Supporting Information

A Boc SPPS - compatible linker for the synthesis of peptide *o*-aminoanilides

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Abbreviations

DCM	Dichloromethane
DIC	*N,N'*-Diisopropylcarbodiimide
DIEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate
HPLC	High performance liquid chromatography
NCL	Native chemical ligation
NHS	*N*-Hydroxysuccinimide
TFA	Trifluoroacetic acid
SPPS	Solid phase peptide synthesis

Reagents

Boc N^{α}-amino acids for peptide synthesis were purchased from Bachem (Bubendorf, Switzerland), HATU was purchased from GL Biochem (Shanghai, China), DIEA (purified by redistillation, 99.5%), DMF (ACS reagent, >99.8%) and DCM (ACS reagent >99.5%) were purchased from Sigma-Aldrich (St. Louis, United States of America).

Solid Phase Peptide Synthesis (SPPS)

All syntheses were conducted using the *in situ* neutralization protocol[1] on MBHA resin (Peptides International 0.59 meq/g). Standard coupling conditions were HATU (5 equiv), Boc-N^{α}-amino acid (5 equiv), DIEA (7.5 equiv) with coupling times of 15 min.

Preparation of Fmoc-Dbz-OH

Fmoc-Dbz-OH was prepared following a previously described protocol.[2] 3,4-Diaminobenzoic acid (0.33 M) was dissolved in a 1:1 mixture of CH_3CN and 100 mM sodium bicarbonate (pH 7.9). The pH was raised back to 7.9 using a 1 M NaOH solution to allow for Dbz to fully dissolve. Fmoc *N*-hydroxysuccinimide ester (1 equiv) was added over 5 hours and the reaction was stirred for an additional 16 h at room temperature. Fmoc-Dbz-OH was precipitated by acidification with 1 M HCl and

the precipitate was filtered and washed using diethyl ether, hexanes, methanol and DCM. The product was dried under vacuum.

Fmoc-Dbz-OH coupling

Fmoc-Dbz-OH was either coupled to deprotected Lys (installed onto the resin using standard SPPS conditions described above) or the resin directly. Prior to coupling of Fmoc-Dbz-OH, the resin was neutralized using 10% (v/v) DIEA in DMF for 5 minutes. Fmoc-Dbz-OH (2.5 equiv), HATU (2.5 equiv), DIEA (2.75 equiv) were used and the coupling was allowed to proceed for 1 h.

Dbz protection and peptide assembly

Fmoc-Dbz-resin was deprotected to Dbz-resin using 20% piperidine in DMF. Then, the first amino acid was coupled using previously described conditions (Non-Gly, non-beta branched amino acid: (4 equivs), HBTU (4 equiv) and DIEA (6 equiv) for 1 h x 2, Fmoc-Gly-OH (4 equivs), HBTU (4 equiv), HOBt (4 equiv) and DIEA (4 equiv for 30 min).[2]

The resin was thoroughly washed with DMF and then DCM. A 10% (v/v) solution of chloroformates (2-chlorobenzyloxycarbonyl (2-ClZ) chloroformate or ethyl chloroformate) (10 equiv) was prepared in DCM, added to the resin and allowed to react overnight at room temperature in a closed reaction vessel. The resin was then thoroughly washed with DCM and then DMF.

The first amino acid was then Boc deprotected (neat TFA) and the remaining amino acids were coupled using the standard SPPS conditions outlined above. The *N*-terminal Boc group was deprotected with neat TFA and the peptide resin was washed with DMF and then neutralized with 10% (v/v) DIEA in DMF. Peptides were cleaved using a 9:1 mixture of anhydrous HF and anisole scavenger for 1 h at 0 °C. Subsequently, the cleavage solution was evaporated and the peptides were precipitated in cold anhydrous diethyl ether. The precipitate was dissolved in 30% acetonitrile, 0.05% TFA and 69.95% H_2O, frozen and lyophilized to dryness.

His(Dnp) deprotection

Dried peptide resin was swollen in DMF and drained. To deprotect His(Dnp), 20% (v/v) of β-mercaptoethanol and 10% (v/v) DIEA in DMF were added, drained

instantly and added again. After 30 minutes at room temperature, the resin was drained and the deprotection cocktail was added one last time for one hour before the resin was thoroughly flow washed with DMF.

Trp(For) deprotection

An ice cold 10% solution of 4-methyl piperidine in DMF was added to the resin, drained instantly and then added again. After one hour on ice, the resin was flow washed with DMF.

Lys biotinylation

Peptides were biotinylated on resin. Boc-Lys(Fmoc)-OH (2 equiv) was coupled with HATU (2 equiv) and DIEA (2.5 equiv) in DMF. The reaction progress was monitored using a Ninhydrin assay.[3,4]

Model peptide **5** (FK(Biotin)GGG-Dbz-K) was completely assembled using Boc SPPS and lysine side chain Fmoc deprotected (20% 4-methyl piperidine in DMF, 2 x 5 min). D-Biotin (2.5 equiv), NHS (2.5 equiv) and DIC (2.5 equiv) were dissolved in DMSO to a concentration of 0.5 mol/L each and the resultant solution was preactivated by stiring for 30 min at room temperature before it was added to the resin and left for 30 min to couple. After 30 minutes, DIEA (2.5 equiv) was directly added to the resin solution and left for an additional 30 minutes at room temperature to force coupling to completion. Reaction progress was monitored using a Ninhydrin assay.

During the synthesis of compound **6** (DTHFPICIFCCK(N^{ε}-Biotin)-Dbz-K-NH_2), Biotin was coupled to Boc-Lys($N^{\varepsilon}H_2$)-Dbz(2-ClZ)-Lys(2ClZ)-MBHA resin before Boc deprotection using the same coupling conditions described above for compound **5**. Following Boc deprotection, the remainder of the peptide was then assembled on Lys(N^{ε}-biotin)-Dbz(2Cl-Z)-Lys(2Cl-Z)-MBHA resin.

Activation and ligation

Peptide-*o*-aminoanilides (10 mM), were dissolved in 6 M guanidinium hydrochloride (GuHCl), 100 mM sodium phosphate, pH 3 and chilled in a salt saturated ice bath to −15 °C. A 0.5 M stock solution of sodium nitrite at 0 °C was added the peptide-*o*-aminoanilide to give a final concentration of 50 mM and kept in the −15 °C ice bath for 3–5 minutes. Subsequently, sodium 2-mercaptoethanesulfonate (Mesna) (20

equiv) was added to the reaction by addition of equal volume of 6 M GuHCl, 200 mM phosphate buffer (pH 8) and 200 mM Mesna. The pH of the reaction was measured and adjusted to 7. The reaction was monitored immediately by analytical HPLC and the resulting peptide-Mesna thioester was isolated by semi preparative HPLC.

Native chemical ligation

N-terminal cysteine and thioester peptides (equimolar, 10 mM) were dissolved in 6 M GuHCl, 200 mM sodium phosphate, 100 mM 4-mercaptophenylacetic acid (MPAA), pH 7.0. at room temperature. Reaction progress was monitored by analytical HPLC and the ligation product was isolated by semi preparative HPLC.

HPLC

Analytical reverse phase high performance liquid chromatography (HPLC) was performed using an Agilent 1100 system with a Jupiter Proteo 4 µm 90 Å column (150 mm x 4.6 mm) at a flow rate of 1 mL/min and using a gradient of 2% B/min (Solution A: 0.05% TFA in H_2O, Solution B: 0.045% TFA in 9:1 ACN/H_2O).

Semipreparative reverse phase HPLC was performed on a Waters Prep LC 4000 System using a Jupiter Proteo 10 µm 90 Å (250 mm x 10 mm) at a flow rate of 5 mL/min using linear gradients of 1% B/min.

Preparative reverse phase HPLC was performed on a Waters Prep LC 4000 System using a Jupiter Proteo 10 µm 90 Å (250 mm x 21.2 mm) at a flow rate of 15 mL/min using linear gradients of 0.5% B/min.

Mass Spectroscopy

Mass spectroscopy data was obtained by manual injection of samples on an API 2000 electrospray ionization quadruple MS/MS mass spectrometer (PE-Sciex). Peptide masses were calculated using the m/z ions.

HPLC and MS data of peptides

W(For)H(Dnp)GGG-Dbz-K-NH_2 (**4a**)

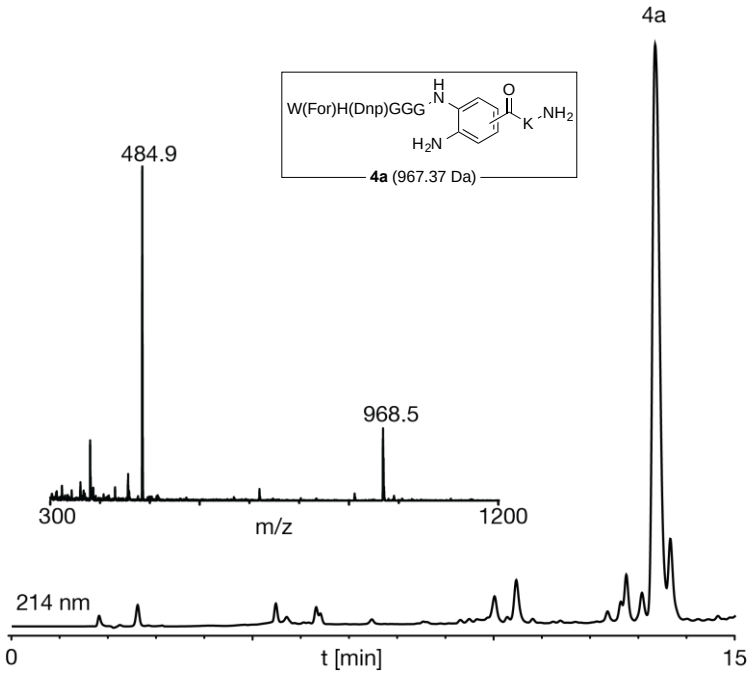

Figure S1. Formyl and Dnp protected model peptide **4a**. Observed: 967.7 (± 0.2) Da, theoretical: 967.4 Da.

WHGGG-Dbz-K-NH_2 (**4b**)

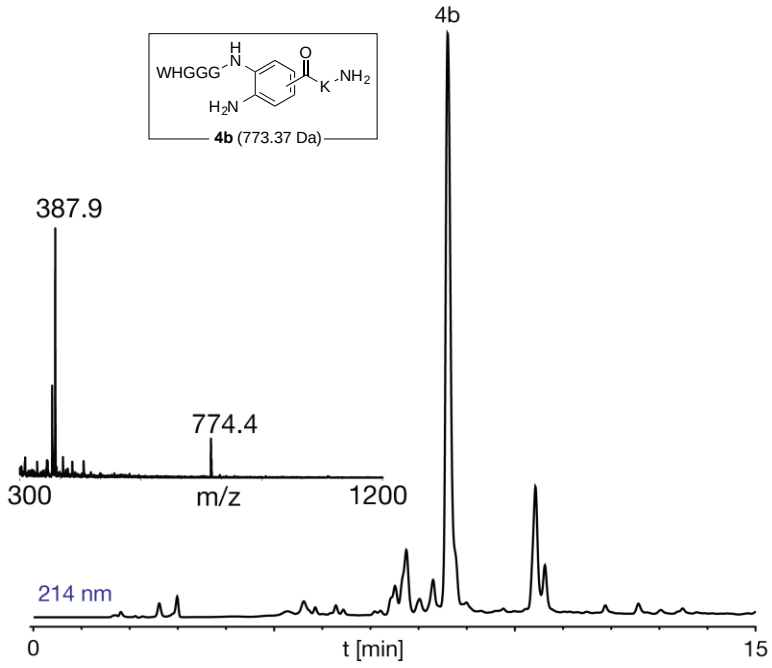

Figure S2. Formyl and Dnp unprotected model peptide **4b**. Observed: 773.6 (± 0.3) Da, theoretical: 773.4 Da.

FK(Biotin)GGG-Dbz-K-NH_2 (**5**)

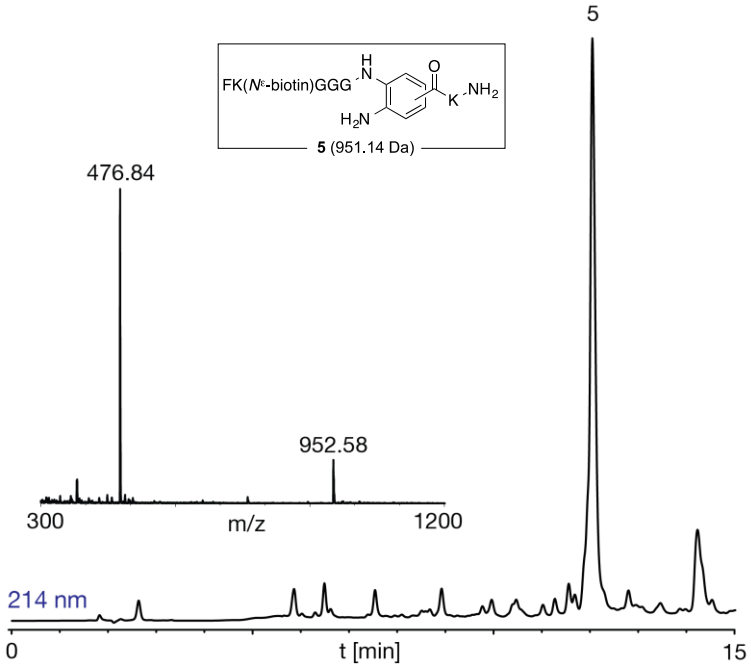

Figure S3. Biotinylated model peptide **5**. Observed: 951.6 (± 0.1) Da, theoretical: 951.1 Da.

DTHFPICIFCCK(N^{ε}-Biotin)-Mesna (**7**)

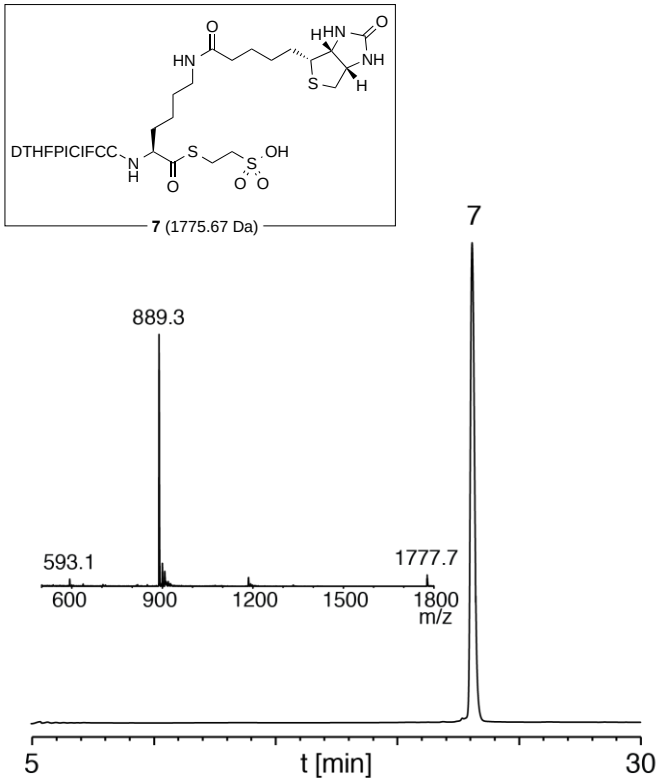

Figure S4. HPLC and MS traces of DTHFPICIFCCK(N^{ε}-Biotin)-Mesna (**7**). Observed: 1776.7 (± 0.1) Da, theoretical: 1775.67 Da.

DTHFPICIFCCK(N^{ε}-Biotin)CCHRSKCGMCCKT-NH_2 (**9**)

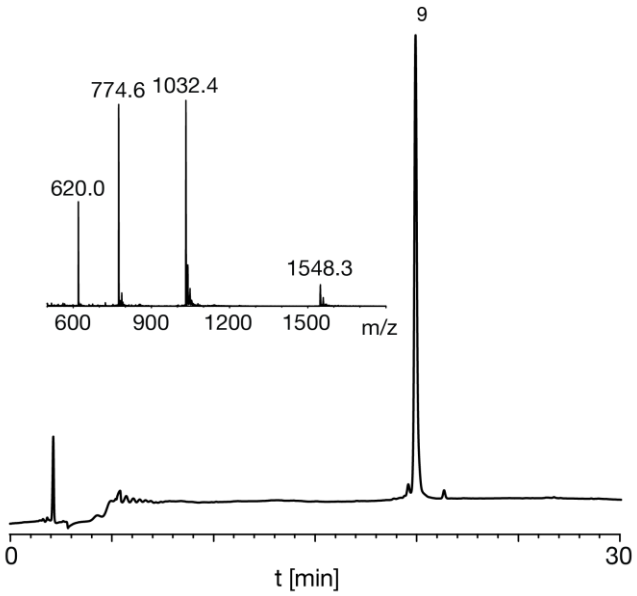

Figure S5. Purified biotinyl Hepcidin. Observed: 3094.6 (± 0.3) Da, theoretical: 3094.8 Da.

References

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(2) Blanco-Canosa, J. B.; Dawson, P. E. *Angew. Chem. Int. Ed.* **2008**, *47*, 6851–6855.

(3) Kaiser, E.; Colescott R. L.; Bossinger, C. D.; Cook, P. I. *Analytical Biochemistry* **1970**, *34*, 595–598.

(4) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Analytical Biochemistry* **1981**, *117*, 147–157.