A multi-ligation strategy for the synthesis of heterofunctionalized glycosylated scaffolds

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General details

All chemical reagents were purchased from Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France) and were used without further purification. Protected amino acids and Fmoc-Gly-Sar resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochemie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). All amino-acids belong to the L-series. PyBOP was purchased from France Biochem. Analytical RP-HPLC was performed on Waters system equipped with a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. Analysis was carried out at 1.0 mL.min⁻¹ (EC 125/3 nucleosil 300-5 C₁₈) with UV monitoring at 214 nm and 250 nm using a linear A–B gradient (solvent A: 0.09% CF₃CO₂H in H₂O; solvent B: 0.09% CF₃CO₂H in 90% acetonitrile). Purifications were carried out at 22.0 mL.min⁻¹ (VP 250/21 nucleosil 100-7 C₁₈) with UV monitoring at 214 nm and 250 nm using a linear A–B gradient (buffer A: 0.09% CF₃CO₂H in H₂O; buffer B: 0.09% CF₃CO₂H in 90% acetonitrile). ESI mass spectra of peptides and glycopeptides were measured on an Esquire 3000 spectrometer from Bruker or on an Acquity UPLC/MS system from Waters equipped with a SQ Detector 2. For 21 and 22, HPLC analyses were performed on a Waters RP-HPLC system with dual wavelength detector on a Nucleosil C₁₈ column (Macherey Nagel, 250 x 4.6 mm, 5 µm) at 1 mL.min⁻¹ using a linear A–B gradient (solvent A: 50 mM triethylammonium acetate (TEAAc) buffer containing 5% acetonitrile; solvent B: acetonitrile containing 5% water). They were purified on a µ-Bondapak C₁₈ column (Macherey-Nagel Nucleosil, 10 x 250 mm, 7 µm) with similar gradients at a flow rate of 4 mL.min⁻¹ on a Gilson HPLC system with dual wavelength detector and a fraction collector (prep Fc). MALDI-ToF mass spectrum for compound 21 was performed on an Autoflex Bruker using hydricolic acid (HPA, 45 mg; ammonium citrate 4 mg in 500 µL H₂O/CH₃CN) as matrix. Quantification was performed at 260 nm using CARY 400 Scan UV-Visible Spectrometer; ε (181100 M⁻¹.cm⁻¹) was estimated according to the nearest neighbor model.

Synthetic scheme for compound 1.
**Compound A.** The linear protected peptide was synthesized manually by solid-phase peptide synthesis (SPPS) using the standard 9-fluorenylmethoxycarbonyl/tertiobutyl (Fmoc/tBu) protection strategy and the Fmoc-Gly-SASRIN resin. The peptide was cleaved with a solution of 1% TFA in CH₂Cl₂, precipitated in diethyl ether and was used without further purification. Analytical RP-HPLC: $R_t = 14.1$ min ($C_{18}$, 214 nm, 5-100% B in 25 min).

![RP-HPLC profile of crude compound A.](image)

ESI⁻-MS: $m/z$ calcd for C$_{78}$H$_{131}$N$_{18}$O$_{21}$: 1656.9; found: 1657.1 [M+H]$^+$. 

S3
**Compound B.** Linear peptide A (370 mg, 0.22 mmol) was dissolved in DMF (0.5 mM). PyBOP (139.5 mg, 0.27 mmol) was added and the pH value was adjusted to 8 by addition of DIPEA. The solution was stirred at room temperature for 1 h and the solvent was removed under reduced pressure. The residue was dissolved in a minimum of CH$_2$Cl$_2$ and diethyl ether was added for precipitation. Peptide B was obtained as a white powder and was used in the next step without further purification. Analytical RP-HPLC: $R_t = 15.8$ min (C$_{18}$, 214 nm, 5-100% B in 25 min).

**RP-HPLC profile of crude compound B.**

ESI$^+$-MS: $m/z$ calcd for C$_{78}$H$_{129}$N$_{18}$O$_{20}$: 1638.9; found: 1639.4 [M+H]$^+$. 
**Compound C.** Compound B (126 mg, 0.077 mmol) was dissolved in dry DMF (6.1 mL), allyl alcohol (14 µL, 0.38 mmol) and hydrazine monohydrate (126 µL, 0.077 mmol) were added. The solution was stirred at room temperature. Analytical HPLC confirmed the complete conversion of B into C within 30 minutes. After evaporation, the product was precipitated in diethyl ether to give C as a white powder. Analytical RP-HPLC: $R_t = 16.5$ min ($C_{18}$, 214 nm, 5-100% B in 25 min).

![RP-HPLC profile of crude compound C.](image)

ESI$^+$-MS: $m/z$ calcd for $C_{70}H_{118}N_{18}O_{19}Cl$: 1549.8; found: 1549.7 [M+H]$^+$. 

S5
**Compound 1.** The crude compound C (20.6 mg, 13.3 µmol) was treated with a cocktail of TFA/TIS/H$_2$O (95:2.5:2.5 v/v/v, 5 mL) and NaIO$_4$ (28.4 mg, 0.13 mmol) was added. The solution was stirred at room temperature for 30 min and the product was directly purified by RP-HPLC, affording pure aldehyde-containing cyclodecapeptide 1 as a white powder after freeze-drying. Analytical RP-HPLC: $R_t = 10.3$ min (C$_{18}$, 214 nm, 5-100% B in 25 min).

![RP-HPLC profile of purified compound 1.](image)

ESI$^+$-MS: $m/z$ calcd for C$_{55}$H$_{91}$N$_{17}$O$_{16}$Cl (hydrated aldehyde): 1280.6; found: 1280.7 [M+H]$^+$. 

S6
Stepwise synthesis of 9.

**Compound 3.** Cyclopeptide 1 (9.6 mg, 7.6 µmol) and β-Glc aminooxy 2 (1.8 mg, 9.1 µmol) were dissolved in 0.1% TFA in H2O (10 mM). After stirring for 30 min at room temperature, analytical HPLC indicated complete conversion. The crude mixture was directly purified by preparative HPLC without additional treatment to give, after freeze-drying compound 3 as a flocculent powder. Yield: 87% (9.5 mg); analytical RP-HPLC: R_t = 9.9 min (C_{18}, 214 nm, 5-100% B in 25 min).

RP-HPLC profile of crude compound 3.

RP-HPLC profile of purified compound 3.

ESI^+MS: m/z calcd for C_{61}H_{100}N_{18}O_{20}Cl: 1439.7; found: 1439.8 [M+H]^+.
**Compound 5.** Glycopeptide 3 (9.5 mg, 6.6 µmol) and β-GlcNAc thiol 4 (4.7 mg, 19.8 µmol) were dissolved in a mixture DMF/H$_2$O (2:1, 1.8 mL) and DPAP (0.5 mg, 2 mmol) was added. The solution was irradiated at 365 nm for 30 min and directly purified to obtain compound 5 as a white foam. Yield: 67% (7.4 mg); analytical RP-HPLC: $R_t = 9.1$ min (C$_{18}$, 214 nm, 5-100% B in 25 min).

RP-HPLC profile of crude compound 5.

RP-HPLC profile of purified compound 5.

ESI$^+$-MS: $m/z$ calcd for C$_{69}$H$_{115}$N$_{19}$O$_{25}$SCl: 1676.8; found: 1676.9 [M+H]$^+$. 
**Compound 7.** To a degassed solution of 5 (7.4 mg, 4.4 µmol) and α-GalNAc propargyl 6 (2.4 mg, 11.1 µmol) in DMF (1.0 mL), CuSO₄ (1.1 mg, 4.4 µmol) in PBS buffer (100 mM, 500 µL) then THPTA (9.5 mg, 22 µmol) and sodium ascorbate (6.1 mg, 30.8 µmol) in PBS buffer (100 mM, 500 µL) were added. The reaction was stirred at room temperature under argon and analytical HPLC indicated complete reaction after 1 h. Chelex resin was then added to remove excess of copper, the reaction mixture was filtered and directly purified by RP-HPLC affording pure compound 7 as a white powder. Yield: 85% (7.2 mg); analytical RP-HPLC: Rᵣ = 8.3 min (C₁₈, 214 nm, 5-100% B in 25 min).

ESI⁺-MS: m/z calcd for C₈₀H₁₃₂N₂₀O₃₁SCl: 1935.9; found: 1936.8 [M+H]⁺.
**Compound 9.** Compound 7 (7.2 mg, 3.7 µmol) and α-Man thiol 8 (3.6 mg, 18.5 mmol) were dissolved in a mixture DMF/H$_2$O (1:1, 1.6 mL), then KI (12.2 mg, 74.1 µmol) and DIPEA (35.4 µL, 0.21 mmol) were added. The suspension was left stirring for 1 h. The reaction mixture was directly purified by RP-HPLC affording 9 as a white powder. Yield: 84% (6.5 mg); analytical RP-HPLC: $R_t = 8.1$ min (C$_{18}$, 214 nm, 5-100% B in 25 min).

RP-HPLC profile of crude compound 9.

RP-HPLC profile of purified compound 9.

ESI$^+$-MS: $m/z$ calcd for C$_{86}$H$_{143}$N$_{26}$O$_{36}$S$_2$: 2096.9; found: 2096.9 [M+H]$^+$. 
One-pot synthesis of 9.

Cyclopeptide 1 (2 mg, 1.6 µmol) and β-Glc aminoxy 2 (0.36 mg, 1.9 µmol) were dissolved in 0.1% TFA in H₂O (200 µL). After stirring for 30 min at room temperature, analytical HPLC indicated complete conversion. To this crude mixture, β-GlcNAC thiol 4 (0.55 mg, 2.3 µmol) and DPAP (0.06 mg, 0.23 mmol) dissolved in DMF (400 µL) were added. The solution was irradiated at 365 nm for 30 min. To this crude mixture, α-GalNAC propargyl 6 (0.68 mg, 3.1 µmol) and CuSO₄ (0.39 mg, 1.6 µmol) in PBS (100 mM, 100 µL) were added followed by a solution of THPTA (3.4 mg, 7.8 µmol) and sodium ascorbate (2.2 mg, 10.9 µmol) in PBS (100 mM, 100 µL). The reaction was stirred at room temperature under argon and analytical HPLC indicated complete reaction after 1 h. To the crude mixture, α-Man thiol 8 (1.5 mg, 7.8 mmol), KI (5.1 mg, 31.2 µmol) and DIPEA (14.9 µL, 85.9 µmol) were added. The solution was stirred for 1 h and was finally purified by semi-preparative HPLC to provide 9 as a white powder. Overall yield: 47% (1.5 mg).
**Compound 11.** Compound 9 (2.0 mg, 0.95 µmol) was dissolved in dry DMF (1 mL) and Biotin-OSu 11 (0.48 mg, 1.4 µmol) was added to the solution. The pH was adjusted at 8 with DIPEA and the solution was stirred at room temperature. After 30 min, the DMF was evaporated under reduced pressure and the crude mixture was purified by semi-preparative RP-HPLC. Yield: 90% (1.9 mg); analytical RP-HPLC: \( R_t = 8.7 \text{ min} \) (C\textsubscript{18}, 214 nm, 5-100% B in 25 min).

![RP-HPLC profile of crude compound 11.](image1)

ESI\(^+\)-MS: \( m/z \) calcd for C\(_{96}H\(_{157}\)N\(_{22}\)O\(_{38}\)S\(_{3}\): 2323.0; found: 2323.0 [M+H]\(^+\).
**Compound 13.** Compound 9 (3.5 mg, 1.7 µmol) was dissolved in dry DMF (1.7 mL) and FITC 12 (0.9 mg, 2.5 µmol) was added to the solution. The pH was adjusted at 8 with DIPEA and the solution was stirred at room temperature. After 30 min, the DMF was evaporated under reduced pressure and the crude mixture was purified by RP-HPLC. Yield: 85% (3.5 mg); analytical RP-HPLC: $R_t = 10.2$ min ($C_{18}$, 214 nm, 5-100% B in 25 min).

RP-HPLC profile of crude compound 13.

ESI⁺-MS: $m/z$ calcd for C₁₀₇H₁₅₄N₂₁O₄₁S₅: 2487.0; found: 2487.1 [M+H]⁺.
**Compound 16.** Compound 9 (3.4 mg, 1.6 µmol) was dissolved in dry DMF (600 µL) and BocCys(NPys)OSu 14 (1.5 mg, 3.2 µmol) was added. The pH was adjusted at 8 with DIPEA and the solution was stirred at room temperature. After 1 h, the DMF was evaporated under reduced pressure and the crude mixture was precipitated in diethyl ether. Analytical RP-HPLC: $R_t = 12.9$ min (C$_{18}$, 214 nm, 5-100% B in 25 min); ESI$^+$-MS: m/z calcd for C$_{99}$H$_{158}$N$_{23}$O$_{41}$S$_4$: 2454.0; found: 2453.7 [M+H]$^+$. The crude compound was subsequently treated with a solution of TFA/CH$_2$Cl$_2$ (1/1, 1 mL). After 30 min, the solution was evaporated and the residue purified by semi-preparative RP-HPLC. Yield: 93% (3.7 mg); analytical RP-HPLC: $R_t = 9.4$ min (C$_{18}$, 214 nm, 5-100% B in 25 min); ESI$^+$-MS: m/z calcd for C$_{94}$H$_{150}$N$_{23}$O$_{39}$S$_4$: 2353.9; found: 2353.0 [M+H]$^+$. This compound (3.7 mg, 1.5 µmol) and the PV peptide 15 (2.8 mg, 1.6 µmol) were dissolved separately in a mixture of sodium acetate buffer 25 mM pH 5 and DMF (1/1, 1.2 mL). Both solutions were degassed under reduced pressure, mixed together and the reaction mixture was stirred at room temperature under argon atmosphere. After 1 h, the crude yellow mixture was purified by semi-preparative RP-HPLC to obtain compound 16 as a pure lyophilized powder. Yield: 85% (4.9 mg); analytical RP-HPLC: $R_t = 13.2$ min (C$_{18}$, 214 nm, 5-100% B in 25 min).
**Compound 18.** 5’diol oligonucleotide (5’X TCC ATG ACG TTC CTG ACG TT3’ with X = (CH₂)₄CH(OH)CH₂OH) was prepared on an ABI 3400 DNA synthesizer (Applied Biosystems) by using standard β-cyanoethyl nucleoside phosphoramidite chemistry at 1 μM scale. 5’ diol functionality was introduced using acetal protected diol phosphoramidite. 1 5’ diol Oligonucleotide (0.426 μmol; 42%) was obtained after RP-HPLC and treatment with 80 % aqueous acetic acid solution for 1 h at room temperature. 5’-diol ODN (0.25 μmol) was subjected to periodate oxidation with a 0.02 M aqueous sodium-ß-periodate solution (300 μL) to obtain 18 after desalting on NAP10 cartridge. Yield: 80% (0.2 μmol); analytical RP-HPLC: *R*<sub>t</sub> = 14.2 min (C<sub>18</sub>, 260 nm, 0-30% B in 20 min).

MALDI-Tof MS (-): *m/z* calcd for C<sub>200</sub>H<sub>261</sub>N<sub>67</sub>O<sub>127</sub>P<sub>20</sub>: 6255.05; found: 6254.55 [M-H]<sup>-</sup>.

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**Compound 19.** Compound 9 (5.7 mg, 2.7 µmol) was dissolved in dry DMF (1.1 mL), then BocAoa-Osu 17 (1.5 eq, 1.2 mg, 4.1 µmol) was added and the pH was adjusted at 8 using DIPEA. After 1 h analytical HPLC indicated complete reaction coupling. The DMF was evaporated under reduced pressure and the crude mixture was precipitated in diethyl ether and used without further purification. Analytical RP-HPLC: $R_t = 11.9$ min (C$_{18}$, 214 nm, 5-100% B in 25 min); ESI$^+$-MS: $m/z$ calcd for C$_{93}$H$_{154}$N$_{21}$O$_{40}$S$_2$: 2270.0; found: 2270.1 [M+H]$^+$. The crude compound was subsequently deprotected using a solution of TFA (60% in CH$_2$Cl$_2$, 1.5 mL) within 30 min. The resulting aminooxylated compound was purified by RP-HPLC. Yield: 93% (5.7 mg); analytical RP-HPLC: $R_t = 8.2$ min (C$_{18}$, 214 nm, 5-100% B in 25 min); ESI$^+$-MS: $m/z$ calcd for C$_{88}$H$_{146}$N$_{21}$O$_{38}$S$_2$: 2270.0; found: 2169.6 [M+H]$^+$. To a solution of 18 (0.2 µmol) in ammonium acetate buffer (200 µL, 0.4 M, pH 4.6) was added this aminooxylated cyclopeptide (0.069 µmol, 0.15 mg) solubilized in water. The reaction was stirred at room temperature during 4 h and monitored by RP-HPLC. The conjugate 19 was obtained after RP-HPLC purification. Yield: 65% (0.045 µmol); analytical RP-HPLC: $R_t = 15.7$ min (C$_{18}$, 260 nm, 0-30% B in 20 min).
**Compound 21.** Compound 20 (0.53 mg, 0.45 µmol) was dissolved in 0.1% TFA in water (10 mM) and the previous aminooxylated compound (5.8 mg, 2.7 µmol) was added. The solution was stirred for 1 h at 37°C and purified by RP-HPLC to obtained compound 21. Yield: 87% (3.8 mg); analytical RP-HPLC: $R_t = 10.3$ min (C$_{18}$, 214 nm, 5-100% B in 25 min).

![RP-HPLC profile of crude compound 21.](image1)

![RP-HPLC profile of purified compound 24.](image2)

ESI$^+$-MS: $m/z$ calcd for C$_{404}$H$_{651}$N$_{98}$O$_{166}$S$_{8}$: 9793.3; found: 9792.8.