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Supporting Information

Expanding the versatility and scope of the oxime ligation: rapid bioconjugation to disulfide-rich peptides

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Materials and Methods

Materials

Fmoc amino acid derivates Fmoc-Gly-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Pro-OH, Fmoc-Cys(Trt)-OH, Boc-Cys(Trt)-OH, Fmoc-His(Boc)-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH and Fmoc-aminooxy acetic acid (Fmoc-Aoa-OH) were purchased from Iris Biotech. The rink amide resin (Polystyrene AM RAM; 0.69 mmol/g) was obtained from RAPP Polymere. Triisopropylsilane (TIPS), 1,2-ethaedithiol (EDT), ammonium bicarbonate (NH_4HCO_3), 2-fluoro-2-deoxy-D-glucose (FDG) and iodine (I_2) were obtained from Sigma-Aldrich. The coupling reagent O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) and N,N`-bis(Boc)-aminooxy acetic acid were purchased from Chem-Impex International; N,N-diisopropylethylamine (DIPEA) from Auspep; dimethylformamide (DMF) and diethyl ether from RCI Labscan Limited; trifluoroacetic acid (TFA), piperidine and methanol (MeOH) from Chem-Supply; and dichloromethane (DCM) and acetonitrile (ACN) from Merck.

Standard Fmoc solid phase peptide synthesis, cleavage, and purification

Peptides were manually synthesised on a Rink-amide resin on 0.125 mmol scale. The resin was washed with DMF and left swelling in DMF overnight. Fmoc deprotection was performed using 30% piperidine (v/v) in DMF 2 x 2 min. Couplings were carried out in DMF using 4 eq relative to the resin loading of Fmoc-amino acid/HCTU/DIEA (1:1:1) for 15 min. Upon completion of the peptide assembly, the resin was washed with DCM and MeOH and dried over a flow of nitrogen. Cleavage from resin and removal of the side chain protecting groups were achieved by treatment with TFA:TIPS:EDT:H_2O (90:2.5:2.5:5) at 25°C for 80 min. TFA was evaporated under a stream of nitrogen. Cleaved peptides were precipitated and washed with ice-cold diethyl ether and lyophilised in 50% ACN, 0.1% TFA, H_2O. The crude peptides were analysed *via* analytical C_{18}-RP-HPLC and LC-MS. HPLC column: Agilent Technology, Zorbex C_{18}, 2.1 x 100 mm, 5 µm, 300 Å. Solvent A: 0.05% TFA in H_2O. Solvent B: 0.043% TFA in 90% ACN. HPLC method: 0–50% B in 50 min, 1% gradient, 0.25 mL/min flowrate.

Synthesis of OTK^8[Aoa]

Gly and Lys(Mtt) were coupled to the Rink amid resin following the standard Fmoc solid phase peptide synthesis (Fmoc-SPPS) protocol as described above. The Mtt group was removed with 1% TFA in DCM, followed by coupling of N,N`-bis(Boc)-aminooxy acetic acid with EEDQ (1:1.2) overnight. Boc-Cys(Trt) was used as the *N*-terminal amino acid. The sequence assembly was completed using the Fmoc-SPPS, followed by TFA cleavage and peptide purification as described above.

Synthesis of linear OTK^{8}[Fmoc-Aoa] and α-CnIG[Fmoc-Aoa]

The peptides were synthesised on a Rink-amide resin on a 0.25 mmol scale using manual Fmoc-SPPS. Coupling reagent was 0.5 M HCTU and coupling time 15 min. Boc-Cys(Trt) was used as the *N*-terminal amino acid. Upon complete assembly, the Mtt group was selectively removed with 1% TFA in DCM (6 x 5 min). N-Fmoc-Aoa-OH was coupled with EEDQ (1:1.2) overnight. TFA cleavage and analysis of the crude peptide were performed according to the SPPS, cleavage and purification protocols described above.

-Sequences of OTK^{8} and α-CnIG:

OTK^{8}: CYIQNCPKG*

α-CnIG: CCHPACGKYFKC*

* C-terminal amide

Directed folding of α-CnIG

The first disulfide bond (Cys^{1}–Cys^{6}) was formed in 0.1 M NH_4HCO_3 (pH 8.2, 1 mg/mL peptide concentration) stirring overnight. The reaction was stopped by lowering the pH to 2 with TFA (1% v/v), followed by purification *via* preparative C_{18}-RP-HPLC. To form the second disulfide bond (Cys^{2}–Cys^{13}), a 1 mg/mL peptide solution in 45% ACN/H_2O, 0.1% TFA was prepared, and an iodine solution (10 mg/mL in MeOH) was added until it turned brown and stirred for 40 min at 25°C. Ascorbic acid was added until the solution became clear to stop the reaction, and the peptide solution was diluted with 0.05% TFA in Millipore water to a final ACN concentration of 15% (v/v), followed by preparative C_{18}-RP-HPLC purification using a linear gradient of 0–50% B (solvent A: 0.05% TFA; B: 90% ACN, 0.043% TFA) in 100 min at 16 mL/min on an Agilent-Eclipse C_{18} column (300 Å, 5 µm, 22.5 x 250 mm) while monitoring UV absorbance at 224 nm.

Analysis of the stability of OTK^{8}[Aoa] and OTK^{8}[Fmoc-Aoa] in commonly used oxidative folding conditions

The following oxidative folding conditions were tested:

0.1 M NH_4HCO_3, pH 8.2

0.1 M NH_4OAc pH 5

30% iPrOH, 0.1 M NH_4HCO_3, pH 8.0

30% DMSO, 0.1 M NH_4HCO_3, pH 8.2

30% DMSO, 0.1 M NH_4OAc, pH 5

30% DMSO, 0.1 M K_2HPO_4/KH_2PO_4, pH 6

6 M GnHCl, 0.1 M Tris, pH 8.5

A 1 mg/mL peptide solution of OTK^{8}[Aoa] or OTK^{8}[Fmoc-Aoa] was prepared in 50% ACN_{aq} and mixed with each oxidative folding buffer to a final peptide concentration of 0.1 mg/mL. Oxidation reactions were stirred at 25°C overnight. 100 µL of each oxidation reaction was sampled and quenched with 20 µL neat TFA. Samples were drawn and analysed by LC-MS and C_{18}-RP-HPLC using a linear gradient 0–50% B (solvent A: 0.05% TFA; B: 90% ACN, 0.043% TFA) on an Agilent-Zorbax C_{18} column (300 Å, 3.5 µm, 2.1 x 50 mm).

Fmoc-removal study

800 µM stock solutions of OTK^8[Fmoc-Aoa] in H_2O, DMF, 80% $EtOH_{aq}$ or 80% ACN_{aq} were prepared. Piperidine was added to the peptide solution to a final concentration of 30% piperidine (v/v). Reactions were stopped by diluting 10 µL aliquots of the reaction mixture in 200 µL 1% TFA/H_2O. Aliquots were taken every 10 s for 4 min and analysed by analytical RP-HPLC and MS.

Kinetic analysis of the oxime ligation of OTK^8[Aoa] with benzaldehyde, acetophenone or D-glucose.

Solutions of OTK^8[Aoa] (2 mM in H_2O or 20 mM in DMF, 80% $EtOH_{aq}$ or 80% ACN_{aq}), ligands (20 mM) and pPDA catalyst (25 mM) were prepared and incubated at 25°C for 2 h or 6 h (D-glucose). The reaction was stopped by adding 5 µL acetone to 10 µL aliquots of the reaction mixture. The aliquots were further diluted with 100 µL 0.05% TFA/H_2O in water and analysed by analytical C_{18}-RP-HPLC and MS.

One-pot reaction: Fmoc-removal and oxime ligation

a) Benzaldehyde and acetophenone

Purified and folded OTK^8[Fmoc-Aoa] was dissolved in 30% (v/v) piperidine in 80% $EtOH_{aq}$ and incubated for 30 s. Equimolar amounts of the ligands (20 mM) and pPDA (25 mM) were added to the reaction mixture and incubated. The reaction was stopped by diluting the reaction mixture 1:100 with 0.05% TFA/H_2O.

b) D-Glucose reaction

Purified and folded OTK^8[Fmoc-Aoa] was dissolved in pre-heated 30% (v/v) piperidine/anhydrous DMF (20 mM peptide concentration) and incubated for 1 min at 75°C. The reaction was quenched with TFA (~30% v/v) followed by the addition of pre-heated aniline (2 eq) and D-glucose (100 eq). The reaction was stopped with 10% acetone in 0.05% TFA/H_2O after 5 min (1:100). OTK^8[Aoa]-D-glucose was purified by analytical RP-HPLC using a linear gradient 5–45% B in 20 min (solvent A: 0.05% TFA/H_2O; solvent B: 90% ACN_{aq}, 0.043% TFA) on an Agilent-Zorbax C_{18} column (300 Å, 3.5 µm, 2.1 x 50 mm).

c) FDG

Purified and folded OTK^8[Fmoc-Aoa] was dissolved in pre-heated (75°C) 30% piperidine/anhydrous DMF (20 mM peptide concentration) and incubated for 1 min at 75°C. The reaction was quenched with TFA (~30% v/v) followed by the addition of pre-heated (75°C) PDA (2 eq) and FDG (100 eq) dissolved in anhydrous DMF. The reaction was quenched with 10% acetone in 0.05% TFA/H_2O) after 5 min (1:100). OTK^8[Aoa-FDG] was purified by analytical C_{18}-RP-HPLC using a linear gradient 5–45% B in 20 min (solvent A: 0.05% TFA/H_2O; solvent B: 90% ACN_{aq}, 0.043% TFA) on an Agilent-Zorbax C_{18} column (300 Å, 3.5 µm, 2.1 x 50 mm).

Results

Stability of OTK[8][Aoa] and OTK[8][Fmoc-Aoa] during oxidative folding

The stability of the unprotected aminooxy group in OTK[8][Aoa] was investigated during disulfide bond formation in five oxidative folding conditions (**Figure S1a**). Folding was monitored by analytical C_{18}-RP-HPLC and LC-MS. OTK[8][Aoa] was highly unstable, resulting in very low yields (~10%) of folded OTK[8][Aoa] (**Figure S1a**). The main side product was the loss of the aminooxy NH_2 group to yield a primary alcohol (1080.4 Da observed with a mass difference of -15 Da to folded OTK[8][Aoa]), which was more pronounced at lower pH. The stability of OTK[8][Fmoc-Aoa] during oxidation was also examined under several conditions (**Figure S1b**). Fully folded OTK[8][Fmoc-Aoa] was obtained in high yields and purity (>90%) for all folding conditions, and no side product formation was observed.

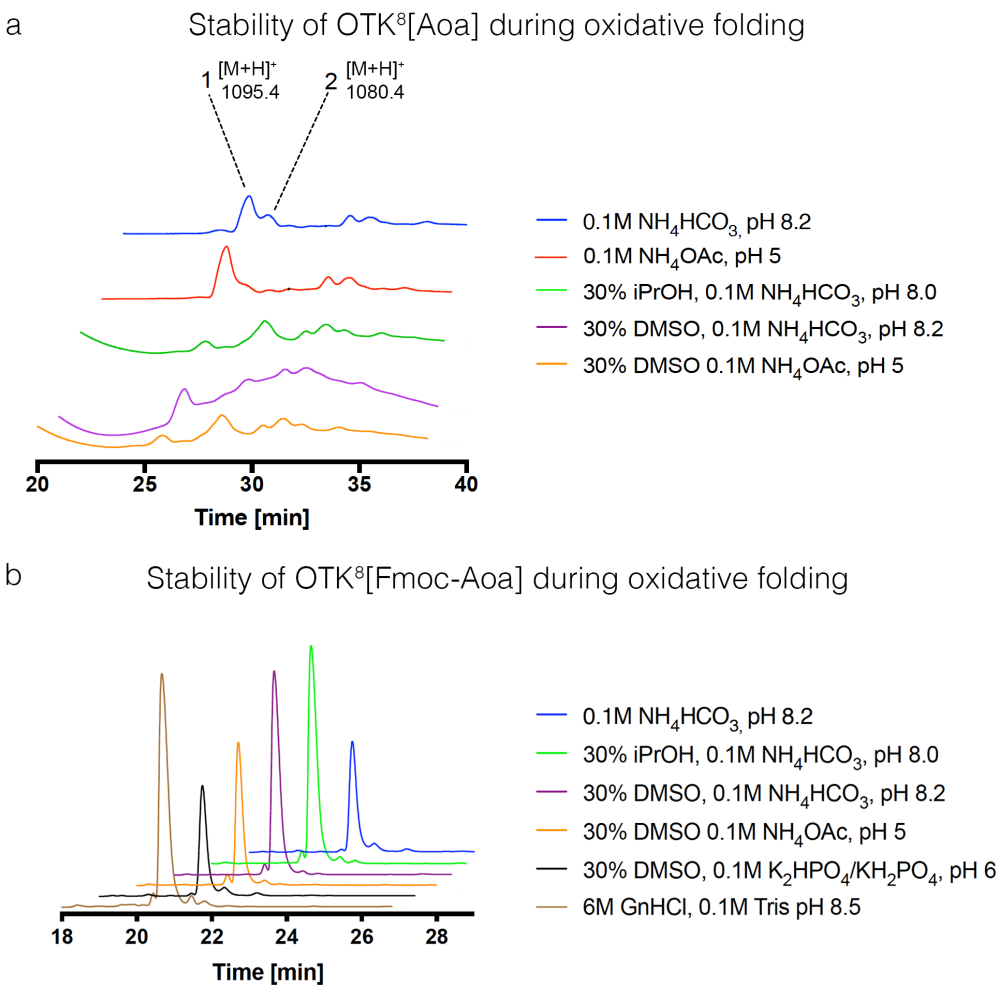

Figure S1. Stability of OTK[8][Aoa] and OTK[8][Fmoc-Aoa] during oxidative folding. (a) Reduced OTK[8][Aoa] was exposed to different oxidative folding conditions for 12 h at 25°C resulting in low yields and decomposition/side product formation. **(b)** Reduced OTK[8][Fmoc-Aoa] folded under all tested folding conditions within 12 h at 25°C in high yields and purity.

Directed disulfide bond formation of α-CnGI[Fmoc-Aoa]

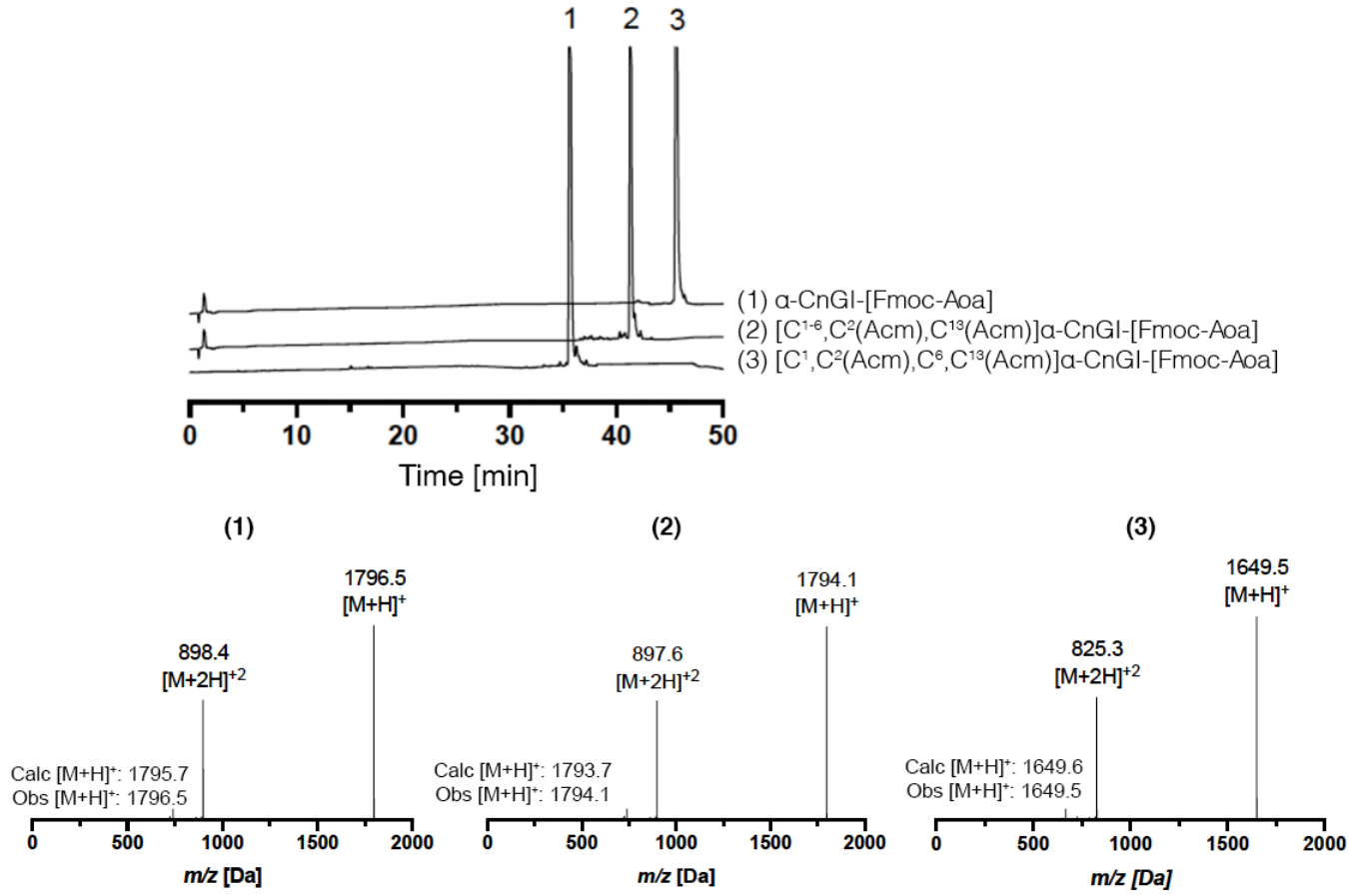

Figure S2. **RP-HPLC and MS data of α-CnGI-[Fmoc-Aoa] oxidised *via* directed disulfide bond formation.** Analytical C_{18}-RP-HPLC traces of α-CnGI-[Fmoc-Aoa] formation. Peak (1): reduced [C^{1},C^{2}(Acm),C^{6},C^{13}(Acm)]-α-CnGI-[Fmoc-Aoa]; Peak (2): partially folded [$C^{1\text{-}6}$,C^{2}(Acm),C^{13}(Acm)]-α-CnGI-[Fmoc-Aoa]; Peak (3): fully folded [$C^{1\text{-}6}$,$C^{2\text{-}13}$]-α-CnGI-[Fmoc-Aoa]. Calculated and observed masses of (1), (2) and (3) along with their MS data are provided below.

Long-term stability study of OTK[8][Fmoc-Aoa]

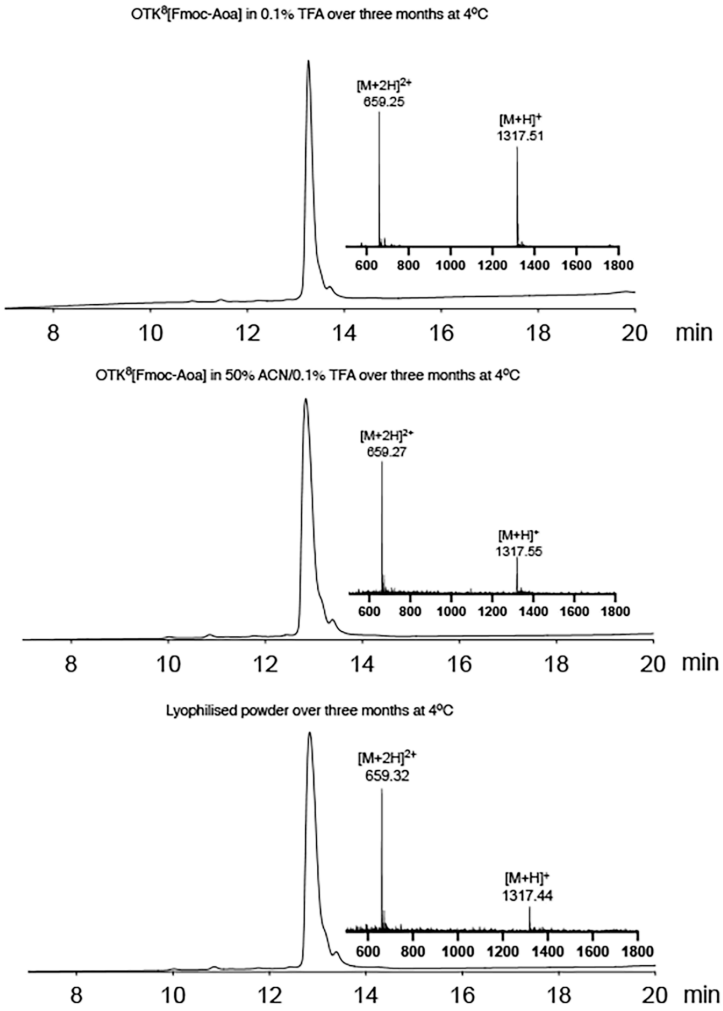

Figure S3. **Long-term stability study of OTK[8][Fmoc-Aoa].** Long-term stability was studied using analytical C_{18}-RP-HPLC and LC-MS for over three months. OTK[8][Fmoc-Aoa] was stable over three months at 4°C when either dissolved in 0.1% TFA/H_2O or 50% ACN_{aq}/0.1% TFA or stored as a lyophilised powder.

Fmoc-removal study

Fmoc removal kinetics were analysed using OTK[8][Fmoc-Aoa] in H_2O, DMF, 80% $EtOH_{aq}$ or 80% ACN_{aq} using 30% (v/v) piperidine. The reaction was monitored *via* RP-HPLC and LC-MS. The Fmoc group was removed within seconds in all tested solvents **(Figure S2)**.

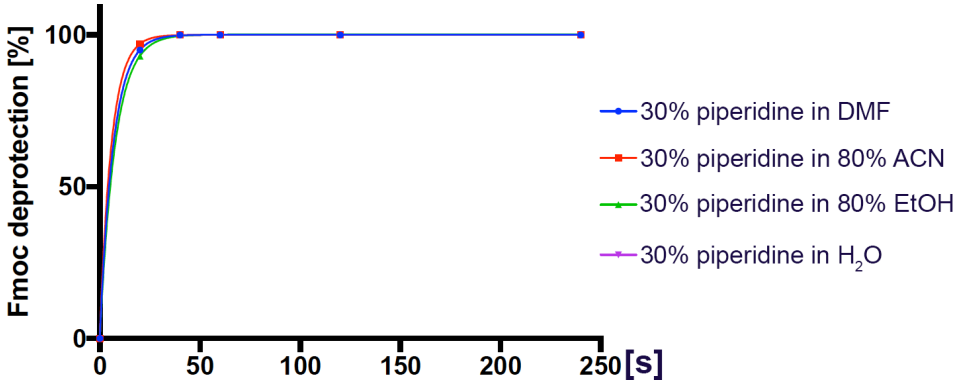

Figure S4. Fmoc-removal of OTK[8][Fmoc-Aoa] in different solvents. OTK[8][Fmoc-Aoa] was dissolved in DMF, 80% $EtOH_{aq}$ or 80% ACN_{aq} and the Fmoc group was removed by adding 30% (v/v) piperidine. Progress of the reaction was monitored by taking aliquots at 0, 20, 40, 60, 120 and 240 s and analysing them by C_{18}-RP-HPLC and LC-MS.

Reaction kinetics in different solvents

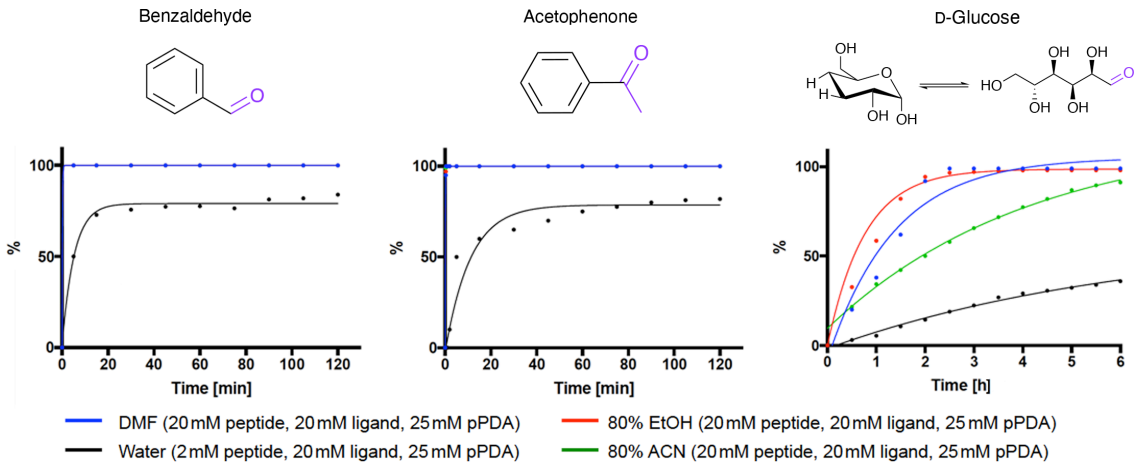

Figure S5. Analysis of oxime ligation reaction kinetics of OTK[8][Aoa] to benzaldehyde, acetophenone or D-glucose. Equimolar amounts (20 mM) of OTK[8][Aoa] and the ligands benzaldehyde, acetophenone or D-glucose were mixed in H_2O, 80% EtOH, 80% ACN or DMF (except for water, where the peptide was at 2 mM due to solubility limitations). The reaction mixture was incubated at 25°C. The reaction process was monitored *via* C_{18}-RP-HPLC and LC-MS.