU in DMF (0.45 M) and 10 equivalents of DIPEA in NMP (2 M). All couplings were performed for 5 min at 25 W with a maximum temperature of 75 °C, except for the following amino acids: Fmoc–Arg(Pbf)–OH which was double coupled using a 25 min room temperature coupling followed by a 5 min period at 25 W / 75 °C; Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH couplings were performed for 5 min at 25 W at a maximum temperature of 50 °C. The peptide was liberated from the resin employing a cocktail of TFA/TIS/H_2O/EDT (94/1/2.5/2.5, *v/v/v/v*). The crude peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in 1:1 (*v/v*) acetonitrile:water and lyophilised to afford the crude peptide. After purification by RP-HPLC and lyophilisation, EPO5 (**12**) peptide was obtained as a fluffy white powder (55 mg, 6.1% yield). For characterisation, see **Figure S5**.

Peptide Manipulations

EPO4-5 (14)

EPO4 (**11**) (50.0 mg, 15.7 µmol, 3 mM) and EPO5 (**12**) (70.4 mg, 15.7 µmol, 3 mM) were dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (5.25 mL) containing MPAA (176 mg, 1.05 mmol, 200 mM) and TCEP.HCl (75.0 mg, 263 µmol, 50 mM) and pH adjusted to 6.8. Monitoring by LC-MS revealed the reaction was complete after 2 h at which time, methoxylamine hydrochloride (65.8 mg, 788 µmol, 150 mM) was added and the pH adjusted to 4.0. After 3 h the Thz^{98} to Cys^{98} conversion was complete and the crude product was purified by reverse phase HPLC to yield EPO4-5 peptide **14** (47.0 mg, 6.28 µmol, 40% yield). The reaction scheme and analytical monitoring are presented in **Figure S6**.

‘Click’ EPO3-5 (15)

EPO3 (**10**) (2.05 mg, 0.58 µmol, 3 mM) and EPO4-5 (**14**) (4.12 mg, 0.55 µmol, 2.85 mM) were dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (195 µL) containing MPAA (0.65 mg, 3.87 µmol,

20 mM) and TCEP.HCl (2.23 mg, 7.74 µmol, 40 mM) and the pH adjusted to 6.8. As monitored by LC-MS, the reaction was complete after 7 h to afford the Thz^{68}-EPO3-5 ligation product upon which time, $CuSO_4$ (3.9 µL of 1 M solution, 3.9 µmol, 20 mM), TCEP.HCl (3.9 µL of 0.5 M solution, 1.95 µmol, 10 mM) and GalNAcα1-*O*-$(CH_2)_3$-N_3 (0.58 mg, 1.74 µmol, 9 mM) were added to the reaction mixture and the temperature elevated to 50 °C. After 5 h, the desired 'click' EPO3-5 *neo*glycopeptide **15** was purified by reverse phase HPLC (0.58 mg, 50.8 nmol, 8.8% yield) (Figure 17). The reaction scheme and analytical monitoring are presented in **Figure 2**. Note: during the click reaction, the analytical HPLC samples needed to be treated with TCEP prior to injection. Without this treatment the chromatogram appeared very poor, with suspected mixed disulfide formation. This reflects the mild oxidizing properties of Cu(I). Before semi-prep purification excess treatment with TCEP.HCl at 50 °C for 30 min was also required. This modification was also used in all subsequent reactions.

'Click' EPO4-5 (**17**)

EPO4 (**11**) (50.0 mg, 15.7 µmol, 3 mM) and EPO5 (**12**) (70.4 mg, 15.7 µmol, 3 mM) were dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (5.25 mL) containing MPAA (176 mg, 1.05 mmol, 200 mM) and TCEP.HCl (75.0 mg, 263 µmol, 50 mM) and the pH adjusted to 6.8. Monitoring by LC-MS revealed completion of reaction after 2.5 h at which time the reaction mixture was loaded onto a C4 semi-prep column and washed with 5% aqueous MeCN for 15 min until all MPAA was removed. Elution with 50% aqueous MeCN followed by lyophilization afforded the crude Thz^{98}-EPO4-5 ligation product (105 mg). This crude mixture of ligation product (105 mg, 3 mM) was dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (4.70 mL) with $CuSO_4$ (188 µL of 1 M solution, 188 µmol, 40 mM) and TCEP.HCl (26.9 mg, 94.0 µmol, 20 mM). The solution pH was adjusted to 7.0, GalNAcα1-*O*-$(CH_2)_3$-N_3 (7.75 mg, 23.5 µmol, 5 mM) was added and the temperature elevated to 40 °C for 2 h. The reaction mixture was then purified by reverse phase HPLC to afford pure Thz^{98} to Cys^{98} converted 'click' EPO4-5 peptide **17** (45.0 mg, 5.79 µmol, 37% yield). The reaction scheme and analytical monitoring are presented in **Figure 3**.

'Click'-EPO3-5 (**16**)

'Click'-EPO4-5 (**17**) (25.0 mg, 3.22 µmol, 3 mM) and EPO3 (**10**) peptide (11.3 mg, 3.22 µmol, 3 mM) were dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (1.07 mL) containing MPAA (18.0 mg, 107 µmol, 100 mM) and TCEP.HCl (12.2 mg, 42.9 µmol, 40 mM). The pH was adjusted to 6.8

and after 2 h LC-MS revealed the reaction was complete. The reaction mixture was loaded onto a C4 semi-pep column and washed with 5% aqueous MeCN for 15 min until all MPAA was removed. Elution with 50% aqueous MeCN then lyophilization afforded the crude monoglycosylated 'click'-Thz^{68}-EPO3-5 ligation peptide (32.0 mg). The crude ligation product (32.0 mg, 3 mM) was dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (943 µL) with $CuSO_4$ (37.7 µL of 1 M solution, 37.7 µmol, 40 mM) and TCEP.HCl (5.39 mg, 18.9 µmol, 20 mM) and the pH adjusted to 7.0. Glu-N_3 (0.97 mg, 4.72 µmol, 5 mM) was added to the reaction mixture, and temperature elevated to 50 ^oC. After 4 h the reaction mixture was purified by reverse phase HPLC to afford bisglycosylated 'click' EPO3-5 peptide **16** (7.80 mg, 0.69 µmol, 21% yield). The reaction scheme and analytical monitoring are presented in **Figure 3**.

EPO1-MPAA (**18**)

Under standard trans-thioesterification conditions at pH 6.8, significant hydrolysis of the thioester moiety was observed. This was ameliorated by lowering the pH to 6.1, in which case hydrolysis was virtually non-existent. EPO1 (**8**) (25.0 mg, 7.1 µmol, 3 mM) was dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (2.35 mL) containing MPAA (79.1 mg, 471 µmol, 200 mM) and TCEP.HCl (26.9 mg, 94.1 µmol, 40 mM) and the pH adjusted to 6.1. After 6 h, approximately 75% conversion was observed and the crude mixture purified by reverse phase HPLC to yield EPO1-MPAA thioester **18** (5.7 mg, 1.61 µmol, 23% yield). The reaction scheme and analytical monitoring are presented in **Figure S7**.

'Click' EPO1-MPAA (**20**)

EPO1 (**8**) (17.0 mg, 4.8 µmol, 3 mM) was dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (1.60 mL) with $CuSO_4$ (32 µL of 1 M solution, 32 µmol, 20 mM) and TCEP.HCl (4.56 mg, 15.9 µmol, 10 mM) and the pH adjusted to 6.3. GalNAc-N_3 (2.0 mg, 8.13 µmol, 5 mM) was added and the click reaction shaken at rt and was complete in 3 h. To the reaction mixture was added MPAA (26.9 mg, 160 µmol, 100 mM), the pH adjusted to 6.0 and the reaction stirred for 7 h. The crude product was purified by reverse phase HPLC to yield 'click' EPO1-MPAA thioester **20** (3.42 mg, 0.91 µmol, 19%). The reaction scheme and analytical monitoring are presented in **Figure S10**.

'Click' EPO1-2 (**21**)

'Click' EPO1 (**20**) (2.01 mg, 0.53 µmol, 3 mM) and EPO2 (**9**) (2.50 mg, 0.53 µmol, 3 mM) were dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (178 µL) and the pH adjusted to 6.3. Monitoring by LC-MS revealed completion of KCl after 6h and sodium 2-mercaptoethanesulfonate (MESNa) (2.51 mg, 17.8 µmol, 100 mM) was added to remove the 'click' EPO1-branched thioester by-product occurring at Cys^{30}, resulting in minor 'click' EPO1-$^{\alpha}COSCH_2CH_2SO_3H$ formation via thioester exchange. After 30 min, $CuSO_4$ (1 M, 3.4 µL of solution, 3.56 µmol, 20 mM), TCEP (0.51 mg, 1.78 µmol, 10 mM), and Gal-N_3 (0.17 mg, 0.89 µmol, 5 mM) were added to the reaction mixture to effect 'one-pot click reaction' at Pra^{38}. After 5 h, the crude product was purified by reverse phase HPLC to yield 'click' EPO1-2 (**21**) (0.35 mg, 0.041 µmol, 7.7% yield) (Figure 13). The reaction scheme and analytical monitoring are presented in **Figure 4**.

'click' EPO1-5 (**22**)

'click'-EPO1-2- (**21**) (4.55 mg, 0.535 µmol, 3 mM) and 'click'-EPO3-5 (**16**) (6.05 mg, 0.535 µmol, 3 mM) were dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (178 µL) containing MPAA (5.99 mg, 35.7 µmol, 200 mM) and TCEP.HCl (2.54 mg, 8.91 µmol, 50 mM). The pH was adjusted to 6.8 and monitored by LC-MS. After 3 h, completion of the reaction was observed and to the reaction mixture was added β-mercaptoethanol (400 µL) and piperidine (250 µL). Completion of deformylation was observed after 10 minutes at which time the reaction mixture was loaded onto a C4 semi-pep column and washed with 5% aqueous MeCN for 15 min until all MPAA was removed. Elution with 50% aqueous MeCN followed by lyophilization afforded the crude 'click'-EPO1-5[$Cys(Acm)^{7,29,33,161}$][$Cys^{30,68,98,128}$] *neo*glycoprotein product **22** (10.0 mg), which was used without further purification. The reaction scheme and analytical monitoring are presented in **Figure 5**.

'Click' EPO1-5 (**23**)

'Click' EPO1-5 (**22**) [$Cys(Acm)^{7,29,33,161}$][$Cys^{30,68,98,128}$] (10.0 mg, 2 mM), was dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 buffer (250 µL) containing TCEP.HCl (7.15 mg, 25 µmol, 100 mM), VA-044 (3.23 mg, 10 µmol, 40 mM) and glutathione (1.54 mg, 5 µmol, 20 mM). The pH was adjusted to 6.5 and the reaction mixture was gently stirred at 40 ^{o}C. After completion at 3 h, the reaction mixture was loaded onto a C4 semi-pep column and washed with 5% aqueous MeCN for 15 min until all non-retentive, material had eluted. Elution with 50% aqueous MeCN

followed by lyophilization afforded the crude desulfurised 'click' EPO1-5[Cys(Acm)^{7,29,33,161}][Ala^{30,68,98,128}] *neo*glycoprotein **23** (8.5 mg). The analytical monitoring is presented in **Figure S11**.

'Click' EPO1-5 (**24**)

'Click' EPO1-5 [Cys(Acm)^{7,29,33,161}] (**23**) (8.5 mg, 2 mM) was dissolved in 1:1 AcOH:H_2O (220 µL) and to this solution was added AgOAc (10.0 mg, 60.0 µmol, 272 mM). The reaction mixture was gently stirred at room temperature. Samples for monitoring by LC-MS required pre-treatment with dithiothreitol (DTT). After completion at 6 h, DTT (2 M in 6 M GnHCl, 250 µL) was added and stirred for 20 minutes, forming an off-white precipitate. The suspension was centrifuged and the supernatant, containing the EPO *neo*glycopeptide, was subjected to reverse phase HPLC purification using a C4 semi-pep column to yield pure 'click' EPO1-5 [Cys^{7,29,33,161}] *neo*glycoprotein **24** (2.10 mg, 21% yield over four steps from the final ligation of **21** and **16**). The analytical monitoring is presented in **Figure S12**.

Attempted folding to afford 'click' EPO (**25**)

A previously documented folding procedure was implemented.[7, 8] Briefly, 'click' EPO1-5 (**24**) (0.58 mg, 30.3 nmol) polypeptide was firstly denatured in a degassed solution of GnHCl (6 M, 5.8 ml) and 100 mM tris (pH = 8.5). The resulting 'click' EPO stock solution (0.1 mg/mL) was then dialyzed against an initial degassed first folding solution containing guanidine (3 M), tris (100 mM), L-cysteine (4 mM) and L-cystine (0.5 mM) adjusted to pH 8.5. After 18 h, the first folding solution was replaced by a second folding solution of guanidine (1 M) and tris (100 mM) adjusted to pH 8.0, and the dialysis allowed to proceed for a further 21 h. The EPO solution was then transferred into a third folding solution of tris (10 mM) adjusted to pH 7.0 and dialyzed for 26 h. After the last dialysis, the 'click' EPO solution was concentrated to approx. 2 mL and purification by RP-HPLC was attempted.

Further dialysis in the subsequent folding solutions included GnHCl (1 M) and tris (100 mM) adjusted to pH = 8.0 for 24 h, then tris (10 mM) adjusted to pH 7. 0 for 24 h.

Characterisation of EPO1-EPO5 peptides

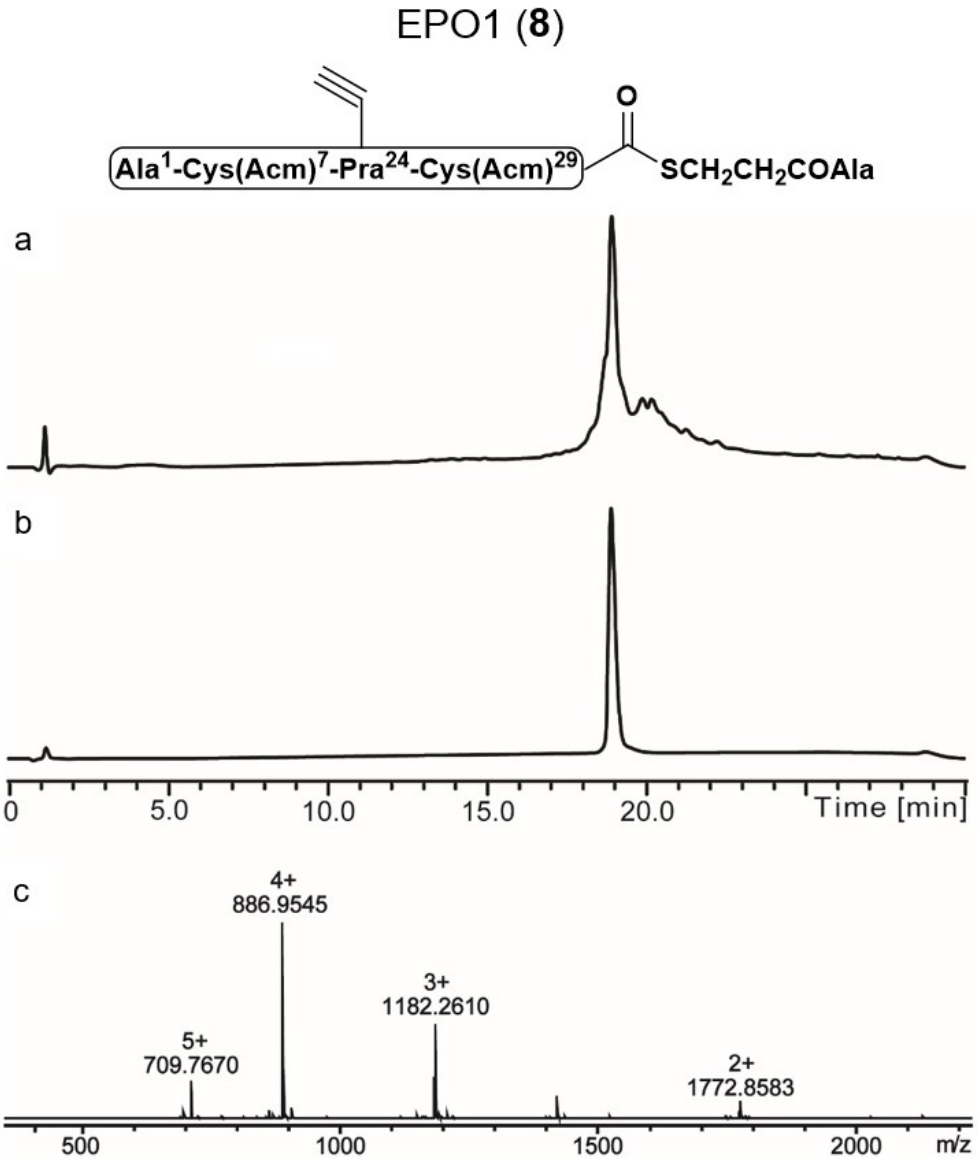

Figure S1: a-b**.** Analytical RP-HPLC traces for the synthesis of EPO1 (**8**) fragment; a. crude peptide; b. purified peptide; c. Mass spectrum of the purified peptide (observed: $[M+2H]^{2+}$= 1772.86 Da, calculated: 1773.07 Da).

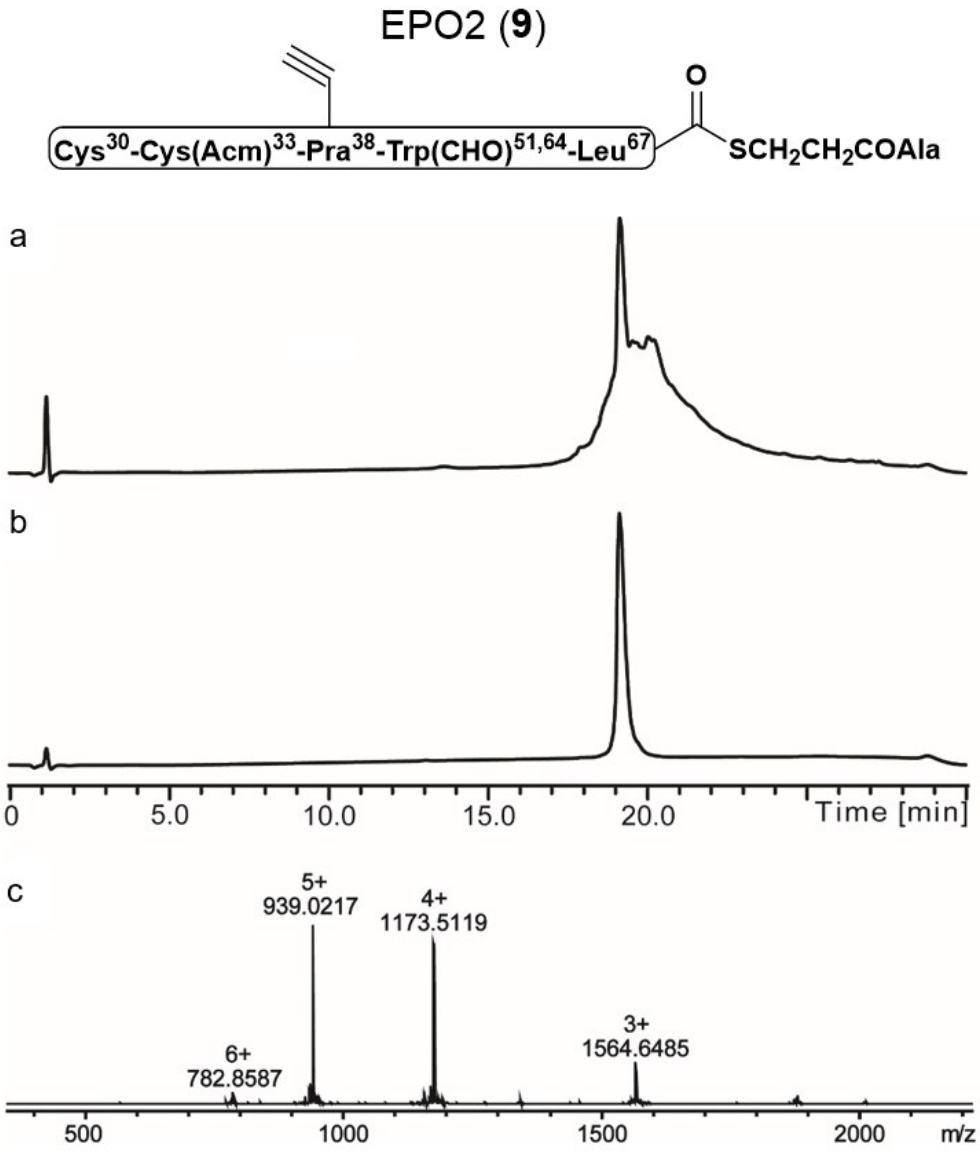

Figure S2: a-b**.** Analytical RP-HPLC traces for the synthesis of EPO1 (**8**) fragment; a. crude peptide; b. purified peptide; c. Mass spectrum of the purified peptide (observed: $[M+3H]^{3+}$= 1564.65 Da, calculated: 1564.77 Da)

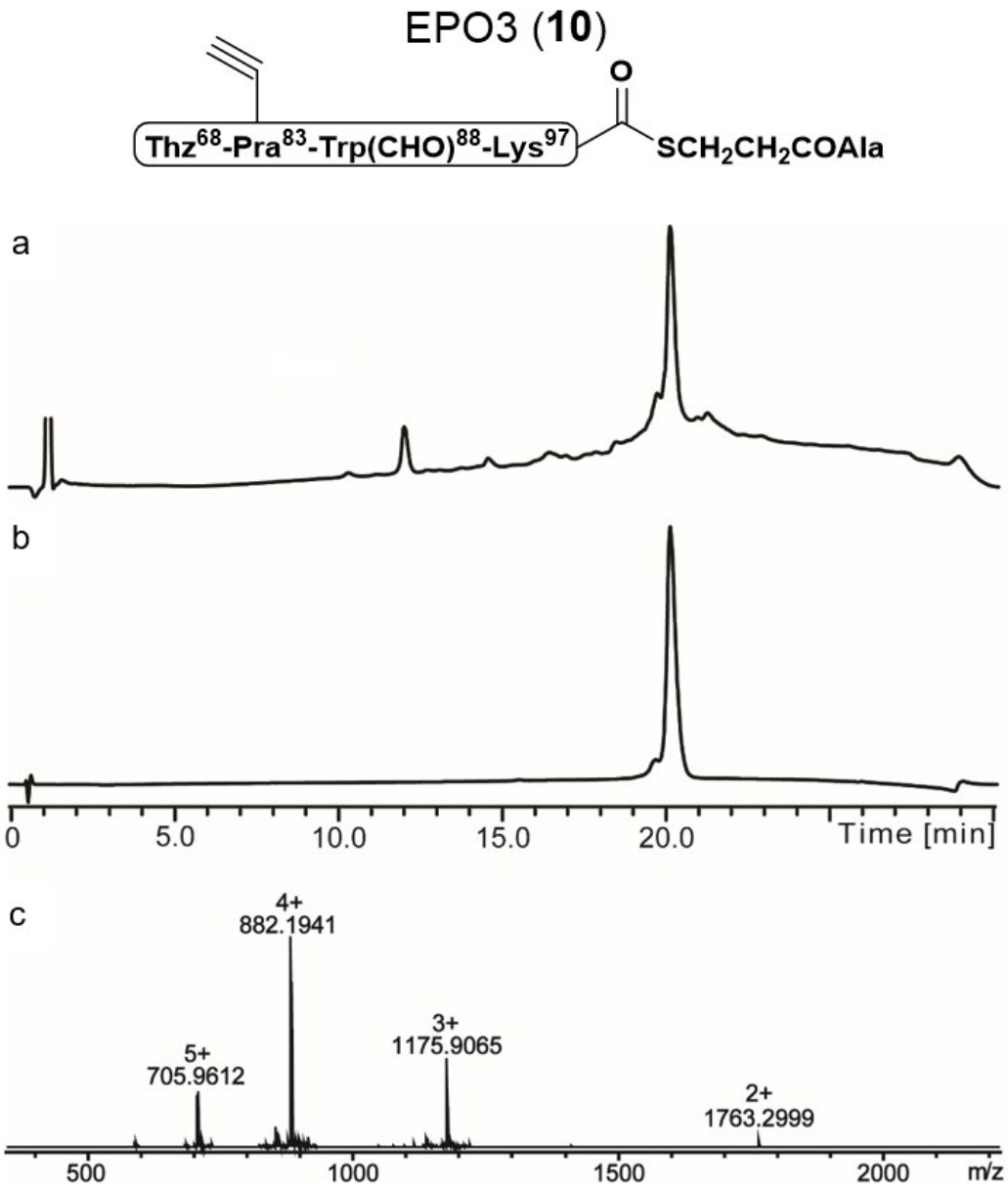

Figure S3: a-b**.** Analytical RP-HPLC traces for the synthesis of EPO1 (**8**) fragment; a. crude peptide; b. purified peptide; c. Mass spectrum of the purified peptide (observed: $[M+2H]^{2+}$= 1763.30 Da, calculated: 1763.56 Da)

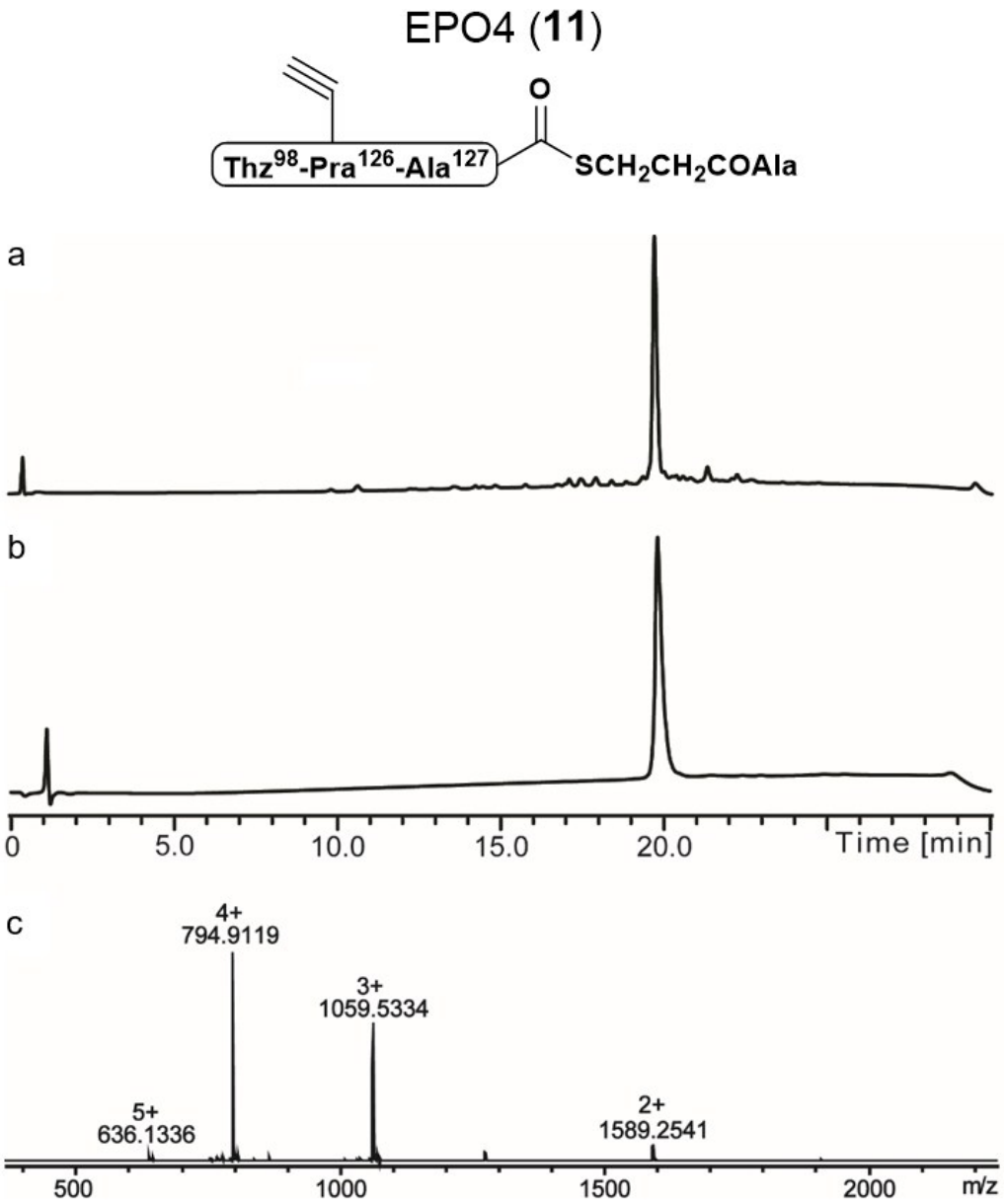

Figure S4: a-b**.** Analytical RP-HPLC traces for the synthesis of EPO1 (**8**) fragment; a. crude peptide; b. purified peptide; c. Mass spectrum of the purified peptide (observed: $[M+2H]^{2+}$= 1589.25 Da, calculated: 1589.36 Da)

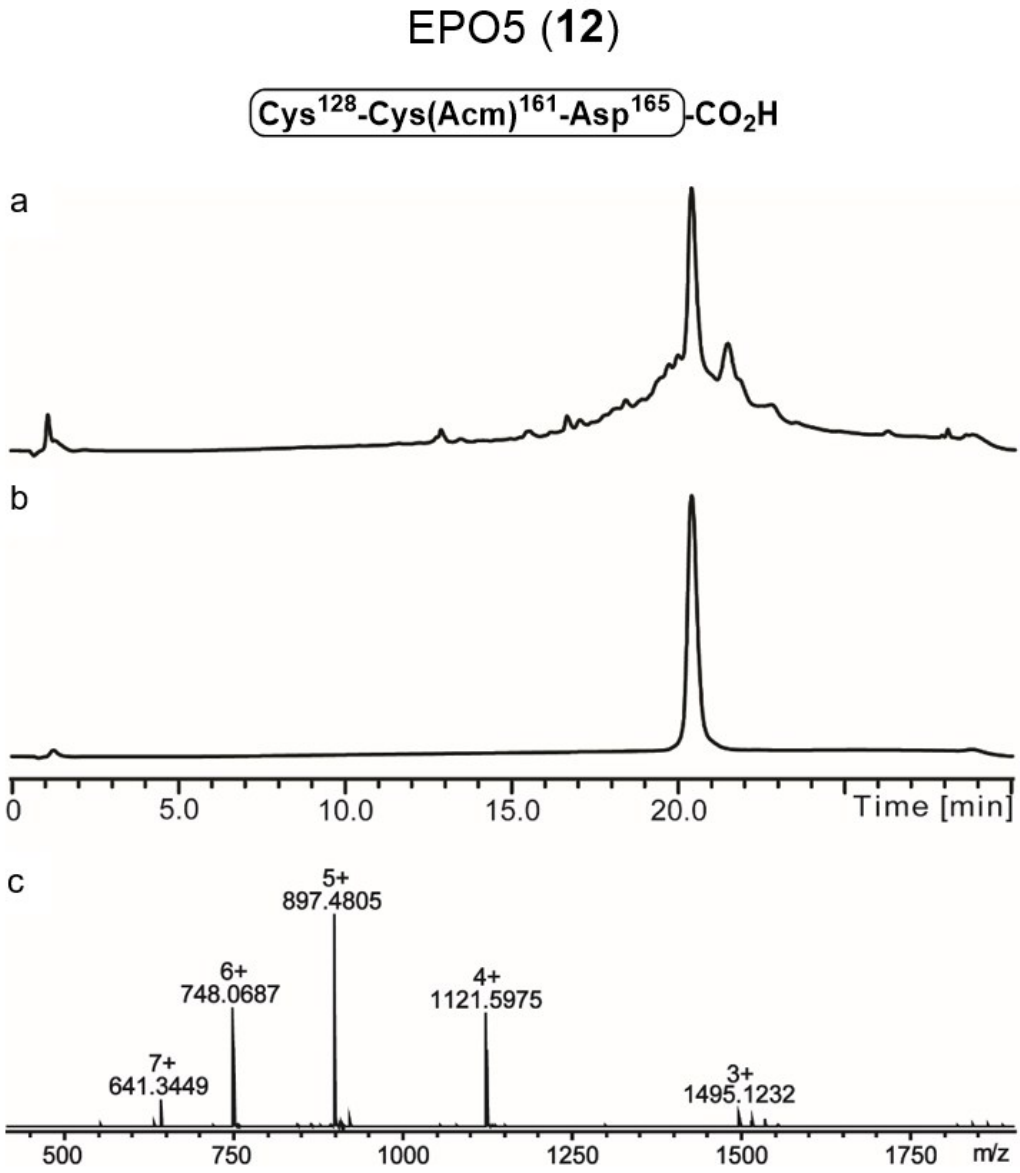

Figure S5: a-b**.** Analytical RP-HPLC traces for the synthesis of EPO1 (**8**) fragment; a. crude peptide; b. purified peptide; c. Mass spectrum of the purified peptide (observed: $[M+3H]^{3+}$= 1495.12 Da, calculated: 1495.42 Da)

Reaction Monitoring

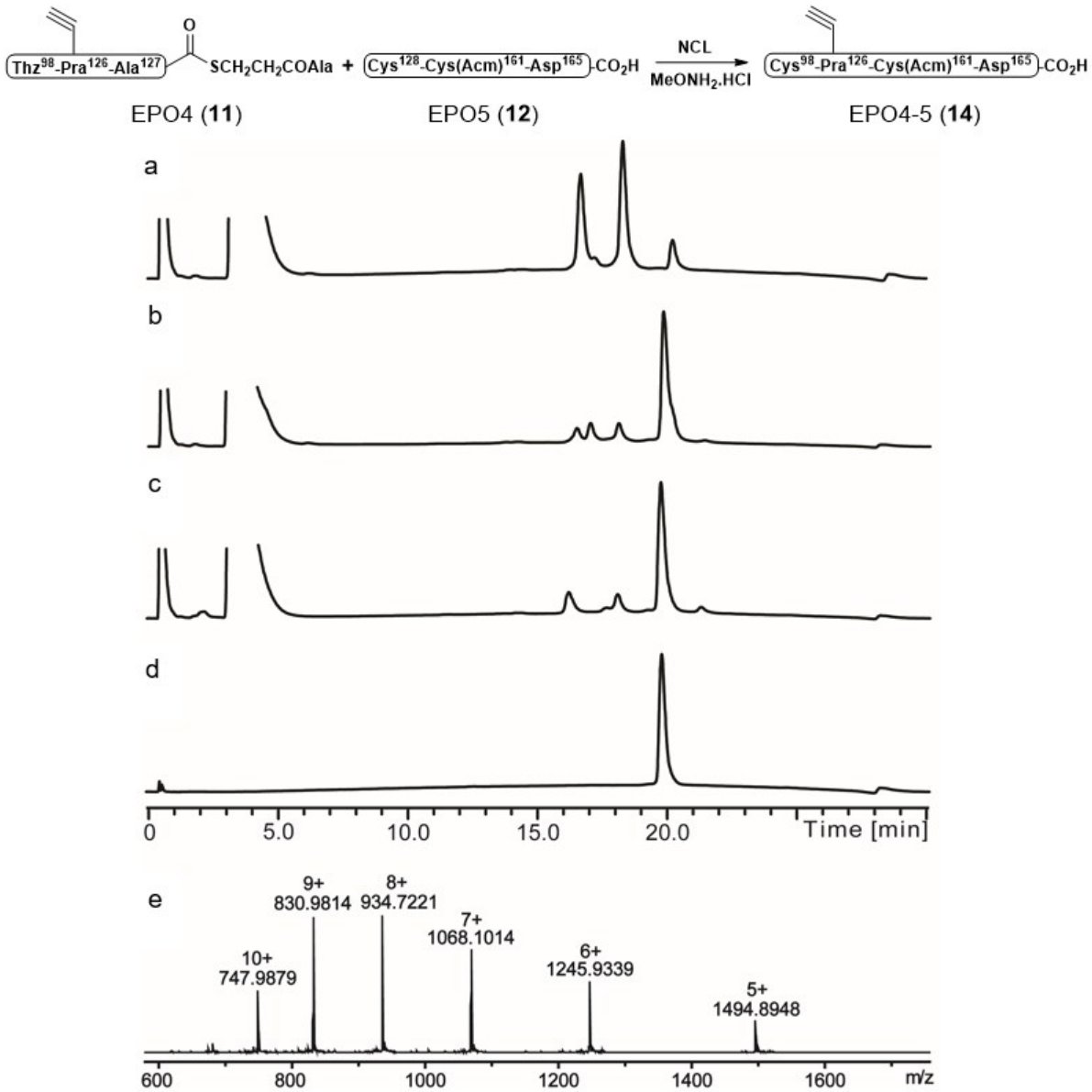

Figure S6. a-b. Analytical LC-MS traces for the NCL between EPO4 (**11**) and EPO5 (**12**) peptides. *Reagents and conditions*: MPAA (200 mM), TCEP (50 mM), 6 M GnHCl/0.2 M Na_2HPO_4, pH 6.8, 2 h; a. t= 5 min; b. t= 2 h, the ligated Thz-EPO4-5 ligation peptide was observed in near quantitative yield; c-e. Analytical LC-MS traces for the Thz to Cys conversion yielding EPO4-5 (**14**). *Reagents and conditions*: $MeONH_2$·HCl (150 mM) in the same pot, pH 4, 3 h; c. Thz^{98} was converted to Cys^{98} to produce EPO4-5 (**14**) after 3 h; d, purified EPO4-5; e. Mass spectrum of EPO4-5 (observed: $[M+5H]^{5+}$= 1494.89 Da, calculated: 1495.14 Da). The analytical monitoring was carried out using an analytical column (Phenomenex Gemini C_{18}, 110 Å, 50 mm x 2.0 mm; 5 µm) with a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

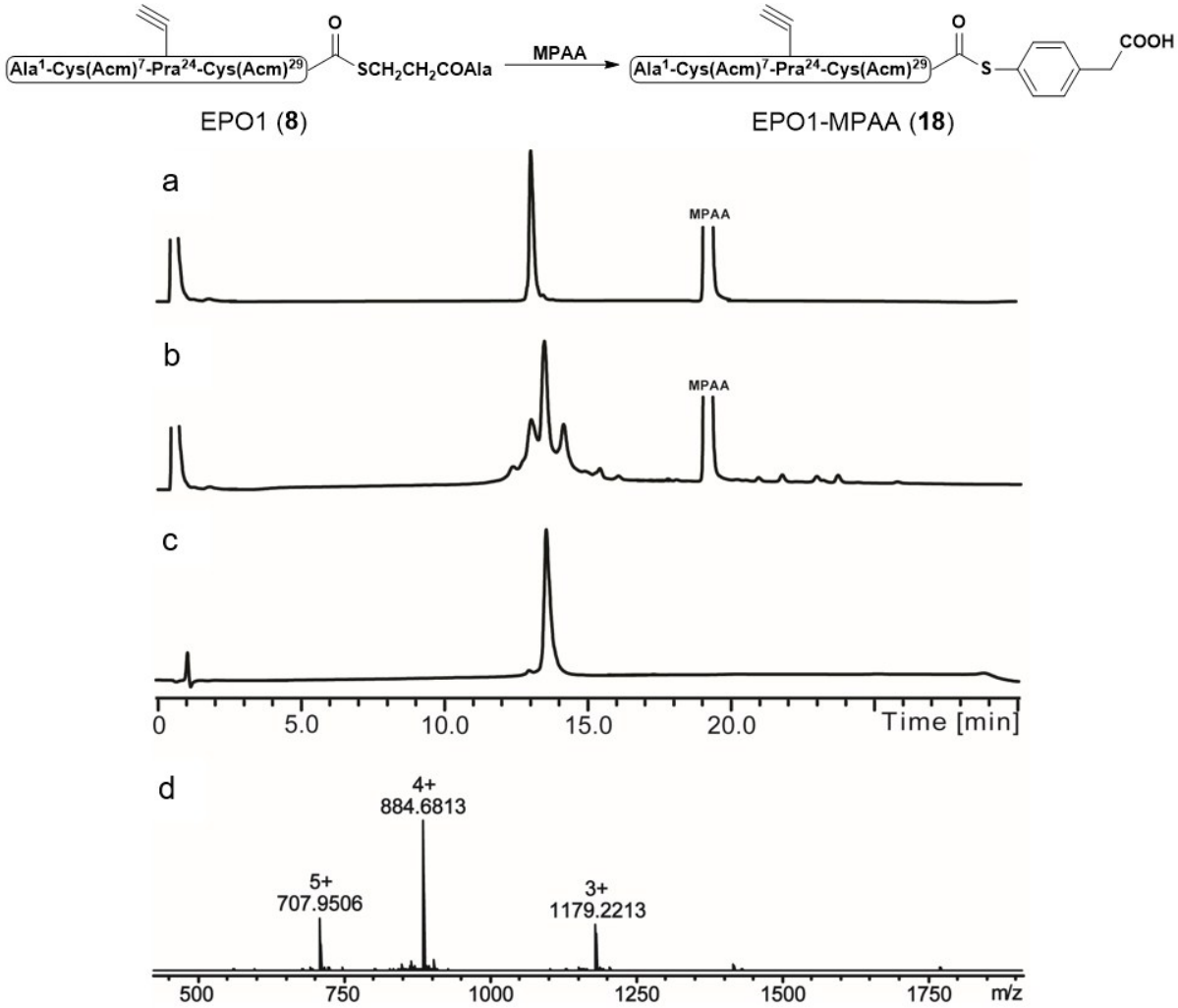

Figure S7. Analytical LC-MS traces for the MPAA thiol exchange of EPO1 (**8**). *Reagents and conditions*: MPAA (200 mM), TCEP (40 mM), 6 M GnHCl/0.2 M Na_2HPO_4, pH 6.1, 6 h; a. t= 5 min; b. t= 6 h, the MPAA exchanged EPO1-MPAA peptide was obtained in approximately 75% conversion; c. purified EPO1-MPAA (**18**) peptide; d. Mass spectrum of EPO1-MPAA peptide (observed: $[M+3H]^{3+}$= 1179.22 Da, calculated: 1179.38 Da). The analytical monitoring was carried out using an analytical column (Phenomenex Gemini C_{18}, 110 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

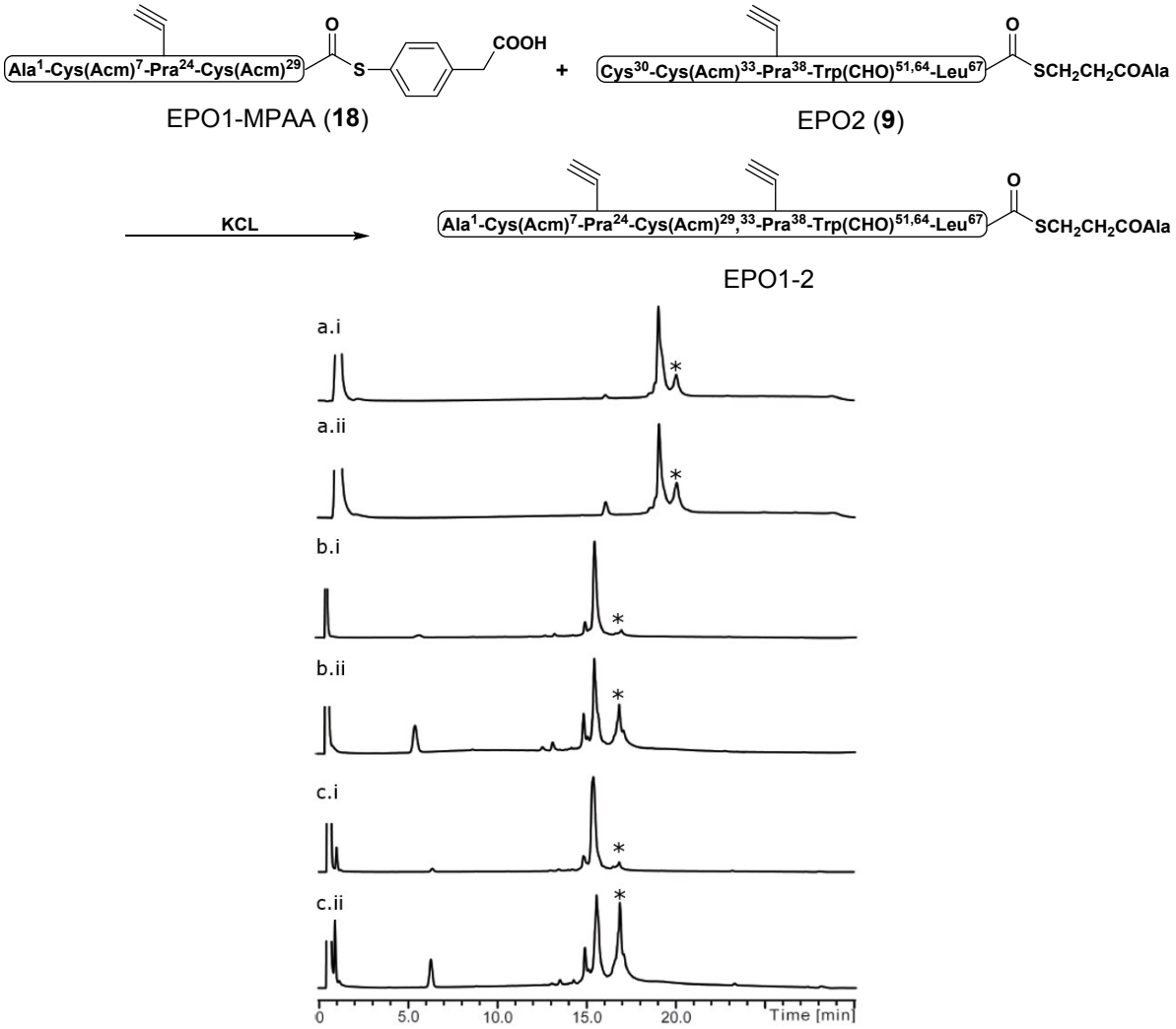

Figure S8. Analytical HPLC traces of KCL trials between EPO1-MPAA (**18**) and EPO2 (**9**) under standard conditions; 6 M GnHCl/0.2 M Na_2HPO_4, with $CuSO_4$ (20 mM), TCEP (10 mM) adjusted to pH 6.1 to avoid thioester hydrolysis as noted during synthesis of **18**; * = ligation product; a.i. t= 1 h. Peptide concentrations = 3 mM. Note: both starting peptides (**18** and **9**) had overlapping t_R. a.ii. t= 7 h. After 7 h, less than 20% of the desired ligated EPO1-2 product was observed; b.i. t= 30 min. Peptide concentrations = 1 mM with the addition of TFE; b.ii. t= 16 h. Less than 30% of the formation of the desired ligated EPO1-2 product was observed; c.i. t= 5 min. Peptide concentrations = 2 mM; c.ii. t= 4 h. This was the best result obtained from a number of trial reactions carried out. However, less than 50% of the desired ligated EPO1-2 product was observed. The analytical RP-HPLC for a. was carried out using an analytical column (Phenomenex Gemini C18, 110 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile). The analytical RP-HPLC for b. and c. were carried out using an analytical column (Phenomenex Jupiter C4, 300 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

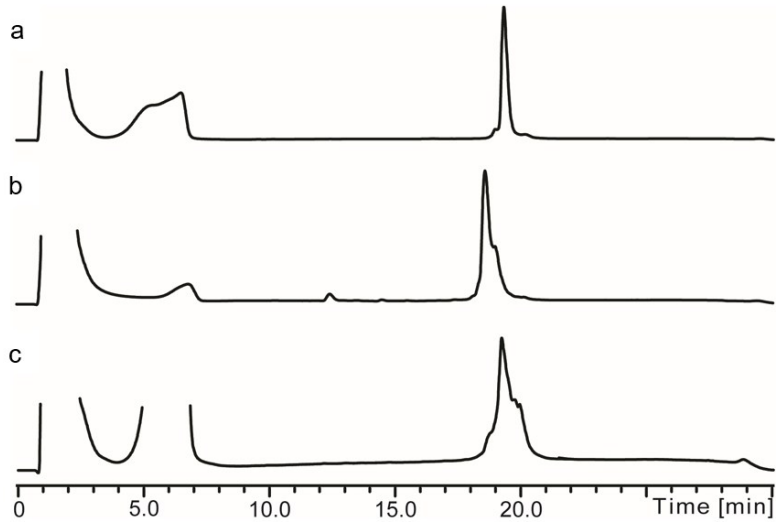

Figure S9. Analytical HPLC traces for EPO1 (**8**) and EPO2 (**9**) (3 mM) subjected to stirring in 6 M GnHCl/0.2 M Na_2HPO_4 buffer containing $CuSO_4$ (20 mM) and TCEP (10 mM); a. EPO1 peptide did not show any Acm deprotection after 6 h, rt; b. EPO1 peptide showed peptides with one and two Acm groups removed after overnight stirring at 55 ^oC; c. EPO2 peptide showed significant Acm removal after 30 min, rt. The analytical RP-HPLC was carried out using an analytical column (Phenomenex Gemini C_{18}, 110 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

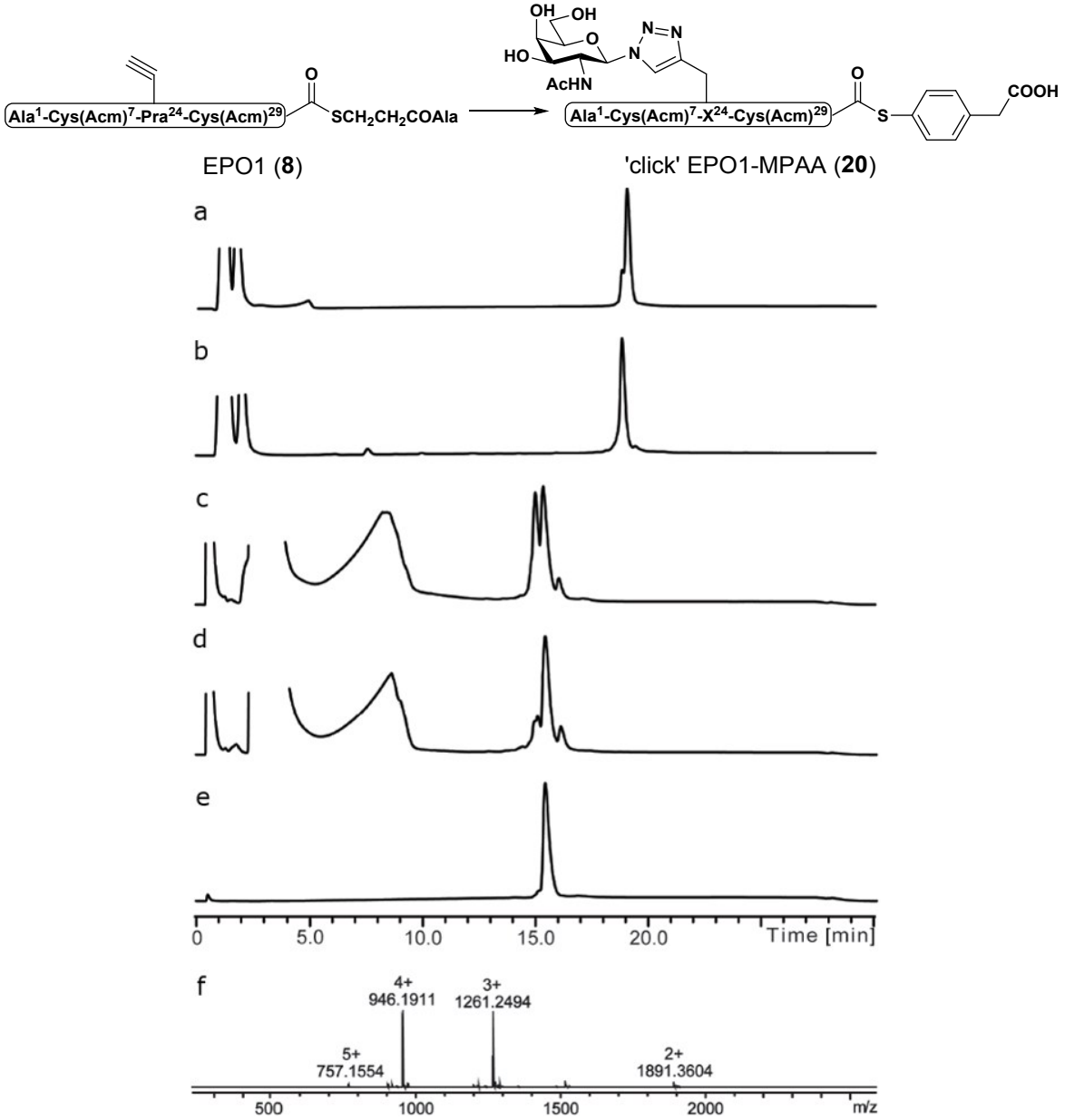

Figure S10.

Analytical LC-MS traces for the one-pot click chemistry and MPAA thiol exchange of EPO1 (**8**) and GalNAc-N_3. *Reagents and conditions*: i) $CuSO_4$ (20 mM) and TCEP (10 mM), 6 M GnHCl/0.2 M Na_2HPO_4, pH 6.3, 4 h; ii) MPAA (100 mM) added to the same pot, pH 6.0, 7 h; a. t= 10 min; b. t= 3 h, the click reaction showed quantitative conversion to yield Click-EPO1; c. t= 1 h after MPAA addition; d. t= 7 h, the MPAA exchange was essentially complete; e. purified 'click' EPO1-MPAA (**20**); f. Mass spectrum of 'click'EPO1-MPAA (**20**) (observed: $[M+2H]^{2+}$= 1891.36 Da, calculated: 1891.67 Da). The analytical RP-HPLC for a. and b. was carried out using an analytical column (Phenomenex Gemini C_{18}, 110 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile). The analytical RP-HPLC for c.-e. was performed using an analytical column (Phenomenex Jupiter C4, 300 Å, 50 mm x 2.0 mm; 5 µm) employing a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

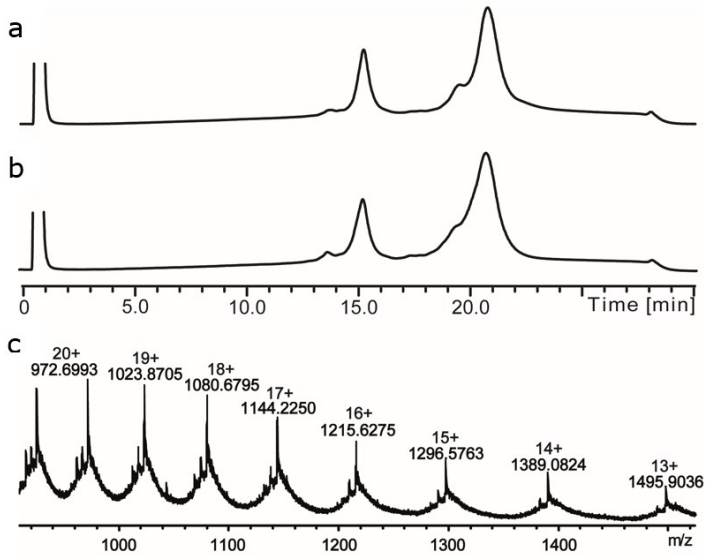

Figure S11. Analytical LC-MS traces for the desulfurization reaction of 'click' EPO1-5 [Cys(Acm)^{7,29,33,161}][Cys^{30,68,98,128}] (**22**). *Reagents and conditions*: 6 M GnHCl/0.2 M Na_2HPO_4, TCEP.HCl (100 mM), VA-044 (40 mM), glutathione (20 mM), pH 6.5, 40 ^oC, 3 h; "solid phase extraction" using C4 semi-pep column and lyophilisation; a. t= 10 min of reaction; b. t= 3 h, the reaction was essentially complete to yield the desulfurised 'click' EPO1-5 [Cys(Acm)^{7,29,33,161}][Ala^{30,68,98,128}] *neo*glycopeptide product **23**; c. Mass spectrum of SPE purified 'click' EPO1-5 (**23**) (observed: [M+14H]$^{14+}$= 1389.08 Da, calculated: 1389.29 Da. Deconvoluted mass observed: 19436.62 Da, calculated: 19436.10 Da). The analytical monitoring was carried out using an analytical column (Phenomenex Jupiter C4, 300 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

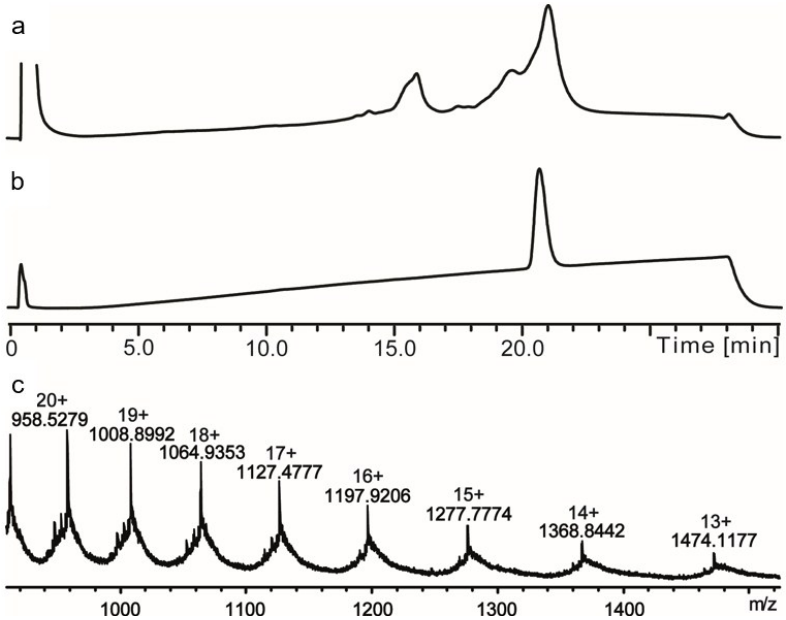

Figure S12. Analytical LC-MS traces for the Acm removal from 'click' EPO1-5[Cys(Acm)^{7,29,33,161}] *neo*glycopeptide. *Reagents and conditions*: AcOH:H_2O (1:1), rt, 6 h, then 2 M DTT in 6 M GnHCl (250 µL); a. t= 6 h of reaction; b. purified 'click' EPO1-5 [Cys^{7,29,33,161}] *neo*glycopeptide **24**; c. Mass spectrum of RP-HPLC purified 'click' EPO1-5 (**24**) (observed: [M+14H]$^{14+}$= 1368.84 Da, calculated: 1368.98 **Da.** Deconvoluted mass observed: 19151.66 Da, calculated: 19151.77 Da). The analytical monitoring was carried out using an analytical column (Phenomenex Jupiter C4, 300 Å, 50 mm x 2.0 mm; 5 µm) using a gradient of 5-65 % buffer B over 30 min (buffer A= 0.1% TFA in H_2O; buffer B= 0.1% TFA in acetonitrile) at 210 nm

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