Supporting Information

Chemical and Enzymatic Synthesis of Sialylated Glycoforms of Human Erythropoietin

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1. General Methods

Solvents were dried according to standard methods. Automated Fmoc-SPPS was performed in 45-mL reaction vessels in a PTI Tribute peptide synthesizer with UV-monitoring and feedback control system. Manual SPPS steps were performed in 2-mL, 5-mL, 10-mL or 20-mL polypropylene syringes equipped with a 25 μm polyethylene filter (Multisyntech, Germany). Fmoc or Boc protected amino acids and coupling reagents were obtained from Novabiochem (Läufelfingen, Switzerland), Iris Biotech (Marktredwitz, Germany) and Sigma Aldrich (Taufkirchen, Germany). 4-(Mercaptomethyl)-benzoic acid (MMBA),[1] and GlcNAc-NH$_2$[2] were synthesized according to published procedures. Trityl-ChemMatrix resin (0.30 mmol/g loading, 100-200 mesh dry) was obtained from PCAS BioMatrix Inc., Canada).

ESI-TOF mass spectra were recorded on a Micromass LCT instrument coupled to an Agilent 1100 HPLC or a Waters ACQUITY UPLC H Class System with a photodiode array detector using solvent A (H$_2$O + 0.1 % HCOOH) and B (MeCN + 0.1 % HCOOH). HR-ESI mass spectra were recorded on a Thermo Q Exactive Orbitrap mass spectrometer. Separations were carried out using [A] YMC-UltraHT Hydrosphere C18 (30 x 2 mm, 2 μm, 120 Å), [B] YMC-UltraHT Hydrosphere C18 (50 x 2 mm, 2 μm, 120 Å), [C] YMC-Triart C8 (30 x 2 mm, 1.9 μm, 120 Å), [D] Supelco Discovery BIO Wide Pore C5 (50 x 2.1 mm, 3 μm, 300 Å), [E] Supelco Discovery BIO Wide Pore C5 (50 x 2.1 mm, 5 μm, 300 Å).

Preparative RP-HPLC was performed on an Äkta Basic HPLC with UV-detection at 214, 254 and 280 nm using solvent C (H$_2$O + 0.1 % TFA) and D (MeCN + 0.1 % TFA). The following columns were used: [F] YMC Pack Pro C8 (250 × 20 mm, 5 μm, 120 Å), [G] Supelco Ascentis C18 (150 × 10 mm, 5 μm, 100 Å), [H] Supelco Discovery BIO Wide Pore C5 (150 × 4.6 mm, 5 μm, 300 Å), [I] Supelco Discovery BIO Wide Pore C5 (150 × 10 mm, 5 μm, 300 Å), [J] YMC Pack Protein RP C4 (150 × 10 mm, 5 μm, 200 Å), [K] YMC Triart C8 (150 × 10 mm, 5 μm, 120 Å), [L] YMC Hydrosphere C18 (150 × 10 mm, 5 μm, 120 Å).

Preparative size exclusion chromatography was performed on an Äkta Purifier HPLC with conductivity detection and UV-detection at 214, 254 and 280 nm using the following columns: [M] Sephadex G-25 (164 × 15 mm, 20 – 50 μm), [N] Sephadex G-25 (155 × 25 mm, 20 – 50 μm), [O] GE Healthcare Superdex Peptide 10/300 GL (310 × 10 mm, 13 μm), [P] GE Healthcare Superdex 75 prep grade 16/600 (600 × 16 mm, 34 μm).

Flash chromatography was performed on a GRACE Reveleris® iES-flash chromatography system with ELS-detector. Thin layer chromatography was performed on coated aluminum plates (silica gel 60 GF$_{254}$, Merck Darmstadt). Spots were detected by UV light or by charring with a 1:1 mixture of 2 N H$_2$SO$_4$/0.2 % resorcinol monomethyl ether in ethanol. Dialysis tubing
Zellutrans V Serie (MW cutoff: 5000, flat width 40 mm) was obtained from Roth (Germany). CD spectra were recorded on a Jasco J-715 spectropolarimeter. A 1 mm cuvette (110-QS, Hellma Analytics, fused silica Suprasil) was used. The ellipticities \(\Delta \Theta\) were corrected by a matrix blank and were normalized as residual molar ellipticities \([\Theta]_{MRW}\): \([\Theta]_{MRW}\) (deg cm\(^2\) dmol\(^{-1}\)) = \(\Delta \Theta\) (mdeg) / (l (mm) × [EPO A] (mol/L) × (N\(_{aa}\) - 1)). l is the layer thickness of the cuvette and N\(_{aa}\) is the number of amino acid residues in the protein.

ICP-OES was measured on an Optima 7300 DV from PerkinElmer with a Meinhard atomizer. CMP-Neu5Ac was kindly provided by Roche Diagnostics GmbH as sodium salt.

\(\alpha\)-2,6-Sialyltransferase from Photobacterium damsela (\(\Delta^{15}\)PD2,6ST)\(^{[3]}\) was obtained from Sigma Aldrich. Alkaline phosphatase from calf intestine was purchased from Sigma Aldrich (10.3 mg/mL with 2145 U/mg, Buffer: 30 mM triethanolamine, pH 7.6, 3 mM NaCl, 1 mM MgCl\(_2\), 0.1 mM ZnCl\(_2\)).

The degree of loading of resins was determined by Fmoc cleavage of the penultimate amino acid.\(^{[4,5]}\) Absorbance measurements were performed on a Specord 2000 spectrophotometer from Analytik Jena. Analytical TFA deprotections were performed by adding 0.5 mg of resin or 0.2 mg of protected peptide to 100 \(\mu\)L of TFA/TIS/H\(_2\)O (95:2.5:2.5) for 60 min. The mixture was dried in high vacuum, the residue was dissolved in MeCN/H\(_2\)O + 0.1 % HCOOH and analyzed by HPLC-MS.

2. General Synthesis Procedures

**Automated Fmoc-SPPS**

Solid-phase synthesis following the Fmoc strategy\(^{[6]}\) was performed automatically in 45 mL reaction vessels on a Tribute peptide synthesizer with IntelliSynth UV Monitoring and Feedback Control System from Protein Technologies Inc. All reactions were performed at ambient temperature under a nitrogen atmosphere and mechanical shaking. Nitrogen was passed through the reaction suspension for additional mixing. Before starting the synthesis, the resin was swollen by washing with CH\(_2\)Cl\(_2\) (5 x 30 s) and DMF (5 x 30 s).

**Automated Removal of N\(^a\)-Fmoc**

To remove N-terminal Fmoc protection a piperidine solution (20 % in DMF) was added to the resin and shaken for 0.5 min. The coupled UV monitoring system of the peptide synthesizer initiated further Fmoc cleavage cycles if the absorbance (\(A_{301}\)) of the filtrate indicated incomplete cleavage. After complete Fmoc removal, the resin was washed with DMF (5 x 30 s).
**Automated Coupling of Fmoc amino acid derivatives**

The amino acid building block and the activation reagent were taken up in a defined volume of DIPEA in DMF (for molarity see specific synthesis) before coupling, mixed for 2 min, and added to the deprotected peptidyl resin. Cysteine derivatives were activated as symmetric anhydrides and coupled manually. At the end of each coupling the resin was washed with DMF (5 x 30 s). The exact coupling conditions are given in the specific synthesis protocols.

**Manual coupling of cysteine derivatives**[^7]

In dried glassware under argon, the cysteine building block (10 equiv.) was dissolved in dry DMF/CH$_2$Cl$_2$ 1:2.5 (c Fmoc/Boc-Cys(PG)-OH = 180 mM) and N,N-diisopropylcarbodiimide (5 equiv.) was added at 0 °C. The reaction mixture was stirred at 0 °C for 5 min and for 30 min at ambient temperature. Subsequently, the CH$_2$Cl$_2$ was removed under vacuum. The suspension was drawn up into a syringe with and added to the deprotected peptidyl resin swollen in DMF. The residue and the vessel were washed three times with an appropriate volume of DMF to give a concentration of 105 mM for the anhydride in the coupling solution. After 3 - 5 h of shaking at ambient temperature, the coupling solution was removed and the resin was washed with CH$_2$Cl$_2$ and DMF (five times each).

**Mild acidic cleavage of protected peptidyl acids from trityl resin**

To cleave protected peptidyl acids from p-carboxytrityl-ChemMatrix resin the peptidyl resin was swelled in CH$_2$Cl$_2$ in a syringe reactor for 20 min. Subsequently 20 % HFIP in CH$_2$Cl$_2$ (approx. 8 mL/g resin) was added and the suspension was shaken at room temperature for 1 min. The resin was treated seven times for 1 min each with the same volume of 20 % HFIP solution and finally washed five more times with CH$_2$Cl$_2$. All the cleavage and washing solutions were collected in a cooled flask (0 °C) containing 25 times the volume of CH$_2$Cl$_2$ used for a single cleavage. The combined cleavage solutions were concentrated under vacuum at 4 °C bath temperature. HFIP was azeotropically removed by adding CH$_2$Cl$_2$ several times. The residue was dried in vacuo and lyophilized from dioxane.

**Conversion of glycopeptide hydrazides to thioesters**

The glycopeptide hydrazide (1.0 equiv.) was dissolved in GdmCl buffer (6 M GdmCl, 0.2 M NaH$_2$PO$_4$, pH 3.0) to a final concentration of 3.5 mM and stirred for 15 min at -15 °C. Subsequently, 0.5 M NaNO$_2$ (6.5 eq) was added under stirring. After 1 h at -15 °C MESNa (65 equiv., 1.0 M in 6 M GdmCl/0.2 M NaH$_2$PO$_4$) was added. The pH value of the MESNa solutions...
was set to 6.6 for EPO 29-67 glycopeptides 21 and 35 and to pH 6.0 for EPO 29-97 glycopeptides S14 and S15. The concentration of the acyl azide in the thiolysis mixture was 2.75 mM. Once the reaction mixture reached ambient temperature a microelectrode was used to readjust the pH using 6 M and 1 M NaOH. At the desired pH the reaction mixture was stirred for 1 h with the microelectrode immersed. Subsequently, the microelectrode and the magnetic stir bar were rinsed with 100 μL of phosphate buffer (0.2 M NaH₂PO₄/6 M GdmCl/pH 3.0). For sialylated compounds 0.2 M NaH₂PO₄/6 M GdmCl/pH 6.6 (EPO 29-67 glycopeptide 35) or pH 6.0 (EPO29-97 glycopeptide S15) were used in the rinsing step. The combined reaction mixtures were desalted by gel filtration.

3. Synthesis of Glycopeptide Hydrazides

3.1 Synthesis of EPO 29-67 Hydrazide 19

Figure S1: Synthesis of EPO 29-67 hydrazide 19

Fmoc-Leu-Trt-CM resin S1 (loading = 276.6 μmol/g) was synthesized according to Ref.[8]. 462.8 mg (128 μmol, 1 eq) of resin S1 were placed in a 45 mL peptide synthesizer reaction vessel and the peptide chain was elongated automatically. For cleavage of the Fmoc group the general procedure was applied and the amino acid building blocks were coupled under the conditions denoted in table S1 and the general procedures section. After complete elongation the resin S2 was washed with DMF (5 x) and CH₂Cl₂ (5x) and was dried in vacuo.

Table S1:

| Position | Building Block                | m (mg) | V (mL) 0.19 M DIPEA in DMF | Coupling time (min) |
|----------|-------------------------------|--------|---------------------------|---------------------|
| Gly-66   | Fmoc-Gly-OH[4]                | 190.3  | 7.0                       | 45                  |
| Gln-65   | Fmoc-Gln(Trt)-OH[4]           | 390.9  | 7.0                       | 45                  |
| Residue | Peptide | Mass (Da) | Solvent | Conjugation |
|---------|---------|-----------|---------|-------------|
| Trp-64  | Fmoc-Ala-OH<sup>a</sup> | 337.0 | 7.0 | 45 |
| Val-63  | Fmoc-Val-OH<sup>a</sup> | 217.2 | 7.0 | 45 |
| Glu-62  | Fmoc-Glu(OtBu)-OH<sup>a</sup> | 272.3 | 7.0 | 45 |
| Val-61  | Fmoc-Val-OH<sup>a</sup> | 217.2 | 7.0 | 45 |
| Ala-60  | Fmoc-Ala-OH<sup>a</sup> | 199.3 | 7.0 | 45 |
| Gln-59  | Fmoc-Gln(Trt)-OH<sup>a</sup> | 390.9 | 7.0 | 45 |
| Gln-58  | Fmoc-Gln(Trt)-OH<sup>a</sup> | 390.9 | 7.0 | 45 |
| Gly-57  | Fmoc-(Dmb)Gly-OH<sup>b</sup> | 171.8 | 7.0 | 90 |
| Val-56  | Fmoc-Val-OH<sup>c</sup> | 434.4 | 14.0 | 90 |
| Glu-55  | Fmoc-Glu(OtBu)-OH<sup>a</sup> | 272.3 | 7.0 | 45 |
| Nle-54  | Fmoc-Nle-OH<sup>a</sup> | 226.2 | 7.0 | 45 |
| Arg-53  | Fmoc-Arg(Pbf)-OH<sup>a</sup> | 415.2 | 7.0 | 45 |
| Lys-52  | Fmoc-Lys(Boc)-OH<sup>a</sup> | 299.9 | 7.0 | 45 |
| Trp-51  | Fmoc-Trp(Boc)-OH<sup>a</sup> | 337.0 | 7.0 | 45 |
| Ala-50  | Fmoc-Ala-OH<sup>a</sup> | 199.3 | 7.0 | 45 |
| Tyr-49  | Fmoc-Tyr(tBu)-OH<sup>a</sup> | 294.1 | 7.0 | 45 |
| Phe-48  | Fmoc-Phe-OH<sup>a</sup> | 248.0 | 7.0 | 45 |
| Asn-47  | Fmoc-Asn(Trt)-OH<sup>a</sup> | 381.9 | 7.0 | 45 |
| Val-46  | Fmoc-Val-OH<sup>a</sup> | 217.2 | 7.0 | 45 |
| Lys-45  | Fmoc-Lys(Boc)-OH<sup>a</sup> | 299.9 | 7.0 | 45 |
| Thr-44, Asp-43 | Fmoc-Asp(OtBu)-Thr(ψ<sub>Me,Me</sub>pro)-OH<sup>b</sup> | 212.2 | 4.5 + 2.5 mL DMF | 90 |
| Pro-42  | Fmoc-Pro-OH<sup>a</sup> | 215.9 | 7.0 | 45 |
| Val-41  | Fmoc-Val-OH<sup>a</sup> | 217.2 | 7.0 | 45 |
| Thr-40, Ile-39 | Fmoc-Ile-Thr(ψ<sub>Me,Me</sub>pro)-OH<sup>b</sup> | 189.9 | 4.5 + 2.5 mL DMF | 90 |
| Asp-38  | Fmoc-Asp(OPhiPr)-OH<sup>b</sup> | 181.8 | 4.5 + 2.5 mL DMF | 90 |
| Glu-37  | Fmoc-Glu(OtBu)-OH<sup>a</sup> | 272.3 | 7.0 | 45 |
| Asn-36  | Fmoc-Asn(Trt)-OH<sup>a</sup> | 381.9 | 7.0 | 45 |
| Leu-35  | Fmoc-Leu-OH<sup>a</sup> | 226.2 | 7.0 | 45 |
| Ser-34  | Fmoc-Ser(tBu)-OH<sup>a</sup> | 245.4 | 7.0 | 45 |
| Cys-33  | Fmoc-Cys(Acm)-OH<sup>d</sup> | 530.5 | - | 210 |
| His-32  | Fmoc-His(Trt)-OH<sup>a</sup> | 396.6 | 7.0 | 45 |
| Glu-31  | Fmoc-Glu(OtBu)-OH<sup>a</sup> | 272.3 | 7.0 | 45 |
| Ala-30  | Fmoc-Ala-OH<sup>a</sup> | 199.3 | 7.0 | 45 |
| Cys-29  | Boc-Cys(Acm)-OH<sup>d</sup> | 374.2 | - | 210 |

<sup>a</sup> Activation of this amino acid (640.0 µmol, 5 equiv.) was performed with HCTU (254.2 mg, 614.4 µmol, 4.8 equiv.)

<sup>b</sup> Activation of this amino acid (384.0 µmol, 3 equiv.) was performed with PyBOP (193.2 mg, 371.2 µmol, 2.9 equiv.)

<sup>c</sup> Activation of this amino acid (1.28 mmol, 10 equiv.) was performed with HCTU (518.9 mg, 1.25 mmol, 9.8 equiv.)
Activation and coupling of cysteine derivatives (1.28 mmol, 10 equiv.) was performed following the general procedure (anhydride formation with DIC (99.1 µL, 640.0 µmol, 5 equiv.) in 7.1 mL DMF/CH₂Cl₂ (1:2.5), coupling in 6.1 mL DMF).

Peptidyl resin S2 (863.2 mg, loading = 91.9 µmol/g, 79.3 µmol) was treated with dilute HFIP according to the general procedure (yield: 478.9 mg crude peptidyl acid S3, 63.3 µmol, 79.8 %, TLC: CH₂Cl₂/MeOH 10:1; Rf (S3) = 0.40). Under argon, 300 mg of the cleaved peptidyl acid (39.6 µmol, 1 equiv.) were dissolved in absolute CH₂Cl₂ (3.3 mL, 12 mM S3) and BocHN-NH₂ (31.4 mg, 237.8 µmol, 6 equiv.), Cl-HOBt (26.9 mg, 158.5 µmol, 4 equiv.) followed by addition of DIPEA (37.1 µL, 217.9 µmol, 5.5 equiv.) at 0 °C. DIC (24.5 µL, 158.5 µmol, 4 equiv.) was added and the mixture was kept at 0 °C until complete conversion (TLC, 10:1 CH₂Cl₂/MeOH). The reaction mixture was diluted with 150 mL of CH₂Cl₂ and extracted with 150 mL of 2 M KHCO₃. The aqueous phase was extracted twice with 150 mL of CH₂Cl₂. The organic phases were combined and dried over MgSO₄. The filtrate was adsorbed onto silica gel 60 (63 - 200 µm) and purified by flash chromatography (Reveleris iES, FlashPure cartridge 40 g (35 - 45 µm), 0-10 % MeOH in CH₂Cl₂ over 40 min, 40 mL/min). The fractions were analyzed by TLC, dried and lyophilized from dioxane.

Yield of 19: 137.4 mg (17.9 µmol, 36.1 % over 3 steps). Rf (19) = 0.53 (CH₂Cl₂/MeOH 10:1).

RP-HPLC-MS: column [C], 95 % MeCN/H₂O for 1.25 min, then 95-100 % MeCN/H₂O + 0.1 % HCOOH over 9 min, 0.5 mL/min, injection volume: 4 µL of 5 mg 19/mL DMF. ESI-MS of 19: m/z (average isotopes) C₄₁₄H₅₄⁷N₅₇O₇₆S₃ (7682.43); calculated: 3853.21 [M+H+Na]²⁺, 3842.22 [M+2H]²⁺, 2561.82 [M+3H]³⁺; found: 3853.92 [M+H+Na]²⁺, 3842.83 [M+2H]²⁺, 2563.06 [M+3H]³⁺.

Deprotection of 19 with TFA/TIS/H₂O (95:2.5:2.5): RP-HPLC-MS: column [A], 10-40 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 19d m/z (average isotopes) C₂₀₅H₃₁₅N₅₇O₆₂S₂ (4634.23); calculated: 2318.12 [M+2H]²⁺, 1545.75 [M+3H]³⁺, 1159.57 [M+4H]⁴⁺; found: 2318.37 [M+2H]²⁺, 1545.68 [M+3H]³⁺, 1159.45 [M+4H]⁴⁺.

Figure S2: RP-HPLC-MS of protected EPO 29-67 hydrazide 19.
3.2 Selective Deprotection of EPO 29-67 Hydrazide 19

The peptide hydrazide 19 (130.1 mg, 16.9 µmol) was dissolved in 8.5 mL of 1 % TFA in CH₂Cl₂ (c 19 = 2 mM). After shaking for 10 min, 85 mL of cold Et₂O (-24 °C) were added and the suspension was kept for 20 min in a cooling bath at –24 °C followed by centrifugation (4000 g, 4 °C, 10 min). The supernatant was decantated and the precipitate was resuspended in cold Et₂O. After centrifugation the hydrazide S₄ was dried in vacuo and subsequently lyophilized from dioxane. Yield of S₄: 124.9 mg (16.5 µmol, 97.6 %). RP-HPLC-MS: column [C], 95 % for 1.25 min, then 95-100 % MeCN/H₂O + 0.1 % HCOOH in 9 min, 0.5 mL/min, injection volume 4 µL of 5 mg S₄/mL DMF. ESI-MS of S₄: m/z (average isotopes) C₄₀H₅₃N₇O₇₉S₃ (7564.25) calculated: 3783.13 [M+2H]²⁺, 2522.42 [M+3H]³⁺; found: 3782.80 [M+2H]²⁺, 2522.02 [M+3H]³⁺.
3.3 Synthesis of EPO 68-97 Hydrazide 22

Fmoc-Lys(Boc)-Trt-CM resin S5 (loading = 264.3 µmol/g) was synthesized according to Ref.\[8\]. 462.8 mg (129.9 µmol, 1 eq) of resin S5 were placed in a 45 mL peptide synthesizer reaction vessel and the peptide chain was elongated automatically. For cleavage of the Fmoc group the general procedure was applied and the amino acid building blocks were coupled under the conditions denoted in table S2 and the general procedure section. After complete elongation the resin S6 was washed with DMF (5x) and CH$_2$Cl$_2$ (5x) and was dried in vacuo.
Then 483.7 mg of the peptidyl resin S6 (loading = 116.1 µmol/g, 56.2 µmol) was treated with dilute HFIP according to the general procedure. Yield of peptidyl acid S7: 276.9 mg, 52.2 µmol,

| Position | Building Block | m (mg) | V (mL) | Coupling duration (min) |
|----------|----------------|--------|--------|-------------------------|
| Asp-96   | Fmoc-Asp(OtBu)-OH<sup>a</sup> | 267.4  | 6.5 + 0.5 mL DMF | 45 |
| Val-95   | Fmoc-Val-OH<sup>a</sup> | 220.6  | 6.5 + 0.5 mL DMF | 45 |
| His-94   | Fmoc-His(Trt)-OH<sup>a</sup> | 402.8  | 6.5 + 0.5 mL DMF | 45 |
| Leu-93   | Fmoc-Leu-OH<sup>a</sup> | 229.7  | 6.5 + 0.5 mL DMF | 45 |
| Gln-92   | Fmoc-Gln(Trt)-OH<sup>a</sup> | 397.0  | 6.5 + 0.5 mL DMF | 45 |
| Leu-91   | Fmoc-Leu-OH<sup>a</sup> | 229.7  | 6.5 + 0.5 mL DMF | 45 |
| Pro-90   | Fmoc-Pro-OH<sup>a</sup> | 219.3  | 6.5 + 0.5 mL DMF | 45 |
| Glu-89   | Fmoc-Glu(OtBu)-OH<sup>a</sup> | 276.6  | 6.5 + 0.5 mL DMF | 45 |
| Trp-88   | Fmoc-Trp(Boc)-OH<sup>a</sup> | 342.3  | 6.5 + 0.5 mL DMF | 45 |
| Pro-87   | Fmoc-Pro-OH<sup>a</sup> | 219.3  | 6.5 + 0.5 mL DMF | 45 |
| Gln-86   | Fmoc-Gln(Trt)-OH<sup>a</sup> | 397.0  | 6.5 + 0.5 mL DMF | 45 |
| Ser-85, Ser-84 | Fmoc-Ser(tBu)-Ser(ψ<sub>Me,Me</sub>pro)-OH<sup>b</sup> | 199.1  | 4.0 + 3.0 mL DMF | 90 |
| Asp-83   | Fmoc-Asp(OAll)-OH<sup>a</sup> | 257.0  | 6.5 + 0.5 mL DMF | 45 |
| Val-82   | Fmoc-Val-OH<sup>a</sup> | 220.6  | 6.5 + 0.5 mL DMF | 45 |
| Leu-81   | Fmoc-Leu-OH<sup>a</sup> | 229.7  | 6.5 + 0.5 mL DMF | 45 |
| Leu-80   | Fmoc-Leu-OH<sup>a</sup> | 229.7  | 6.5 + 0.5 mL DMF | 45 |
| Ala-79   | Fmoc-Ala-OH<sup>a</sup> | 202.4  | 6.5 + 0.5 mL DMF | 45 |
| Gln-78   | Fmoc-Gln(Trt)-OH<sup>a</sup> | 397.0  | 6.5 + 0.5 mL DMF | 45 |
| Gly-77   | Fmoc-Gly-OH<sup>a</sup> | 193.3  | 6.5 + 0.5 mL DMF | 45 |
| Arg-76   | Fmoc-Arg(Pbf)-OH<sup>a</sup> | 421.7  | 6.5 + 0.5 mL DMF | 45 |
| Leu-75   | Fmoc-Leu-OH<sup>a</sup> | 229.7  | 6.5 + 0.5 mL DMF | 45 |
| Val-74   | Fmoc-Val-OH<sup>a</sup> | 220.6  | 6.5 + 0.5 mL DMF | 45 |
| Ala-73   | Fmoc-Ala-OH<sup>a</sup> | 202.4  | 6.5 + 0.5 mL DMF | 45 |
| Glu-72   | Fmoc-Glu(OtBu)-OH<sup>a</sup> | 276.6  | 6.5 + 0.5 mL DMF | 45 |
| Ser-71, Leu-70 | Fmoc-Leu-Ser(ψ<sub>Me,Me</sub>pro)-OH<sup>b</sup> | 187.4  | 4.0 + 3.0 mL DMF | 90 |
| Leu-69   | Fmoc-Leu-OH<sup>a</sup> | 229.7  | 6.5 + 0.5 mL DMF | 45 |
| Cys-68   | Boc-Cys(StBu)-OH<sup>c</sup> | 402.3  | -     | 180 |

<sup>a</sup> Activation of this amino acid (649.5 µmol, 5 equiv.) was performed with HCTU (258.1 mg, 623.5 µmol, 4.8 equiv.)

<sup>b</sup> Activation of this amino acid (389.7 µmol, 3 equiv.) was performed with PyBOP (196.2 mg, 376.7 µmol, 2.9 equiv.)

<sup>c</sup> Activation and coupling of cysteine derivatives (1.30 mmol, 10 equiv.) was performed following the general procedure (anhydride formation with DIC (100.7 µL, 649.5 µmol, 5 equiv.) in 7.2 mL DMF/CH<sub>2</sub>Cl<sub>2</sub> (1:2.5), coupling in 3.8 mL DMF).
92.9 %, \( R_f (S7) = 0.41 \) (CH\(_2\)Cl\(_2\)/MeOH 10:1).

The cleaved peptidyl acid S7 (186.6 mg, 35.2 \( \mu \)mol, 1 equiv.) was dissolved in 1.9 mL of absolute CH\(_2\)Cl\(_2\) followed by addition of BocHN-NH\(_2\) (27.9 mg, 211.2 \( \mu \)mol, 6 equiv.), Cl-HOBT (23.9 mg, 140.8 \( \mu \)mol, 4 equiv.), and DIPEA (32.9 \( \mu \)L, 193.6 \( \mu \)mol, 5.5 equiv.) at 0 °C. DIC (21.8 \( \mu \)L, 140.8 \( \mu \)mol, 4 equiv.) was added and the mixture was kept at 0 °C until complete conversion (TLC, CH\(_2\)Cl\(_2\)/MeOH 10:1). The reaction mixture was diluted with 150 mL of CH\(_2\)Cl\(_2\) and washed once with 150 mL of 2 M KHCO\(_3\). The aqueous phase was extracted twice with 100 mL of CH\(_2\)Cl\(_2\). The organic phases were combined and dried over MgSO\(_4\). The filtrate was adsorbed onto silica gel 60 (63 - 200 \( \mu \)m) and purified by flash chromatography (Reveleris iES, FlashPure cartridge 40 g (35 - 45 \( \mu \)m), 0-10 \% MeOH in CH\(_2\)Cl\(_2\) over 40 min, 40 mL/min). The fractions were analyzed by TLC, dried and lyophilized from dioxane. Yield: 163.7 mg (30.2 \( \mu \)mol, 79.9 \% over 3 steps). \( R_f (22) = 0.53 \) (CH\(_2\)Cl\(_2\)/MeOH 10:1). C\(_{287}\)H\(_{400}\)N\(_{42}\)O\(_{55}\)S\(_3\) (5414.78).

Deprotection of 22 with TFA/TIS/H\(_2\)O (95:2.5:2.5): RP-HPLC-MS: column [A], 10-50 \% MeCN/H\(_2\)O + 0.1 \% HCOOH. ESI-MS of 22d \( m/z \) (average isotopes) C\(_{156}\)H\(_{256}\)N\(_{42}\)O\(_{44}\)S\(_2\) (3488.13) calculated: 3489.14 [M+H]\(^+\), 1745.07 [M+2H]\(^{2+}\), 1163.72 [M+3H]\(^{3+}\), 873.04 [M+4H]\(^{4+}\); found: 3488.98 [M+H]\(^+\), 1744.47 [M+2H]\(^{2+}\), 1163.32 [M+3H]\(^{3+}\), 872.98 [M+4H]\(^{4+}\).

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Figure S7: RP-HPLC-MS of deprotected EPO 68-97 hydrazide 22d.
3.4 Selective Deprotection of EPO 68-97 Hydrazide 22

![Diagram of EPO 68-97 Hydrazide](image)

**Figure S8:** Synthesis of deallylated EPO 68-97 hydrazide S8.

To a solution of peptide hydrazide 22 (82.8 mg, 15.3 μmol, 1 equiv.) in 510 μL of 10 % MeOH in CH$_2$Cl$_2$ were added phenylsilane (28.3 μL, 229.5 μmol, 15 eq), 2-[(dicyclohexylphosphino)ethyl]trimethyl-ammonium chloride (DCHT) (8.81 mg, 27.5 μmol, 1.8 eq) and Pd$^{II}$EnCat 30 (11.48 mg, loading: 0.4 mmol/g, 0.3 eq) under an argon atmosphere and exclusion of light. The suspension was stirred slowly until complete conversion of the allyl ester 22 (TLC: CH$_2$Cl$_2$/MeOH 15:1; $R_f = 0.38$ (22); 0.32 (S8)). The reaction mixture was filtered and the residue was washed with CH$_2$Cl$_2$. The filtrate was extracted twice with H$_2$O and the combined aqueous phases were extracted twice with CH$_2$Cl$_2$. The combined organic phases were dried over MgSO$_4$ and the filtrate was adsorbed to silica gel 60 (63 - 200 μm) and purified by flash chromatography (Reveleris iES, FlashPure cartridge 12 g (35 - 45 μm), 0-10 % MeOH in CH$_2$Cl$_2$ over 30 min, 28 mL/min). The fractions containing S8 were combined and concentrated. The peptide hydrazide S8 was taken up in 500 μL of CH$_2$Cl$_2$ and precipitated with cold Et$_2$O (10 mL) for 30 min at -24 °C. After centrifugation the supernatant was decanted and the residue was suspended in cold Et$_2$O (-24 °C) and centrifuged. The precipitate was dried in vacuo and lyophilized from dioxane. Yield: 60.7 mg (11.3 μmol, 73.9 %). $R_f$ (S8) = 0.32 (CH$_2$Cl$_2$/MeOH 15:1). C$_{284}$H$_{396}$N$_{42}$O$_{55}$S$_3$ (5374.71).

Deprotection of S8 with TFA/TIS/H$_2$O (95:2.5:2.5): RP-HPLC-MS: column [A], 10 % for 1.25 min, then 10-50 % MeCN/H$_2$O + 0.1 % HCOOH over 9 min, 0.5 mL/min. ESI-MS of S8d m/z (average isotopes) C$_{153}$H$_{252}$N$_{42}$O$_{44}$S$_2$ (3448.07) calculated: 3449.08 [M+H]$^+$, 1725.04 [M+2H]$^{2+}$, 1150.36 [M+3H]$^{3+}$, 863.02 [M+4H]$^{4+}$; found: 3445.75 [M+H]$^+$, 1722.80 [M+2H]$^{2+}$, 1148.86 [M+3H]$^{3+}$, 862.17 [M+4H]$^{4+}$. 

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3.5 Synthesis of EPO 29-67 Glycopeptide Hydrazide 20

Nonasaccharide azide S9[^1] was taken up in 100 - 200 µL H₂O and lyophilized prior to reduction. Under an argon atmosphere the glycan azide S9 (9.08 mg, 5.45 µmol, 1 equiv.) was dissolved in absolute MeOH (606 µL, 9 mM S9) followed by addition of DIPEA (abs., 18.54 µL, 109.0 µmol, 20 equiv.) and 1,3-propanedithiol (65.5 µL, 654.0 µmol, 120 equiv.). The solution was shaken at ambient temperature. After complete conversion (TLC: iPrOH/1 M NH₄OAc 2:1; R_t S9 = 0.47; R_t 16 = 0.20), the solvent was removed in vacuo. The residue was suspended in 1.5 mL of cold Et₂O (-24 °C) and the glycosylamine was precipitated for 10 min at -24 °C in a cooling bath. After centrifugation (20 000g, 3 min), the supernatant was discarded.

[^1]: Nonasaccharide azide S9 was taken up in 100 - 200 µL H₂O and lyophilized prior to reduction. Under an argon atmosphere the glycan azide S9 (9.08 mg, 5.45 µmol, 1 equiv.) was dissolved in absolute MeOH (606 µL, 9 mM S9) followed by addition of DIPEA (abs., 18.54 µL, 109.0 µmol, 20 equiv.) and 1,3-propanedithiol (65.5 µL, 654.0 µmol, 120 equiv.). The solution was shaken at ambient temperature. After complete conversion (TLC: iPrOH/1 M NH₄OAc 2:1; R_t S9 = 0.47; R_t 16 = 0.20), the solvent was removed in vacuo. The residue was suspended in 1.5 mL of cold Et₂O (-24 °C) and the glycosylamine was precipitated for 10 min at -24 °C in a cooling bath. After centrifugation (20 000g, 3 min), the supernatant was discarded.
and the residue was triturated with cold Et₂O twice in an analogous manner. Finally, the glycosylamine 16 was dried in vacuo and used directly for aspartylation.

The aspartyl peptide S4 (51.54 mg, 6.81 µmol, 1.25 equiv.) was dissolved in 151.6 µL of an activation mixture (5.18 mg of HATU (13.63 µmol, 2.5 equiv.) and 7.42 µL of DIPEA (43.60 µmol, 8 equiv.) in DMF/DMSO (abs, 1:1)). After 10 min, the activated peptide was added to the glycosylamine 16 and the vial was rinsed three times with 63 µL each of DMF/DMSO (abs., 1:1). The reaction mixture was shaken at ambient temperature for 17 h. Subsequently, 19.5 mL of precooled TFA/TIS/H₂O (95:2.5:2.5) were added. The solution was shaken for 2.5 h, concentrated in vacuo to 2 mL followed by addition of cold Et₂O (30 mL, -24 °C) for 20 min in a cooling bath at -24 °C. After centrifugation (5000 g, 3 min), the supernatant was discarded. The residue was triturated again with cold Et₂O, dried in vacuo, taken up in 25 % MeCN/H₂O + 0.1 % TFA and lyophilized. The lyophilizate was taken up in 25 % MeCN/H₂O + 0.1 % TFA, and the glycopeptide hydrazide 20 was purified by RP-HPLC (column [F], 25-35 % MeCN/H₂O + 0.1 % TFA, 8 mL/min) and lyophilized. Yield of 20: 14.51 mg (2.32 µmol, 42.6 %). RP-HPLC-MS: column [A], 10-30 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 20 m/z (average isotopes) C_{267}H_{418}N_{62}O_{106}S_{2} (6256.73) calculated: 3129.37 [M+2H]^{2+}, 2086.58 [M+3H]^{3+}, 1565.19 [M+4H]^{4+}; found: 3129.88 [M+2H]^{2+}, 2086.77 [M+3H]^{3+}, 1565.26 [M+4H]^{4+}.

**Figure S11**: RP-HPLC-MS of EPO 29-67 glycopeptide hydrazide 20 a) crude and b) after purification by RP-HPLC.
3.6 Synthesis of EPO 68-97 Glycopeptide Hydrazide 23

Nonasaccharide azide S9 \[^9\] was taken up in 100 - 200 µL H₂O and lyophilized prior to reduction. Under an argon atmosphere the glycan azide S9 (9.23 mg, 5.54 µmol, 1 equiv.) was dissolved in absolute MeOH (615 µL, 9 mM S9) followed by addition of DIPEA (abs., 18.84 µL, 110.8 µmol, 20 equiv.) and 1,3-propanedithiol (66.6 µL, 664.7 µmol, 120 equiv.). The solution was shaken at ambient temperature. After complete conversion (TLC: iPrOH/1 M NH₄OAc 2:1; Rₙ S9 = 0.47; Rₙ 16 = 0.20), the solvent was removed in vacuo. The residue was suspended in 1.5 mL of cold Et₂O (-24 °C) and the glycosylamine was precipitated for 10 min at -24 °C in a cooling bath. After centrifugation (20 000 g, 3 min), the supernatant was discarded and the residue was triturated with cold Et₂O twice in an analogous manner. Finally, the glycosylamine 16 was dried in vacuo and used directly for aspartylation.

The aspartyl peptide S8 (37.21 mg, 6.92 µmol, 1.25 equiv.) was dissolved in 153.9 µL of an activation mixture (5.27 mg of HATU (13.85 µmol, 2.5 equiv.), 1.89 mg of HOAt (13.85 µmol, 2.5 equiv.) and 4.71 µL of DIPEA (27.70 µmol, 5 equiv.) in DMF/DMSO (abs., 1:1)). After 10 min, the activated peptide was added to the glycosylamine 16 and the vial was rinsed three times with 25.6 µL each of DMF/DMSO (abs., 1:1). The reaction mixture was shaken at ambient temperature for 17 h. Subsequently, 19.8 mL of precooled TFA/TIS/H₂O (95:2.5:2.5) were added to the aspartylation solution. The solution was shaken for 2.5 h, concentrated in vacuo to 2 mL followed by addition of cold Et₂O (30 mL, -24 °C) for 20 min in a cooling bath at -24 °C. After centrifugation (5000 g, 3 min), the supernatant was discarded. The residue was
triturated with cold Et₂O, dried *in vacuo*, taken up in taken up in 25 % MeCN/H₂O + 0.1 % TFA and lyophilized. The lyophilizate was taken up in 3.0 mL of 25 % MeCN/H₂O + 0.1 % TFA, and the glycopeptide hydrazide 23 was purified by RP-HPLC (column [F], 25-40 % MeCN/H₂O + 0.1 % TFA, 8 mL/min) and lyophilized. Yield of 23: 10.38 mg (2.05 µmol, 37.0 %). RP-HPLC-MS: column [A], 10-50 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 23 m/z (average isotopes) C₂₁₅H₃₅₅N₄₇O₈₈S₂ (5070.57 g/mol) calculated: 2536.29 [M+2H]²⁺, 1691.20 [M+3H]³⁺, 1268.65 [M+4H]⁴⁺; found: 2534.65 [M+2H]²⁺, 1689.85 [M+3H]³⁺, 1267.92 [M+4H]⁴⁺.

**Figure S13:** RP-HPLC-MS of EPO 68–97 glycopeptide hydrazide 23 a) crude and b) after purification by RP-HPLC.
4. Synthesis of the C-terminal Fragments

4.1 Synthesis of EPO 98-127 Thioester 4

**Figure S14**: Synthesis of EPO 98-127 thioester 4.

Fmoc-Ala-Trt-CM resin S10 (loading = 370 µmol/g) was synthesized according to Ref.[8]. 351.4 mg (130 µmol, 1 eq) of resin S10 were placed in a 45 mL peptide synthesizer reaction vessel and the peptide chain was elongated automatically. For cleavage of the Fmoc group the general procedure was applied and the amino acid building blocks were coupled under the conditions listed in table S3 and the general procedure section. After complete elongation the resin S11 was washed with DMF (5x) and CH₂Cl₂ (5x) and was dried in vacuo.

**Table S3:**

| Position   | Building Block                | m (mg) | V (mL)         | Coupling duration (min) |
|------------|-------------------------------|--------|----------------|-------------------------|
| Ser-126, Ala-125 | Fmoc-Ala-Ser(ψMe₂Me₃pro)-OH[^a] | 144.7  | 1.5 + 1.5 mL DMF | 60                      |
| Ala-124    | Fmoc-Ala-OH[^a]               | 171.2  | 3.0            | 45                      |
| Asp-123    | Fmoc-Asp(OtBu)-OH[^a]         | 226.3  | 3.0            | 45                      |
| Pro-122    | Fmoc-Pro-OH[^a]              | 185.6  | 3.0            | 45                      |
| Pro-121    | Fmoc-Pro-OH[^a]             | 185.6  | 3.0            | 45                      |
| Ser-120    | Fmoc-Ser(tBu)-OH[^a]         | 210.9  | 3.0            | 45                      |
Peptidyl resin **S11** (247.6 mg, loading = 110 μmol/g, 27.2 μmol, 1 eq) was swelled with 5 mL of CH₂Cl₂ for 5 min and then treated with 7 mL of 1.5 % TFA in CH₂Cl₂ containing 2 % Et₃SiH (10 x, 2 min each). Subsequently, the resin was washed with CH₂Cl₂ (6 x, 7 mL each). All solutions were combined in a flask containing 2.0 mL of DIPEA in 20 mL of CH₂Cl₂ (11.84 mmol, 1.1 eq based on TFA). Further 2.0 mL of DIPEA were added and the solution was concentrated in vacuo to a final volume of 6 mL. n-Butyl 3-mercaptopropionate (132.7 mg, 817.2 μmol, 30 eq), Cl-HOBt (92.4 mg, 544.8 μmol, 20 eq) and DIC (84.9 μL, 544.8 μmol, 20 eq) were added. After 8 d 25 mL of TFA/Et₃SiH/H₂O/nBu-3-MeP (95:2:2:1) were added. After 2 h, the reaction mixture was concentrated in vacuo to 4 mL and precipitated with cold Et₂O (40 mL) in a cooling bath at −24 °C for 24 h. After centrifugation (5000 g, 3 min), the supernatant was discarded. The residue was triturated twice with Et₂O (40 mL each, −24 °C, 30 min), dried in vacuo and lyophilized from 50 % MeCN/H₂O + 0.1 % HCOOH. The crude product was dissolved in 6 mL of 50 % MeCN/H₂O + 0.1 % HCOOH and 4 was isolated by
two consecutive RP-HPLC purifications (1. Column [F], 20-50 % MeCN/H₂O + 0.1 % TFA, 8 mL/min; 2. Column [L], 20-50 % MeCN/H₂O + 0.1 % TFA, 4 mL/min) and was lyophilized. Yield of 4: 36.2 mg (11.48 µmol, 42.2 %). RP-HPLC-MS: column [A], 20-60 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 4: m/z (average isotopes) C₁₃₆H₂₃₄N₃₈O₄₃S₂ (M = 3153.71) calculated: 3154.71 [M+H]⁺, 1577.86 [M+2H]²⁺, 1052.24 [M+3H]³⁺; found: 3154.79 [M+H]⁺, 1577.39 [M+2H]²⁺, 1051.91 [M+3H]³⁺.

**Figure S15:** RP-HPLC-MS of purified EPO 98-127 Thioester 4.

### 4.2 Synthesis of EPO 128-166 Peptidyl Acid 5

Fmoc-Arg(Pbf)-Trt-CM resin S₁₂ (loading = 250 µmol/g) was synthesized according to Ref.¹⁸. 300.0 mg (75 µmol, 1 eq) of resin S₁₂ were placed in a 45 mL peptide synthesizer reaction vessel and the peptide chain was elongated automatically. For cleavage of the Fmoc group the general procedure was applied and the amino acid building blocks were coupled under the conditions denoted in table S4 and the general procedure section. After complete elongation the resin S₁₃ was washed with DMF (5 x) and CH₂Cl₂ (5 x) and was dried in vacuo (yield: 533.4 mg, loading = 50.3 µmol, 67.1 %).
| Position | Building Block | m (mg) | V (mL) 0.4 M DIPEA in DMF | Coupling duration (min) |
|----------|----------------|--------|---------------------------|-------------------------|
| Asp-165  | Fmoc-Asp(tBu)-OH[^a] | 154.3  | 2.0                        | 45                      |
| Gly-164  | Fmoc-Gly-OH[^a]    | 111.5  | 2.0                        | 45                      |
| Thr-163  | Fmoc-Thr(tBu)-OH[^a] | 149.1  | 2.0                        | 45                      |
| Arg-162  | Fmoc-Arg(Pbf)-OH[^a] | 243.3  | 2.0                        | 45                      |
| Cys-161  | Fmoc-Cys(Acm)-OH[^c] | 310.7  | -                          | 180                     |
| Ala-160  | Fmoc-Ala-OH[^a]    | 116.7  | 2.0                        | 45                      |
| Glu-159  | Fmoc-Glu(tBu)-OH[^a] | 159.6  | 2.0                        | 45                      |
| Gly-158  | Fmoc-Gly-OH[^a]    | 111.5  | 2.0                        | 45                      |
| Thr-157, Tyr-156 | Fmoc-Tyr(tBu)-Thr(ψMe,Me,Glu)-OH[^b] | 225.3  | 2.0                        | 45                      |
| Leu-155  | Fmoc-Leu-OH[^a]    | 132.5  | 2.0                        | 45                      |
| Lys-154  | Fmoc-Lys(Boc)-OH[^a] | 175.7  | 2.0                        | 45                      |
| Leu-153  | Fmoc-Leu-OH[^a]    | 132.5  | 2.0                        | 45                      |
| Lys-152  | Fmoc-Lys(Boc)-OH[^a] | 175.7  | 2.0                        | 45                      |
| Gly-151  | Fmoc-Gly-OH[^a]    | 111.5  | 2.0                        | 45                      |
| Arg-150  | Fmoc-Arg(Pbf)-OH[^a] | 243.3  | 2.0                        | 45                      |
| Leu-149  | Fmoc-Leu-OH[^a]    | 132.5  | 2.0                        | 45                      |
| Phe-148  | Fmoc-Phe-OH[^a]    | 145.3  | 2.0                        | 45                      |
| Asn-147  | Fmoc-Asn(tBu)-OH[^a] | 223.8  | 2.0                        | 45                      |
| Ser-146, Tyr-145 | Fmoc-Tyr(tBu)-Ser(ψMe,Me,Glu)-OH[^b] | 220.0  | 2.0                        | 45                      |
| Val-144  | Fmoc-Val-OH[^a]    | 127.3  | 2.0                        | 45                      |
| Arg-143  | Fmoc-Arg(Pbf)-OH[^a] | 243.3  | 2.0                        | 45                      |
| Phe-142  | Fmoc-Phe-OH[^a]    | 145.3  | 2.0                        | 45                      |
| Leu-141  | Fmoc-Leu-OH[^a]    | 132.5  | 2.0                        | 45                      |
| Lys-140  | Fmoc-Lys(Boc)-OH[^a] | 175.7  | 2.0                        | 45                      |
| Arg-139  | Fmoc-Arg(Pbf)-OH[^a] | 243.3  | 2.0                        | 45                      |
| Phe-138  | Fmoc-Phe-OH[^a]    | 145.3  | 2.0                        | 45                      |
| Thr-137, Asp-136 | Fmoc-Asp(tBu)-Thr(ψMe,Me,Glu)-OH[^b] | 207.1  | 2.0                        | 45                      |
| Ala-135  | Fmoc-Ala-OH[^a]    | 116.7  | 2.0                        | 45                      |
| Thr-134  | Fmoc-Thr(tBu)-OH[^a] | 149.1  | 2.0                        | 45                      |
| Ile-133  | Fmoc-Ile-OH[^a]    | 132.5  | 2.0                        | 45                      |
| Thr-132  | Fmoc-Thr(tBu)-OH[^a] | 149.1  | 2.0                        | 45                      |
| Arg-131  | Fmoc-Arg(Pbf)-OH[^a] | 243.3  | 2.0                        | 45                      |
| Leu-130  | Fmoc-Leu-OH[^a]    | 132.5  | 2.0                        | 45                      |
| Pro-129  | Fmoc-Pro-OH[^a]    | 126.5  | 2.0                        | 45                      |
| Cys-128  | Fmoc-Cys(tBu)-OH[^c] | 232.1  | -                          | 180                     |

[^a] Activation of this amino acid (374.9 µmol, 5 equiv.) was performed with HCTU (155.1 mg, 374.9 µmol, 5 equiv.)
b) Activation of this amino acid (374.9 µmol, 5 equiv.) was performed with PyBOP (195.1 mg, 374.9 µmol, 5 equiv.)

[c] Activation and coupling of cysteine derivatives (749.8 µmol, 10 equiv.) was performed following the general procedure (anhydride formation with DIC (58.4 µL, 374.9 µmol, 5 equiv.) in 4.2 mL DMF/CH₂Cl₂ (1:2.5), coupling in 3.6 mL DMF).

A portion of peptidyl resin S13 (364.8 mg, loading = 94.3 µmol/g, 34.4 µmol) was suspended in 4.3 mL of TFA/TIS/H₂O (95:2.5:2.5) and shaken for 2.5 h. The cleavage solution was drained and the resin was washed three times for 10 min with TFA/TIS/H₂O (95:2.5:2.5, 1.5 mL each). The filtrates were combined, concentrated in vacuo to 2.0 mL and precipitated with Et₂O (20 mL) in a cooling bath at −24 °C for 20 min, followed by centrifugation (5000 g, 3 min). The precipitate was treated analogously with Et₂O (20 mL each) twice. The residue was dried in vacuo, taken up in 25 % MeCN/H₂O + 0.1 % TFA, filtered through a PTFE filter (0.2 µm) and lyophilized. The crude product (162.8 mg) was dissolved in 16.2 mL of 20 % MeCN/H₂O + 0.1 % TFA and purified by RP-HPLC (column [F], 20-50 % MeCN/H₂O + 0.1 % TFA, 8 mL/min) and lyophilized.

Yield of 5: 94.3 mg (19.9 µmol, 57.8 %). RP-HPLC-MS: column [A], 10-50 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 5: m/z (average isotopes) C₂₁₀H₃₄₂N₆₀O₅₆S₃ (M = 4727.60 g/mol, Mₘᵢ = 4724.4982 Da) calculated: 946.53 [M+5H]⁵⁺, 788.94 [M+6H]⁶⁺, 676.38 [M+7H]⁷⁺; found: 2364.55 946.29 [M+5H]⁵⁺, 788.64 [M+6H]⁶⁺, 676.08 [M+7H]⁷⁺.

**Figure S17:** RP-HPLC-MS of purified EPO 128-166 5.
5. Synthesis of EPO A by Sequential NCL

5.1 Synthesis of EPO 29-67 Glycopeptide Thioester 21

According to the general procedure for thioester formation, the glycopeptide hydrazide 20 (4.00 mg, 639.3 nmol, 1 equiv.) was dissolved in 182.7 µL of 6 M GdmCl buffer and reacted with 8.3 µL of 0.5 M NaNO₂ (4.16 µmol, 6.5 equiv.). After 1 h, 41.6 µL of the MESNa stock solution (41.56 µmol, 65 equiv.) were added. The pH value was adjusted to pH 6.6 and the reaction mixture was desalted after 1 h by gel filtration (column [O], 10-30 % MeCN/H₂O + 0.1 % TFA, 0.45 mL/min). The target fraction was lyophilized.

Yield of 21: 3.94 mg (618.8 nmol, 96.8 %). RP-HPLC-MS: column [A], 10-30 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 21: m/z (average isotopes) C₅₆₆H₄₂₀N₁₆O₁₀₉S₄ (6366.87) calculated: 3184.44 [M+2H]²⁺, 2123.30 [M+3H]³⁺, 1592.72 [M+4H]⁴⁺, 1274.38 [M+5H]⁵⁺; found: 3182.67 [M+2H]²⁺, 2122.02 [M+3H]³⁺, 1591.74 [M+4H]⁴⁺, 1273.69 [M+5H]⁵⁺.

Figure S18: Synthesis of EPO 29-67 glycopeptide thioester 21.

Figure S19: RP-HPLC-MS of EPO 29-67 glycopeptide thioester 21.
5.2 Synthesis of EPO 29-97 Glycopeptide Hydrazide 25

The ligation buffer (6 M GdmCl, 0.2 M NaH$_2$PO$_4$, 40 mM TCEP, 0.1 M MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.9. Glycopeptide hydrazide 23 (6.29 mg, 1.24 µmol, 1 equiv.) was dissolved in 620 µL of ligation buffer and added to the thioester 21 (7. 89 mg, 1.24 µmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (28 h, LC-MS control, LC-MS samples were prepared with 20 mM TCEP in water). Prior to purification, the mixture was treated with TCEP x HCl (7.11 mg, 24.8 µmol, 20 equiv.) for 30 min. The glycopeptide hydrazide 25 was purified by RP-HPLC (column [G], 20-60 % MeCN/H$_2$O + 0.1 % TFA 3 mL/min).

Yield of 25: 10.01 mg (893.2 nmol, 72.0 %) C$_{478}$H$_{761}$N$_{107}$O$_{194}$S$_{3}$ (11207.08). RP-HPLC: column [A], 20-60 % MeCN/H$_2$O + 0.1 % HCOOH. HR-MS of 25: m/z (exact mass) C$_{478}$H$_{761}$N$_{107}$O$_{194}$S$_{3}$ (11200.2134) calculated: 1601.0378 [M+7H]$^7^+$, 1401.0340 [M+8H]$^8^+$, 1245.4754 [M+9H]$^9^+$, 1121.0286 [M+10H]$^{10^+}$, found: 1601.0373 [M+7H]$^7^+$, 1401.0343 [M+8H]$^8^+$, 1245.4735 [M+9H]$^9^+$, 1121.0275 [M+10H]$^{10^+}$.

Figure S20: Synthesis of EPO 29-97 hydrazide 25

Figure S21: RP-HPLC-MS of EPO 29-97 glycopeptide 25 a) crude after 28 h and b) after RP-HPLC.
5.3 Synthesis of EPO 29-97 Glycopeptide Thioester S14

According to the general procedure for thioester formation, the glycopeptide hydrazide 25 (10.01 mg, 893.2 nmol, 1 equiv.) was dissolved in 255.2 µL of 6 M GdmCl buffer and reacted with 11.62 µL of 0.5 M NaNO₂ (5.81 µmol, 6.5 equiv.). After 1 h, 58.1 µL of the MESNa stock solution (58.06 µmol, 65 equiv.) were added. The pH was adjusted to a value of 6 and the reaction mixture was desalted after 1 h by gel filtration (column [M], 10 % MeCN/H₂O + 0.1 % TFA, 0.5 mL/min). The fraction containing S14 was lyophilized.

Yield of S14: 9.42 mg (832.4 nmol, 93.2 %) C₄₈₀H₇₆₃N₁₀₅O₁₉₇S₅ (11317.22). RP-HPLC-MS: column [A], 20-60 % MeCN/H₂O + 0.1 % HCOOH.

HR-MS of S14: m/z (exact mass) C₄₈₀H₇₆₃N₁₀₅O₁₉₇S₅ (11310.1518 Da) calculated: 1886.0326 [M+6H]⁶⁺, 1616.7432 [M+7H]⁷⁺, 1414.7763 [M+8H]⁸⁺, found: 1886.0195 [M+6H]⁶⁺, 1616.7414 [M+7H]⁷⁺, 1414.7638 [M+8H]⁸⁺.

Figure S22: Synthesis of EPO 29-97 thioester S14

Figure S23: RP-HPLC-MS of purified EPO 29-97 glycopeptide thioester S14.
5.4 Synthesis of EPO 98-166 26

![Chemical structure](attachment:structure.png)

Figure S24: Synthesis of EPO 98-166 26 by NCL.

The ligation buffer (6 M GdmCl, 0.2 M NaH₂PO₄, pH 6.9, 40 mM TCEP, 0.1 M MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.9. Peptide 5 (15.00 mg, 3.17 µmol, 1.2 equiv.) was dissolved in 1.59 mL of ligation buffer and added to the thioester 4 (8.34 mg, 2.64 µmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (13 h, LC-MS control). Subsequently, MeONH₂·HCl (26.51 mg, 317.4 µmol) was added and the pH was adjusted to pH = 3.5 using a microelectrode. Ring opening of the thiazolidine was complete after 24 h according to LC-MS (LC-MS samples were prepared with 20 mM TCEP in 20 % MeCN/H₂O). Prior to purification, the mixture was treated with TCEP·HCl (45.5 mg, 158.7 µmol, 50 equiv.) for 30 min. Peptide 26 was purified by RP-HPLC (column [F], 25-45 % MeCN/H₂O + 0.1 % TFA, 8 mL/min).

Yield of 26: 17.9 mg (2.35 µmol, 89.0 %). RP-HPLC-MS: column [A], 20-50 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 26: m/z (average isotopes) C₃₃₄H₅₅₄N₁₀₀O₉₇S₃ (7618.89) calculated: 1089.42 [M+7H]$^7^+$, 953.37 [M+8H]$^8^+$, 847.55 [M+9H]$^9^+$, found: 1089.47 [M+7H]$^7^+$, 953.34 [M+8H]$^8^+$, 847.52 [M+9H]$^9^+$. 
Figure S25: RP-HPLC-MS of EPO 98-166 26: a) reaction mixture after NCL, b) Thz opening and c) after purification by RP-HPLC.
5.5 Synthesis of EPO 29-166 Glycopeptide 27

The ligation buffer (6 M GdmCl, 0.2 M NaH2PO4, 40 mM TCEP, 0.1 M MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.7. Peptide 26 (3.96 mg, 519.8 nmol, 1.2 equiv.) was dissolved in 217 µL of ligation buffer and added to the thioester S14 (4.90 mg, 433.0 nmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (27 h, LC-MS control, LC-MS samples were prepared with 100 mM TCEP in water).

Prior to purification, the mixture was treated with TCEP x HCl (6.2 mg, 21.6 µmol, 50 equiv.) for 30 min. Peptide 27 was purified by RP-HPLC (column [J], 30-50 % MeCN/H2O + 0.1 % TFA, 3 mL/min) and lyophilized.

Yield of 27: 5.93 mg (315.5 nmol, 72.9 %). RP-HPLC-MS: column [D], 20-60 % MeCN/H2O + 0.1 % HCOOH. HR-MS of 27: m/z (exact mass) C812H1311N205O291S6 (18782.2414) calculated: 1879.2314 [M+10H]10+, 1708.4838 [M+11H]11+, 1566.1941 [M+12H]12+, found: 1879.2380 [M+10H]10+, 1708.4827 [M+11H]11+, 1566.1968 [M+12H]12+.
Figure S27: RP-HPLC-MS of EPO 29-166 glycopeptide 27: a) reaction mixture after NCL and b) purified.

5.6 Desulfurization of EPO 29-166 Glycopeptide 27

Under anaerobic conditions, a buffer (6 M GdmCl, 0.2 M NaH$_2$PO$_4$) was prepared and adjusted to pH = 7.0. A TCEP stock solution was prepared with this buffer (0.5 M TCEP x HCl) and the pH was adjusted to pH 7.0. Furthermore, a glutathione stock solution (160 mM glutathione, giving pH 4.7) and a VA-044 stock solution (80 mM VA-044, giving pH 6.9) were prepared with the GdmCl buffer. The glycopeptide 27 (8.47 mg, 450.7 nmol, 1 equiv.) was dissolved in
the 450.7 µL of the TCEP stock solution (500 equiv.) followed by the glutathione and VA-044 stock solutions (225.3 µL each). The reaction mixture (final pH 6.6) was shaken under anaerobic conditions at 37 °C until completion (3 h according to HRMS). Subsequently, the reaction mixture was desalted by gel filtration (column [M], 10 % MeCN/H2O + 0.1 % TFA, 1.5 mL/min) and lyophilized.

Yield of 28: 8.07 mg (431.6 nmol, 95.8 %). RP-HPLC-MS: column [D], 20–60 % MeCN/H2O + 0.1 % HCOOH. HR-MS of 28: m/z (exact mass) C_{812}H_{1311}N_{205}O_{291}S_{3} (18686.3252) calculated: 1869.6398 [M+10H]^{10+}, 1699.7641 [M+11H]^{11+}, 1558.2010 [M+12H]^{12+}, found: 1869.6413 [M+10H]^{10+}, 1699.7632 [M+11H]^{11+}, 1558.2028 [M+12H]^{12+}.

**Figure S29:** RP-HPLC-MS of EPO 29-166 glycopeptide 28 after SEC.

### 5.7 Cleavage of Acm groups from EPO 29-166 Glycopeptide 28

A buffer (6 M GdmCl, 0.2 M NaH2PO4) was prepared and adjusted to pH = 6.9. The buffer was degassed with argon for 30 min. Subsequently, anhydrous PdCl2 (2.25 mg, 12.69 µmol) was
suspended in 940 µL of this buffer and shaken for 1 h under argon for complete dissolution (13.5 mM PdII). 181 µL of the PdII stock solution (284.5 µg PdCl2, 2.44 µmol, 30 equiv.) were added to the Acm-protected glycopeptide 28 (1.52 mg, 81.3 nmol, 1 equiv.) under argon. Under the exclusion of light the mixture was incubated for 2h (HPLC-MS control after adding 100 equiv. of DTT/ equiv. of Pd to an aliquot). Subsequently, DTT (195 mg) was added to the reaction mixture. The mixture was shaken for 2 h and purified by RP-HPLC (column [H], 10-60 % MeCN/H2O + 0.1 % HCOOH, 1 mL/min). The target fractions were lyophilized. The samples for HPLC-MS of the purified glycopeptide 29 were prepared with 100 mM DTT in water.

Yield of 29: 1.25 mg (67.62 nmol, 83.2 %). RP-HPLC-MS: column [D], 20-60 % MeCN/H2O + 0.1 % HCOOH. HR-MS of 29: m/z (exact mass) C80H1296N202O288S3 (18473.2138) calculated: 1848.3287 [M+10H]10+, 1680.3904 [M+11H]11+, 1540.4418 [M+12H]12+, found: 1848.3244 [M+10H]10+, 1680.3842 [M+11H]11+, 1540.4391 [M+12H]12+.

Figure S31: Synthesis of EPO 29-166 glycopeptide 29: zoom of mass spectra (RP-HPLC-MS) a) before and b) 2 h after addition of PdCl2, c) RP-HPLC-MS of 29 after RP-HPLC.
Figure S31-Pd: Deprotection of 28 with Pd(II) in GdmCl: a) analytical RP-HPLC-MS after quenching an aliquot with insufficient amounts of DTT (6 equiv. of DTT / equiv. of Pd); b) RP-HPLC-MS after quenching an aliquot with excess DTT (100 equiv. of DTT / equiv. of Pd); c) RP-HPLC-MS comparison of tested scavengers.
5.8 Synthesis of EPO 1-28 Glycopeptide Thioester 18

According to the general procedure for thioester formation, the glycopeptide hydrazide 17\(^{[10]}\) (5.00 mg, 1.02 µmol, 1 eq) was dissolved in 292.6 µL of 6 M GdmCl buffer and reacted with 13.31 µL of 0.5 M NaNO\(_2\) (6.66 µmol, 6.5 equiv.). After 1 h, 66.5 µL of the MESNa stock solution (66.56 µmol, 65 equiv.) were added. The pH was adjusted to a value of 6.2 and the reaction mixture was purified after 1 h by RP-HPLC (column [G], 20-35 % MeCN/H\(_2\)O + 0.1 % TFA 3 mL/min). The target fractions were lyophilized.

Yield of 18: 3.02 mg (604.6 µmol, 59.3 %). RP-HPLC-MS: column [A], 0-50 % MeCN/H\(_2\)O + 0.1 % HCOOH. ESI-MS of 18: \(m/z\) (average isotopes) C\(_{205}\)H\(_{343}\)N\(_{43}\)O\(_{91}\)S\(_{4}\) (4994.45) calculated: 2498.23 [M+2H]\(^{2+}\), 1665.82 [M+3H]\(^{3+}\), found: 2498.94 [M+2H]\(^{2+}\), 1666.42 [M+3H]\(^{3+}\).

Figure S32: Synthesis of EPO 1-28 glycopeptide thioester 18.

Figure S33: RP-HPLC-MS of purified EPO 1-28 glycopeptide Thioester 18.
5.9 Final Ligation and Folding of EPO 1-166 (EPO A)

The ligation buffer (8 M GdmCl, 0.2 M NaH$_2$PO$_4$, 40 mM TCEP, 0.1 M MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.9. The glycopeptide thioester 18 (0.67 mg, 133.96 nmol, 2.5 equiv.) was dissolved in 27 µL of ligation buffer and added to glycopeptide 29 (1.01 mg, 53.59 nmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (6 d, LC-MS control). The ligation mixture was diluted with 12.46 mL of the dilution buffer (Table S5) and transferred to a tubular dialysis membrane (ZelluTrans/Roth V series, cutoff = 5 kDa, 40 mm flat width, thickness 20-30 µm, regenerated cellulose), and then dialyzed sequentially against the buffers shown in Table S5 (500 mL each) with constant agitation. The dialysis conditions are listed in Table S6.

Table S5:

| Buffer          | Components          |
|-----------------|---------------------|
| Dilution Buffer | 8 M GdmCl 0.1 M NaH$_2$PO$_4$ 0.5 M l-Arg 0.1 M DTT |
| (degassed, pH 7.5) | (98 %) (98.5 %) |
| Reduction Buffer| 4 M GdmCl 0.1 M NaH$_2$PO$_4$ 0.5 M l-Arg 0.01 M DTT |
| (degassed, pH 8.5) | (98 %) (98.5 %) |
| Oxidation Buffer| 4 M GdmCl 0.1 M NaH$_2$PO$_4$ 0.5 M l-Arg 4 mM l-Cys 0.5 mM l-Cystine |
| (pH 8.5)        | (98 %) (98.5 %)     |

Figure S34: Synthesis and refolding of EPO A
**Table S6:**

| Buffer                  | Conditions                  | Dialysis Duration |
|-------------------------|-----------------------------|-------------------|
| Reduction Buffer        | anaerobic (≤ 5 ppm O₂), 26 °C | 12 h              |
| Oxidation Buffer 4 M    | air, 4 °C                   | 12 h              |
| Oxidation Buffer 2 M    | air, 4 °C                   | 12 h              |
| Folding Buffer 1        | air, 4 °C                   | 8 h               |
| Folding Buffer 2        | air, 4 °C                   | 8 h               |
| Folding Buffer 2        | air, 4 °C                   | 8 h               |
| Folding Buffer 2        | air, 4 °C                   | 8 h               |

After completion of the dialysis the protein solution was concentrated to a volume of 1.25 mL (Amicon 8010 stirred cell with membrane UP010 P from MICRODYN-NADIR, cutoff = 10 kDa). Monomeric EPO A was isolated by size exclusion chromatography (column [P], 0.15 M NaCl, 50 mM NaH₂PO₄, 0.5 M l-arginine, pH 7.5, 1 mL/min). The eluate was concentrated by ultrafiltration to a volume of 1.25 mL, purified by RP-HPLC (column [H], 30-50 % MeCN/H₂O + 0.1 % TFA, 1 mL/min) and lyophilized.

Yield of EPO A: 0.54 mg (23.2 nmol, 43.3 %).

ICP-OES (λ (nm) = 340.458, 363.470, 324.270, 248.892): Pd ≤ 0.01 ppm.

RP-HPLC-MS: column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH.

HR-MS of EPO A: m/z (exact mass) C₁₀₀₂H₁₆₂₁N₂₄₅O₃₇₆S₄ (23230.4137) calculated: 1660.3225 [M+14H]¹⁴⁺, 1549.7015 [M+15H]¹⁵⁺, 1452.9081 [M+16H]¹⁶⁺, found: 1660.3261 [M+14H]¹⁴⁺, 1549.7033 [M+15H]¹⁵⁺, 1452.9103 [M+16H]¹⁶⁺.

The CD spectrum was recorded with 250 µL of EPO A (0.22 mg EPO A/mL) in ultrapure water at 23 °C.
Figure S35: Synthesis and purification of EPO A: a) RP-HPLC-MS of crude NCL mixture after 2.5 h, b) RP-HPLC-MS and SDS-PAGE of crude NCL mixture after 6 d, c) Separation of mono- and oligomers by gelfiltration (Superdex 75), d) RP-HPLC-MS of EPO A, e) HRMS of EPO A, f) found and simulated HR-isotope patterns of [M+15H]^{15+} charge state, g) SDS-PAGE of EPO A reduced and oxidized.
6. Synthesis of Sialylated EPO S by Sequential NCL

6.1 Synthesis of Sialylated EPO 1-28 Glycopeptide Hydrazide 31

The glycopeptide hydrazide 17 (3.0 mg, 0.61 µmol, 1.0 equiv.) was dissolved in 36.6 µL of water and 49.2 µL of a stock solution containing 1.5 mg CMP-Neu5Ac (2.4 µmol, 3.9 eq) in 250 mM Tris, pH 9 was added. Subsequently, 37.1 µL of α-2,6-sialyltransferase (107.59 µg) from P. damselae were added. The solution was incubated at 30 °C. After 2 h 30.7 µL of the CMP-Neu5Ac stock solution were added. After incubation for 1 h at 30 °C, 31 was isolated from the mixture by RP-HPLC (column [G], 5-30 % MeCN/H$_2$O + 0.1 % HCOOH, 3 mL/min). The target fractions were neutralized with Na$_2$CO$_3$ (1 M, approximately 20 µL/mL of eluate) and lyophilized. The residue was dissolved in 1.0 mL of 5 % EtOH/water and desalted (column [N], 5 % EtOH/water, 2 mL/min). The target fraction was lyophilized. Yield of 31: 2.25 mg (0.41 µmol, 67.2 %). RP-HPLC-MS: column [A], 10-50 % MeCN/H$_2$O + 0.1 % HCOOH. HR-MS of 31: m/z (exact mass) C$_{225}$H$_{375}$N$_{47}$O$_{104}$S$_2$ (5463.4941) calculated: 1364.8663 [M−4H]$^{4−}$, 1820.1574 [M−3H]$^{3−}$, 2730.7398 [M−2H]$^{2−}$; found: 1364.8630 [M−4H]$^{4−}$, 1820.1544 [M−3H]$^{3−}$, 2730.7117 [M−2H]$^{2−}$, 5462.4869 [M−H]$^{−}$;

Figure S36: Synthesis of sialylated EPO 1-28 glycopeptide hydrazide 31
6.2 Synthesis of Sialylated EPO 29-67 Glycopeptide Hydrazide 32

The glycopeptide hydrazide 20 (8.00 mg, 1.28 µmol, 1 equiv.) was dissolved in 1.017 mL of Tris buffer (250 mM, pH 9) and 701.4 µL of a stock solution containing CMP-Neu5Ac (23.0 mg, 30.7 µmol, 24 equiv.). Subsequently, 11.58 µL of calf intestinal alkaline phosphatase (100 mU/µL) and 57.33 µL of α-2,6-sialyltransferase (166.3 µg) from *P. damselae* were added. The solution was incubated at 30 °C. After 26 h the reaction mixture was desalted (column [M], 10 % MeCN/H₂O, 0.5 mL/min), lyophilized, and the sialoglycopeptide 32 was purified by RP-HPLC (column [G], 10-30 % MeCN/H₂O + 0.1 % HCOOH, 3 mL/min). The target fractions were neutralized with Na₂CO₃ (1 M, approximately 20 µL/mL of eluate) and lyophilized. The residue was dissolved in 1.5 mL of 10 % MeCN/H₂O and desalted (column [M], 10 % MeCN/H₂O, 0.5 mL/min). The target fraction was lyophilized.
Yield of 32: 3.97 mg (0.58 µmol, 45.3 %). RP-HPLC-MS: column [A], 10-40 % MeCN/H$_2$O + 0.1 % HCOOH. ESI-MS of 32: m/z (average isotopes) C$_{289}$H$_{452}$N$_{64}$O$_{122}$S$_2$ (6839.24) calculated: 2278.74 [M-3H]$^-$, 1708.80 [M-4H]$^4$, 1366.84 [M-5H]$^5$; found: 2281.07 [M-3H]$^-$, 1710.54 [M-4H]$^4$, 1368.14 [M-5H]$^5$.

a) UPLC-MS of reaction mixture

![Reaction mixture UPLC-MS](image1)

b) UPLC-MS of purified 32

![Purified 32 UPLC-MS](image2)

Figure S39: Enzymatic Sialylation of EPO 29-67 glycopeptide hydrazide 20: RP-HPLC-MS of a) crude reaction mixture and b) EPO 29-67 sialoglycopeptide 32 after RP-HPLC.

Figure S39a: a) Comparison of the hydrophilicity of EPO glycopeptide hydrazides 1-28 (17), 29-67 (20) and 68-97 (23) by RP-HPLC-MS (column [A], 10-50 % MeCN/H$_2$O + 0.1 % HCOOH); b) Superposition of CD-spectra of EPO glycopeptide hydrazides 1-28 (17), 29-67 (20) and 68-97 (23).
Table S7: conditions tested for the optimization of the sialylation of glycopeptide 20

|   | [20] | CMP-Neu5Ac | PdST6 | product distribution after 16 h of reaction time from integrated HPLC-trace (UV) |
|---|------|------------|-------|---------------------------------------------------------------------------------|
| 1) | 5 mM | 4 + 4 + 2 eq. | 130 ng/nmol | 20 32-Sia 32 |
| 2) | 4 mM | 1 x 24 eq. | 130 ng/nmol | 26 % 22 % 52 % |
| 3) | 1 mM | 1 x 24 eq. | 130 ng/nmol | 14 % 20 % 66 % |
| 4) | 1 mM | 6 x 4 eq./h | 130 ng/nmol | 12 % 19 % 69 % |
| 5) | 1 mM | 1 x 24 eq. | 260 ng/nmol | 30 % 15 % 55 % |
| 6) | 0.5 mM | 1 x 24 eq. | 130 ng/nmol | 6 % 7 % 87 % |
| 7) | 0.5 mM | 1 x 12 eq. | 130 ng/nmol | 12 % 8 % 80 % |

6.3 Synthesis of Sialylated EPO 68-97 Glycopeptide Hydrazide 33

![Diagram of synthesis](image)

Figure S40: Synthesis of sialylated EPO 68-97 glycopeptide hydrazide 33.

The glycopeptide hydrazide 23 (2.0 mg, 394.4 nmol, 1 equiv.) was dissolved in 29.65 µL of water and 31.56 µL of a stock solution containing 1.15 mg CMP-Neu5Ac (1.58 µmol, 4 equiv.) in 250 mM Tris, pH 9 was added. Subsequently, 17.68 µL of α-2,6-sialyltransferase (51.27 µg) from *P. damselae* were added. The solution was incubated at 30 °C. After 14h 1.15 mg of CMP-Neu5Ac (1.58 µmol, 4 equiv.) in 9 µL of 250 mM Tris pH 9 were added. The solution was incubated for 3 and subsequently 33 was purified by RP-HPLC (column [G], 20-50 % MeCN/H2O + 0.1 % HCOOH, 3 mL/min). The target fractions were neutralized with Na2CO3 (1 M, approximately 20 µL/mL of eluate) and lyophilized. The residue was dissolved in 1.2 mL of 10 % MeCN/H2O and desalted (column [M], 10 % MeCN/H2O, 0.5 mL/min). The target fraction was lyophilized.

Yield of 33: 1.82 mg (322 nmol, 81.6 %). RP-HPLC-MS: column [A], 10-50 % MeCN/H2O +
0.1 % HCOOH. ESI-MS of 33: m/z (average isotopes) C_{237}H_{389}N_{49}O_{104}S_{2} (5653.08) calculated: 1883.35 [M-3H]^3-, 1412.26 [M-4H]^4-; found: 1880.54 [M-3H]^3-, 1410.15 [M-4H]^4-.

Figure S41: Enzymatic Sialylation of EPO 68-97 hydrazide 21: RP-HPLC-MS of a) crude reaction mixture and b) EPO 68-97 sialoglycopeptide 33 after RP-HPLC.

6.4 Synthesis of EPO 1-28 Sialoglycopeptide Thioester 34

According to the general procedure for thioester formation, the sialoglycopeptide hydrazide 31 (3.55 mg, 649.4 nmol, 1 equiv.) was dissolved in 185.5 µL of 6 M GdmCl buffer and reacted with 8.44 µL of 0.5 M NaNO₂ (4.22 µmol, 6.5 equiv.). After 1 h, 42.2 µL of the MESNa stock solution (42.21 µmol, 65 equiv.) were added. The pH was adjusted to a value of 5.9 and the reaction mixture was purified after 1 h by RP-HPLC (column [G], 25-35 % MeCN/H₂O + 0.1 % HCOOH, 3 mL/min). The target fractions were immediately frozen and lyophilized.
Yield of 34: 2.42 mg (433.9 nmol, 66.8 %). RP-HPLC-MS: column [A], 0-50 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 34: m/z (average isotopes) C₂₂₇H₃₇₇N₄₅O₁₀₇S₄ (5576.96) calculated: 1857.98 [M-3H]⁻, 1393.23 [M-4H]⁴⁺, found: 1858.67 [M-3H]⁻, 1393.77 [M-4H]⁴⁺.

Figure S43: RP-HPLC-MS of purified EPO 1-28 sialoglycopeptide thioester 34.

6.5 Synthesis of EPO 29-67 Sialoglycopeptide Thioester 35

According to the general procedure for thioester formation, the sialoglycopeptide hydrazide 32 (3.97 mg, 580.5 nmol, 1 equiv.) was dissolved in 165.9 µL of 6 M GdmCl buffer and reacted with 7.55 µL of 0.5 M NaNO₂ (3.77 µmol, 6.5 equiv.). After 1 h, 37.73 µL of the MESNa stock solution (42.21 µmol, 65 equiv.) were added. The pH was adjusted to a value of 6.5 and the reaction mixture was purified after 1 h by gel filtration (column [M], 10 % MeCN/H₂O, 0.5 mL/min). The target fraction was lyophilized.

Yield of 35: 3.83 mg (551.1 nmol, 94.9 %). RP-HPLC-MS: column [A], 10-40 % MeCN/H₂O + 0.1 % HCOOH. ESI-MS of 35: m/z (average isotopes) C₂₉₁H₄₅₄N₆₂O₁₂₅S₄ (6949.38) calculated: 2315.45 [M-3H]⁻, 1736.34 [M-4H]⁴⁺, 1388.87 [M-5H]⁵⁻; found: 2315.78 [M-3H]⁻, 1736.67 [M-4H]⁴⁺, 1388.89 [M-5H]⁵⁻.
6.6 Synthesis of EPO 29-97 Sialoglycopeptide Hydrazide 36

The ligation buffer (6 M GdmCl, 0.2 M NaH₂PO₄, 40 mM TCEP, 0.1 M MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.9. The sialoglycopeptide hydrazide 33 (3.12 mg, 551.1 nmol, 1 equiv.) was dissolved in 275 µL of ligation buffer and added to the thioester 35 (3.12 mg, 551.1 µmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (14 h, LC-MS control, LC-MS samples were prepared with 100 mM DTT in water).

Prior to purification, 4.63 mg of DTT in 24.4 µL of 6 M GdmCl, 0.2 M NaH₂PO₄, pH 6.9 was added to the reaction mixture. The glycopeptide hydrazide 36 was isolated by RP-HPLC (column [G], 20-50 % MeCN/H₂O + 0.1 % HCOOH, 3 mL/min). Target fractions were neutralized with 1 M Na₂CO₃ (approximately 20 µL/mL eluate) and lyophilized. The residue was taken up in 900 µL of 10 % MeCN/H₂O containing 100 mM DTT, and desalted (column [M], 10 % MeCN/H₂O, 0.5 mL/min). The target fraction was lyophilized.

Yield of 36: 4.45 mg (359.7 nmol, 65.3 %). RP-HPLC-MS: column [A], 20-60 % MeCN/H₂O
+ 0.1 % HCOOH. HR-MS of 36: m/z (exact mass) C_{522}H_{829}N_{111}O_{226}S_{3} (12364.5951) calculated: 1374.8512 [M+9H]^{9+}, 1237.4668 [M+10H]^{10+}, 1125.0614 [M+11H]^{11+}, found: 1374.8576 [M+9H]^{9+}, 1237.4743 [M+10H]^{10+}, 1125.0667 [M+11H]^{11+}.

Figure S47: RP-HPLC-MS of EPO 29-97 sialoglycopeptide thioester 36 a) crude after 14 h, b) after RP-HPLC.

6.7 Synthesis of EPO 29-97 Sialoglycopeptide Thioester S15

According to the general procedure for thioester formation, the glycopeptide hydrazide 36 (5.50 mg, 444.6 nmol, 1 equiv.) was dissolved in 127.0 µL of 6 M GdmCl buffer and reacted with 5.78 µL of 0.5 M NaNO₂ (2.89 µmol, 6.5 equiv.). After 1 h, 28.9 µL of the MESNa stock solution (28.90 µmol, 65 equiv.) were added. The pH was adjusted to a value of 5.9 and the reaction mixture was purified after 1 h by gel filtration (column [M], 10 % MeCN/H₂O, 0.5 mL/min). The target fraction was lyophilized. LC-MS samples were prepared with 40 mM TCEP (water, pH 7).

Yield of S15: 5.29 mg (423.8 nmol, 95.3 %). RP-HPLC-MS: column [A], 20-60 % MeCN/H₂O + 0.1 % HCOOH. HR-MS of S15: m/z (exact mass) C_{524}H_{831}N_{109}O_{229}S_{5} (12474.5335)
calculated: 1783.0835 [M+7H]$^+$, 1560.3240 [M+8H]$^+$, 1387.0666 [M+9H]$^+$, 1248.4606 [M+10H]$^{10+}$, found: 1783.0852 [M+7H]$^+$, 1560.3240 [M+8H]$^+$, 1387.0648 [M+9H]$^+$, 1248.4578 [M+10H]$^{10+}$.

**Figure S49:** RP-HPLC-MS of EPO 29-97 sialoglycopeptide thioester S15 after SEC.

### 6.8 Synthesis of EPO 29-166 Sialoglycopeptide 38

The ligation buffer (6 M GdmCl, 0.2 M NaH$_2$PO$_4$, 40 mM TCEP, 0.1 mM MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.7.

The peptide 26 (1.12 mg, 147.1 nmol, 1.2 equiv.) was dissolved in 61.3 µL of ligation buffer and added to the thioester S15 (1.53 mg, 122.6 nmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (3d, LC-MS control, LC-MS samples were prepared with 100 mM DTT in water).

Subsequently, TCEP x HCl was added (0.44 mg, 1.53 µmol, 12.5 equiv.) and the reaction mixture was purified after 30 min by RP-HPLC (column [I], 30-45 % MeCN/H$_2$O + 0.1 %
HCOOH 3 mL/min). The target fractions were neutralized with 1 M Na$_2$CO$_3$ (approximately 20 µL/mL of eluate) and lyophilized. The residue was taken up in taken up in 1.2 mL of 10 % MeCN/H$_2$O containing 100 mM DTT, and desalted (column [M], 10 % MeCN/H$_2$O, 0.5 mL/min). The target fraction was lyophilized.

Yield of 38: 1.44 mg (72.2 nmol, 58.9 %). RP-HPLC-MS: column [D], 20-60 % MeCN/H$_2$O + 0.1 % HCOOH. HR-MS of 38: m/z (exact mass) C$_{856}$H$_{1379}$N$_{209}$O$_{323}$S$_6$ (19946.6230) calculated: 1995.4680 [M+10H]$^{10+}$, 1814.1534 [M+11H]$^{11+}$, 1663.0579 [M+12H]$^{12+}$, found: 1995.4706 [M+10H]$^{10+}$, 1814.1560 [M+11H]$^{11+}$, 1663.0594 [M+12H]$^{12+}$.

**Figure S51:** RP-HPLC-MS EPO 29-166 sialoglycopeptide 38 via NCL a) crude, b) after RP-HPLC.

### 6.9 Desulfurization of EPO 29-166 Sialoglycopeptide 38

**Figure S52:** Desulfurization of EPO 29-166 glycopeptide 38.
Under anaerobic conditions, a buffer (6 M GdmCl, 0.2 M NaH₂PO₄) was prepared and adjusted to pH = 7.0. A TCEP stock solution was prepared with this buffer (0.5 M TCEP x HCl) and the pH was adjusted to pH 7.0. Furthermore, a glutathione stock solution (160 mM glutathione, giving pH 4.7) and a VA-044 stock solution (80 mM VA-044, giving pH 6.9) were prepared with the GdmCl buffer. The glycopeptide 38 (2.01 mg, 100.7 nmol, 1 equiv.) was dissolved in the 100.7 µL of the TCEP stock solution (500 equiv.) followed by the glutathione and VA-044 stock solutions (50.4 µL each). The reaction mixture (final pH 6.6) was shaken under anaerobic conditions at 37 °C until completion (3 h according to HRMS, LC-MS samples were prepared with 150 mM DTT in water. Subsequently, the reaction mixture was diluted with 200 µL of 20 % MeCN/H₂O + 0.2 % HCOOH, desalted by gel filtration (column [M], 10 % MeCN/H₂O + 0.1 % TFA, 1.5 mL/min) and lyophilized.

Yield of 37: 1.27 mg (63.9 nmol, 63.5 %). RP-HPLC: column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH. HR-MS of 37: m/z (exact mass) C₈₅H₁₃₇₈N₂₀₆O₁₃₂S₃ (19850.7068) calculated: 2206.6414 [M+9H]⁹⁺, 1986.0780 [M+10H]¹⁰⁺, 1805.6170 [M+11H]¹¹⁺, 1655.2328 [M+12H]¹²⁺, found: 1986.0911 [M+9H]⁹⁺, 1805.6192 [M+11H]¹¹⁺, 1655.2354 [M+12H]¹²⁺.  

![Figure S53: RP-HPLC-MS EPO 29-166 sialoglycopeptide 37 after SEC.](image)

### 6.10 Cleavage of Acm groups from EPO 29-166 Sialoglycopeptide 37

![Figure S54: Deprotection of EPO 29-166 glycopeptide 37.](image)
A buffer (6 M GdmCl, 0.2 M NaH2PO4) was prepared and adjusted to pH = 6.9. The buffer was
degassed with argon for 30 min. Subsequently, anhydrous PdCl2 (2.25 mg, 12.69 µmol) was
suspended in 940 µL of this buffer and shaken for 1 h under argon for complete dissolution
(13.5 mM Pd(II)). 62.64 µL of the Pd(II) stock solution (150.0 µg PdCl2, 845.7 nmol, 30 equiv.)
were added to the Acm-protected glycopeptide 37 (0.56 mg, 28.2 nmol, 1 equiv.) under argon.
Under the exclusion of light the mixture was incubated for 2h. Subsequently, DTT (67.6 mg)
was added to the reaction mixture after 2 h. The mixture was shaken for 2 h and purified by
RP-HPLC (column [H], 10-60 % MeCN/H2O + 0.1 % HCOOH, 1 mL/min). The target fractions
were lyophilized. LC-MS samples were prepared with 100 mM DTT in water.
Yield of S16: 0.42 mg (21.38 nmol, 75.8 %). RP-HPLC-MS: column [D], 20-60 % MeCN/H2O
+ 0.1 % HCOOH. HR-MS of S16: m/z (exact mass) C847H1364N206O320S3 (19637.5955)
calculated: 1964.7668 [M+10H]10+, 1786.2432 [M+11H]11+, 1637.4736 [M+12H]12+, found:
1964.7778 [M+10H]10+, 1786.2514 [M+11H]11+, 1637.4817 [M+12H]12+.

![Figure S55](image)

**Figure S55:** RP-HPLC-MS of EPO 29-166 sialoglycopeptide S16 after RP-HPLC.

### 6.11 Synthesis and Refolding of EPO S

![Figure S56](image)

**Figure S56:** Synthesis of EPO S.
The ligation buffer (8 M GdmCl, 0.2 M NaH₂PO₄, 40 mM TCEP, 0.1 M MPAA) was mixed under anaerobic conditions and adjusted to pH = 6.9. The glycopeptide thioester 34 (0.53 mg, 95.42 nmol, 2.5 equiv.) was dissolved in 19.1 µL of ligation buffer and added to glycopeptide S16 (0.75 mg, 38.17 nmol, 1 equiv.). The mixture was incubated under anaerobic conditions until complete conversion of the thioester (6 d, LC-MS control). The ligation mixture was diluted with 9.54 mL of the dilution buffer (Table S5) and transferred to a tubular dialysis membrane (ZelluTrans/Roth V series, cutoff = 5 kDa, 40 mm flat width, thickness 20-30 µm, regenerated cellulose), and then dialyzed sequentially against the buffers shown in Table S5 (390 mL each) with constant agitation. The dialysis conditions are listed in Table S6.

After completion of the dialysis the protein solution was concentrated to a volume of 1.6 mL (Amicon 8010 stirred cell with membrane UP010 P from MICRODYN-NADIR, cutoff = 10 kDa). Monomeric EPO S was isolated by size exclusion chromatography (column [P], 0.15 M NaCl, 50 mM NaH₂PO₄, 0.5 M L-arginine, pH 7.5, 1 mL/min). The eluate was concentrated by ultrafiltration to a volume of 1.6 mL, purified by RP-HPLC (column [H], 30-50 % MeCN/H₂O + 0.1 % TFA, 1 mL/min) and lyophilized.

Yield of EPO S: 0.35 mg (14.0 nmol, 36.7 %). RP-HPLC-MS: column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH. HR-MS of EPO S: m/z (exact mass) C₁₀₆₈H₁₇₂₃N₂₅₁O₃₂₄S₄ (24976.9862) calculated: 1785.0777 [M+14H]¹⁴⁺, 1666.1397 [M+15H]¹⁵⁺, 1562.0689 [M+16H]¹⁶⁺, found: 1785.0852 [M+14H]¹⁴⁺, 1666.1468 [M+15H]¹⁵⁺, 1562.0761 [M+16H]¹⁶⁺.
Figure S57: Synthesis and purification of EPO S: a) RP-HPLC-MS of crude NCL mixture after 66 h, b) RP-HPLC-MS of crude NCL mixture after 6 d, c) Separation mono- and oligomers by gel filtration (Superdex 75), d) RP-HPLC-MS of EPO S, e) HRMS of EPO S, f) found and simulated HR-isotope patterns of [M+15H]^{15+} charge state, g) SDS-PAGE of EPO S reduced and oxidized, and h) superposition of CD-spectra of EPO A and S.

The CD spectrum was recorded with 250 µL of EPO S (0.16 mg EPO S/mL) in ultrapure water at 23 °C.
7. Enzymatic Sialylation of Glycopeptides

7.1 Sialylation of EPO 29-97 Glycopeptide Hydrazide 25

The glycopeptide hydrazide 25 (0.25 mg, 22.3 nmol, 1 equiv.) was dissolved in 1.68 µL of water. CIAP (0.40 µL, 8.92 U) and 1.7 µL of a stock solution containing CMP-Neu5Ac (133.6 µg, 178.5 nmol, 8 equiv.) in 250 mM Tris buffer, pH 9. Subsequently, 1 µL of α-2,6-sialyltransferase from *P. damselae* was added (2.9 µg). The solution was incubated at 30 °C (c 25 = 5 mM). After 12 h, an aliquot of 0.5 µL was diluted with 4.5 µL of 250 mM DTT in 100 mM Tris, pH 9 and analyzed by LC-MS. Subsequently, 8 equiv. of CMP-Neu5Ac were added. After incubation for 5 and 13 h further CMP-Neu5Ac (16 equiv. each) was added. RP-HPLC-MS: column [B], 20-60 % MeCN/H₂O + 0.1 % HCOOH.

**Figure S58**: Sialylation of EPO 29-97 glycopeptide hydrazide 25.

**Figure S59**: Enzymatic sialylation of EPO 29-97 hydrazide 25: RP-HPLC-MS a) after 12 h, b) after 30 h and c) after 40 h.
7.2 Enzymatic α-2,6-Sialylation of EPO 29-166 Glycopeptide 28

![Diagram of sialylation process]

**Figure S60**: Sialylation of EPO 28-166 glycopeptide 28.

The glycopeptide 28 (50 µg, 2.67 nmol, 1 equiv.) was dissolved in 4.12 µL of water containing CMP-Neu5Ac (16.0 µg, 21.39 nmol, 8 equiv.) Subsequently, 0.51 µL of 250 mM Tris buffer pH 7.5 were added, followed by addition of 0.48 µL of CIAP (2 U/µL) and 0.24 µL of α-2,6-sialyltransferase from *P. damselae* was added (0.7 µg). The solution was incubated at 30 °C (c25 = 0.5 m). After 1.5 h, an aliquot of 0.21 µL was diluted with 19.79 µL H2O and analyzed by HRMS. Subsequently, and after 1.5 h further CMP-Neu5Ac (8 equiv. each) were added. After a total reaction time of 31 h further CMP-Neu5Ac (16 equiv.) was added.

**Figure S61**: Enzymatic Sialylation of EPO 29-166 28: RP-HPLC-MS of reaction mixture a) after 1.5 h (8 equiv. of CMPNeu5Ac), b) after 31 h (24 equiv. of CMPNeu5Ac) and c) after 50 h (40 equiv. of CMPNeu5Ac).
7.3 Preparative Sialylation of EPO A

Purified EPO A (230 µg, 9.89 nmol, 1 equiv.) was dissolved in 13.7 µL of 25 mM Tris buffer, pH 7.5 and mixed with 1.79 µL of CIAP (2 U/µL). Subsequently, 1.33 µL of α-2,6-sialyltransferase from P. damselae (3.86 µg) and 2.96 µL of an aqueous stock solution containing CMP-Neu5Ac (88.9 µg, 118.7 nmol, 12 equiv.) were added. The reaction mixture was incubated at 30 °C. After 1.5 h, 3h and 4.5h three further additions of 0.15 µL of a stock solution containing 88.9 µg of CMP-Neu5Ac, 118.7 nmol, 12 equiv. in 25 mM Tris buffer, pH 7.5 were made. The reaction mixture was incubated for another 17.5 h at 30 °C, diluted to a volume of 200 µL with H₂O and purified by RP-HPLC (column [E], 5-95 % MeCN/H₂O + 0.1 % HCOOH, 0.25 mL/min). The eluate was immediately frozen and lyophilized.

Yield of EPO S: 0.12 mg (4.80 nmol, 48.5 %). RP-HPLC-MS: column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH.

HR-MS of EPO S: m/z (exact mass) C₁₀₆₈H₁₇₂₃N₂₅₁O₄₂₄S₄ (24976.9862) calculated: 1785.0777 [M+14H]¹⁴⁺, 1666.1397 [M+15H]¹⁵⁺, 1562.0689 [M+16H]¹⁶⁺, found: 1785.0863 [M+14H]¹⁴⁺, 1666.1463 [M+15H]¹⁵⁺, 1562.0728 [M+16H]¹⁶⁺.

Figure S62: Sialylation of folded EPO A.

Figure S63: Enzymatic sialylation of EPO A: RP-HPLC-HRMS of crude reaction mixture a) after 1.5 h (12 equiv. CMPNeu5Ac), b) after 3 h (24 equiv. of CMPNeu5Ac) and c) after 22 h (48 equiv. of CMPNeu5Ac).
Figure S64: Sialylated EPO S: a) RP-HPLC-MS of crude enzymatic sialylation mixture after 22 h, b) RP-HPLC-MS of purified EPO S, c) HRMS of EPO S, d) found and simulated HR-isotope patterns of [M+15H]^{15+} charge state, e) SDS-PAGE.
8. Expression of EPOR and Complexations with EPO Glycoforms

8.1 Overexpression and Purification of EPO

**Amino acid sequence of His6-SUMO-EPOR(25-250)**

| Amino Acid | Sequence |
|------------|----------|
| 0-40       | MGSSHHHHHHH GSGLVPRGSA SMSDSEVNQE AKPEVKPEVK PETHINLKV |
| 41-80      | DGSSEIFFKI KKTTPRRLRM EAFAKRQGKE MDSLRLYDYG IRIQADQTP |
| 81-120     | DLMEDNDII EAHREQIGGA PPPNLDPKF ESKAALLAR GPEELLCFTE |
| 121-160    | RLEDLVCWFEE EAASAGVPGP NYSFSYQLED EPWKLCRLHQ APTARGAVRF |
| 161-200    | WCSLPTADTS SFVPLELRVT AASGAPRYHR VIHINEVVL DAPVGLVARL |
| 201-240    | ADESCHVVLRL WPPLLPPETMT SHIRYEVDVS AGNGAGSROVEITLEGRTEC |
| 241-280    | VLSNLGRGTR YTFAVRARMA EPSFGGWASA WSEPVSLLTP SDLDP |

*Figure S65: Recombinant expression and refolding of EPOR.*
LB-medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7
lysis buffer: 0.5 M NaCl, pH 8.5
binding buffer: 6 M GdmCl, 20 mM NaH₂PO₄, 20 mM imidazole, 0.5 M NaCl, pH 7.4
elution buffer: 6 M GdmCl, 20 mM NaH₂PO₄, 250 mM imidazole, 0.5 M NaCl, pH 7.4

The EPO receptor was expressed as a His₆-SUMO-EPOR fusion protein (40). The vector pET11a_His₆-SUMO-EBP25-250 was obtained from Genscript. E. coli BL21 (DE3) containing this vector were grown in 5 L flasks containing 1.25 L of LB-medium at 37 °C. The overexpression of 40 was induced after reaching an OD₆₀₀ of 0.6 by addition of 1.25 mL of 1 M IPTG (final concentration 1 mM). The cells were harvested by centrifugation (10000 g, 4 °C, 10 min) after 4 h of incubation. Yield: 4.67 g of cell pellet from 1.25 L of LB-medium.

The cells were suspended in 93 mL of lysis buffer and lysed with a Sonopuls ultrasonic homogenizer HD 2070 (15x 20 s, 50 % cycle, 60 % power). The crude lysate was pelleted by centrifugation (5000 g, 4 °C, 10 min). The pellet was resuspended in 93 mL of lysis buffer and pelleted by centrifugation (2 cycles). The pelleted inclusion bodies were suspended in 47 mL of binding buffer (10 mL/g). The suspension was centrifuged (4 °C, 40000 g, 30 min) and the supernatant filtered through a 0.45 µm PVDF filter (Roth, Germany). The filtrate was loaded onto a Protino Ni²⁺-NTA-Agarose column (20 mL, GE Healthcare, Sweden) with a peristaltic pump (1 mL/min, P-1, GE Healthcare, Sweden). The column was washed with binding buffer (3 mL/min) until the absorption at 280 nm was constant. His₆-SUMO-EPOR 40 was eluted with elution buffer (3 mL/min), precipitated by dialysis (against 1 L of H₂O, 3x) and collected by centrifugation (25000 g, 4 °C, 3 min). Yield: 56.43 mg of 40 from 1.25 L of LB-medium.

RP-HPLC-MS of 40: column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH.

a)
Figure S66: a) SDS-PAGE of the overexpression and purification of 40. Lane 1: molecular weight standard; lane 2: cells before addition of IPTG; lane 3: cells 2 h post addition of IPTG; lane 4: cells 4 h post addition of IPTG; lane 5: soluble fraction after cell lysis; lane 6: insoluble fraction after cell lysis; lane 7: molecular weight standard; lane 8: breakthrough (Ni²⁺-IMAC); lane 9: washing step (Ni²⁺-IMAC); lane 10: eluate of 40 (Ni²⁺-IMAC), b) RP-HPLC-MS of 40.

8.2 Refolding and Purification of EPOR

dissolving buffer I: 0.1 M Tris, 3.5 M urea, 10 mM Lys, 10 mM DTT, pH 8.5

dissolving buffer II: 0.1 M Tris, 6 M urea, 10 mM Lys, 10 mM DTT, pH 8.5

refolding buffer I: 50 mM Tris, 10 mM L-lysine, pH 8.5

refolding buffer II: 0.1 M Tris, 4.5 M urea, 10 mM Lys, 2 mM MPAA, 0.4 mM GSH, pH 8.5

refolding buffer III: 0.1 M Tris, 3 M urea, 10 mM Lys, 2 mM MPAA, 0.4 mM GSH, pH 8.5

refolding buffer IV: 0.1 M Tris, 1.5 M urea, 10 mM Lys, 2 mM MPAA, 0.4 mM GSH, pH 8.5

refolding buffer V: 0.1 M Tris, 0.5 M urea, 10 mM Lys, 2 mM MPAA, 0.4 mM GSH, pH 8.5

refolding buffer VI: 50 mM Tris, 200 mM NaCl, pH 7.5

6 mg of His₆-SUMO-EPOR(25-250) 40 (1 eq, 0.16 µmol) and 1.9 mg of L-cysteine (100 eq, 15.8 µmol) were dissolved (600 rpm, 1 h) in 0.3 mL of dissolving buffer I. After addition of 2.7 mL of refolding buffer I, the cleavage of the His₆-SUMO tag was induced by addition of 7.3 µL of SUMO protease SenP2 (11.08 mg/mL). After complete cleavage (1 d) the liberated peptide EPOR(25-250) was precipitated by dialysis (1 L of H₂O, 3 x), collected by centrifugation (25000 g, 4 °C, 3 min), washed with H₂O (1 mL, 3 x) and lyophilized. Yield of crude EPOR(25-250): 5.79 mg (0.23 µmol, 89 %).

3 mg of crude EPOR(25-250) were dissolved in 30 mL of dissolving buffer II in an anaerobic chamber. After 6 h the solution was removed from the anaerobic chamber and dialysed against refolding buffers II - VI (750 mL, 12 h each). The refolding solution was concentrated (Amicon...
stirred cell, 10 kDa cut off, 50 mL) and purified by gel filtration (HiLoad 16/600 Superdex 75 pg, 60.5 x 1.6 cm, GE Healthcare, 50 mM Tris/200 mM NaCl, pH 7.5, 1 mL/min). The monomeric fraction (69 min) was concentrated (Amicon centrifugal filters ultracel, 10 kDa cut off, 0.5 mL, 14000 g). The yield was determined by absorption at 280 nm and the purity was controlled by LC-MS and HR-MS.

RP-HPLC-MS of EPOR: column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH.

HR-MS of EPOR: direct injection in water. 150 µg of EPOR in 100 µL 50 mM Tris/200 mM NaCl, pH 7.5 were loaded onto a PD Mini Trap G25 column (GE healthcare) followed by 400 µL of water. Elution with 1 mL of water gave EPOR in a concentration of 0.1 mg/mL (A²⁸₀).

Yield of EPOR: 0.77 mg (0.031 µmol, 26 %) ESI-MS: m/z (average isotopes): C₁₁₁₃H₁₇₁₅N₃₀⁷O₃₂₅S₇, (24821.21); calculated: (M+24H)²⁴⁺ 1035.22, (M+23H)²³⁺ 1080.18, (M+22H)²²⁺ 1129.24; found: 1035.31, 1080.29, 1129.34. ESI-HRMS: m/z (exact mass): C₁₁₁₃H₁₇₁₅N₃₀⁷O₃₂₅S₇, (24805.52); calculated: (M+24H)²⁴⁺ 1035.1554, (M+23H)²³⁺ 1080.1184, (M+22H)²²⁺ 1129.1689; found: 1035.1911, 1080.1123, 1129.1627.

The CD spectrum was recorded with 200 µL of EPOR (0.13 mg EPOR/mL) in 50 mM Tris, 200 mM NaCl, pH 7.5 at 20 °C.
Figure S67: Recombinant expression of EPOR: a) SEC of refolding mixture b) RP-HPLC-MS of purified EPOR b) RP-HPLC-MS of purified EPO S, c) SDS-PAGE of EPOR reduced (red) or without prior reduction (ox) d) CD-spectrum of EPOR. e) HRMS of EPO S, found and simulated HR-isotope patterns of [M+18H]^{18+} charge state.
8.3 Complexation of EPO glycoforms with EPOR

The biological recognition of the synthetic EPO glycoforms and commercial reference EPO from CHO cells (PeproTech, Germany) was tested by complexation with the expressed EPO receptor EPOR and analyzed by size exclusion chromatography (Superdex 75 PC, 3.2 mm x 300 mm, 13 µm, GE Healthcare, 75 µL/min, PBS buffer pH 7.4, Agilent 1100 HPLC).

MW (calcd.): EPOR 24825.17 Da, EPO A 23244.57, EPO S 24992.11, EPOcho 37000.

3.7 µL of EPOR (8.5 µg, 0.34 nmol, 2.3 mg EPOR/mL in 50 mM Tris/200 mM NaCl, pH 7.5) were incubated for 30 minutes at room temperature with either 8 µL of EPO A (8 µg, 0.34 nmol, 1 mg EPO A/mL in water) or 9.5 µL of EPO S (8.6 µg, 0.34 nmol, 0.9 mg EPO S/mL in water). Furthermore 1.4 µL of EPOR (3.2 µg, 0.13 nmol, 2.3 mg EPOR/mL in 50 mM Tris/200 mM NaCl, pH 7.5) were incubated for 30 minutes at room temperature with 4.8 µL of EPOcho (4.8 µg, 0.13 nmol, 1 mg EPOcho/mL in water). The total volume of each complexation was adjusted to 12 µL with PBS pH 7.4 and 10 µL of the mixtures were analyzed by SEC. Fractions containing the respective EPO/EPOR complexes (~100 µL total) were collected and analysed by SDS-PAGE (10-20 % Tris/Tricine-gradient gel). For LC-MS analysis 50 µL of each EPO/EPOR complex fraction were incubated with 10 µL of MeCN containing 3 % TFA for 1 h prior to analysis (column [D], 20-60 % MeCN/H₂O + 0.1 % HCOOH).
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