Supporting Information

Non-reducible disulfide bond replacement implies that disulfide exchange is not required for hepcidin-ferroportin interaction

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Content

1. General Information

Materials

O-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HCTU), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU), (7-Azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP), 1-Hydroxy-7-azabenzotriazole (HOAt) and 1-hydroxy-benzotriazole (HOBt) were purchased from GL Biochem (Shanghai, China). Fmoc-amino acids were purchased from C S Bio, GL Biochem (Shanghai, China). *N*, *N'*-Diisopropyl-carbodiimide (DIC), N, N-Diisopropylethylamine (DIPEA) and Triisopropylsilane (TIPS) were purchased from Energy-chemical (Shanghai, China). GSSG and GSH were purchased from Aladdin (Shanghai, China). Acetonitrile (HPLC grade) was purchased from J. T. Baker (Phillipsburg, NJ, USA). N, N-Dimethylformamide (DMF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), N-methyl-2-pyrrolidone (NMP) and anhydrous diethyl ether were purchased from Sinopharm Chemical Reagent. Thioanisole and trifluoroacetic acid (TFA) (HPLC grade) were purchased from J&K Scientific (Beijing, China). All resins were purchased from Hecheng Technology (Tianjing, China).

HPLC, mass spectrometry (MS)

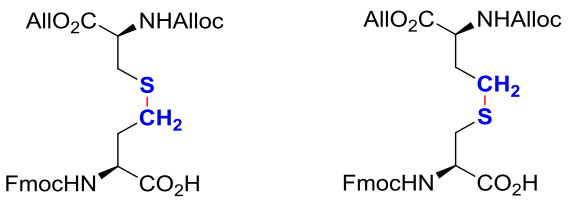
Reversed phase HPLC was performed on Shimadzu Prominence HPLC systems. For peptide analysis, Vydac C18 (4.6 × 250 mm) column was used, with a flow rate of 1.0 mL/min. For peptide purification, Vydac C18 (10 × 250 mm) column was used. Water (with 0.1% TFA) and acetonitrile (with 0.1% TFA) were used as the mobile phase, with a flow rate of 4.0 mL/min. ESI-MS spectra were recorded on a Shimadzu Prominence HPLC system with LC-MS2020.

General procedure for Fmoc-SPPS

All peptides were manually synthesized by Fmoc-SPPS. Rink amide AM resin (0.32 mmol/g) was swollen in a mixture of CH_2Cl_2 /DMF (1/1) for 60 min. Fmoc group was removed by 20% piperidine/DMF solution (5 min + 10 min). Then the resin was washed with DMF (5 times), DCM (5 times) and DMF (5 times). For natural amino acid coupling, a solution of the Fmoc-amino acid (4 equiv.), HCTU (3.8 equiv.) and DIEA (8 equiv.) in DMF was added to the resin for reacting 30 min. Then, double coupling was carried out for 30 min. For diaminodiacid coupling, a solution of the diaminodiacid (2 equiv.), HATU (1.9 equiv.) and DIEA (4 equiv.) in DMF was added to the resin for reacting 3 hours. For Alloc/Allyl deprotection on the resin, a mixture of $Pd(PPh_3)_4$ (1 equiv.) and $PhSiH_3$(5 equiv.) in DMF/ CH_2Cl_2 (2 mL/2 mL) was added to the resin to remove the Alloc/Allyl group of diaminodiacid (1 hours, 2 times). For intramolecular cyclization, a solution of the PyAOP (5 equiv.), HOAt (5 equiv.)

and NMM (10 equiv.) in NMP was added to the resin for reacting 3 hours. This step was repeated again. After assembly of peptide, the rink amide AM resin was treated with cleavage cocktail K (TFA/phenol/water/thioanisole/EDT, 82.5:5:5:5:2.5) for 3 h. The combined TFA solutions were concentrated by nitrogen blowing. The concentrated TFA solutions were precipitated with cold Et_2O. The crude peptides were isolated by centrifugation. Analysis and purification were carried out using HPLC and MS. After lyophilization, the desired peptide was obtained as a white powder.

2. S-C bridged and C-S bridged diaminodiacids



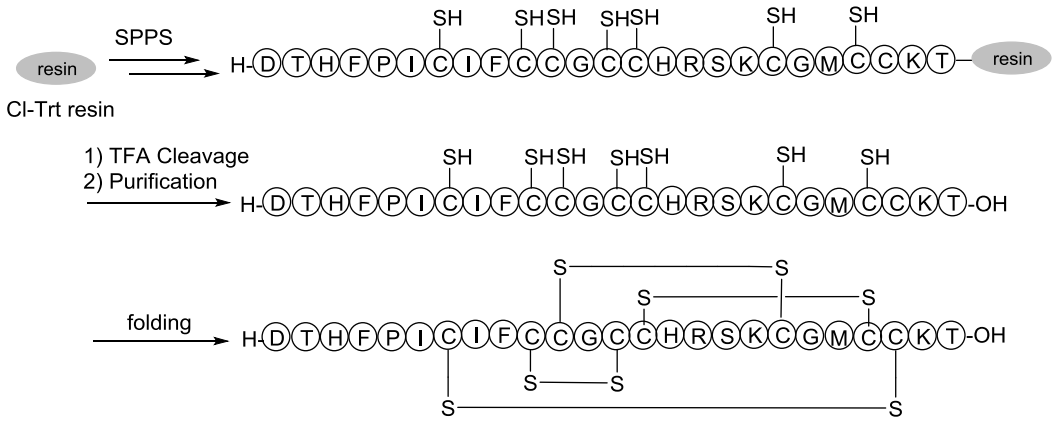
S-C bridged diaminodiacid **C-S bridged diaminodiacid**

Those two diaminodiacids were prepared according to our previously reported papers [1]

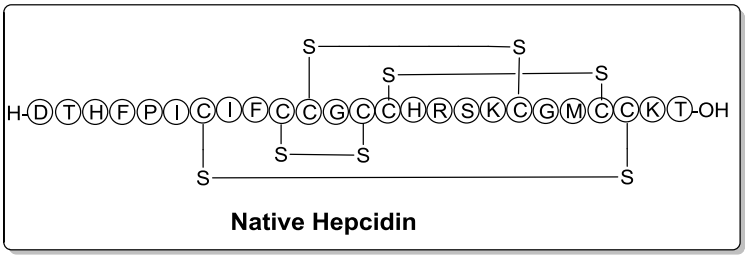
[1] H. K. Cui, Y. Guo, Y. He, F. L. Wang, H. N. Chang, Y. J. Wang, F. M. Wu, C. L. Tian, L. Liu, *Angew. Chem. Int. Ed.*, 2013, **52**, 9558-9562.

3. Synthesis of hepcidin

3.1. Synthesis of native hepcidin



Scheme S1. The route for chemical synthesis of native hepcidin



Native hepcidin

2-Chlorotrityl Chloride resin (160 mg, initial

substitution 0.40 mmol/g) was used to prepare hepcidin. The resin was swollen in a mixture of DCM (5 mL) for 30 min. After washing with DCM (5 times), the first amino acid was anchored to the Cl-Trt resin by treating resin with a mixture solution of Fmoc-L-Thr(tBu)-OH (53 mg, 2.0 equiv), DIPEA (62 μL, 4.0 equiv) in 4 mL DCM containing 0.2 mL DMF, for 4h. After the coupling of the remaining amino acids by Fmoc-based SPPS, the peptide was cleaved from resin by cocktail K reagent (TFA/phenol/water/thioanisole/EDT, 82.5:5:5:5:2.5). After 3 h, the combined TFA solution was concentrated by nitrogen blowing. The remaining TFA solution was precipitated by cold Et_2O, and the peptide was isolated by centrifugation.

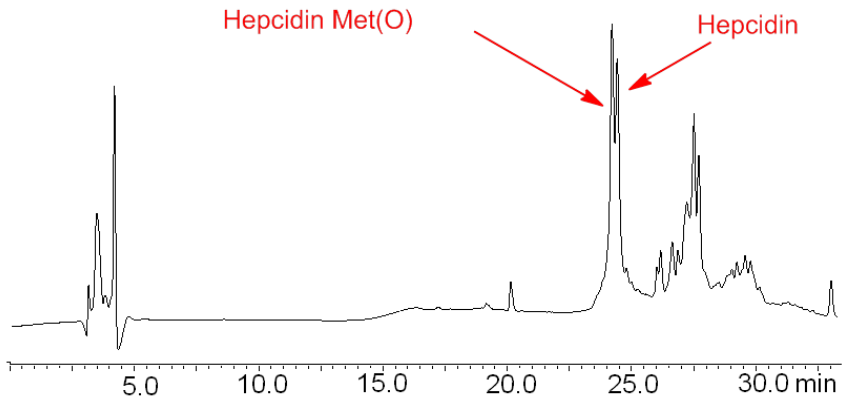
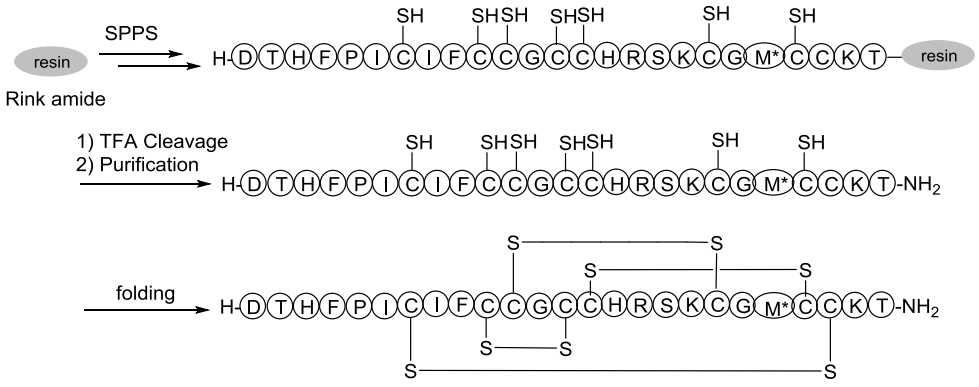
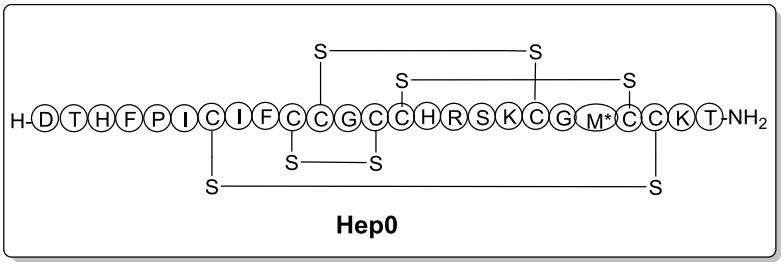


Figure S1. HPLC trace of the linear Hepcidin after TFA cleavage. The oxidation of Met during Fmoc-based SPPS makes the purification of target peptide difficult.

3.2. Synthesis of Hep0



Scheme S2. The route for chemical synthesis of **Hep0**



Hep0

Rink amide AM resin (200 mg, initial substitution 0.32 mmol/g) was used to prepare **Hep0**. The

resin was swollen in a mixture of DMF/DCM (5 mL/5 mL) for 30min, followed by Fmoc deprotection with 20% piperidine/DMF solution (5 mL) (twice: 5 min and 10 min). Then, the resin was washed with DMF (5 times), DCM (5 times) and DMF (5 times). The first amino acid was anchored by treating the resin with a pre-activated solution of Fmoc-L-Thr(tBu)-OH (105 mg, 4.0 equiv), HCTU (148.7 mg, 4.0 equiv) and DIPEA (124.5 µL, 8.0 equiv) in DMF (4 mL) for 2h. After the assembly of hepcidin, the peptide was cleaved from resin by cocktail K reagent (TFA/phenol/water/thioanisole/EDT, 82.5:5:5:5:2.5), for 3 h. The combined TFA solution was concentrated by nitrogen blowing. The remaining solution was precipitated with cold Et_2O, and the peptide was isolated by centrifugation. Analysis and purification of the crude peptide was carried out with HPLC (a linear gradient from 15 % to 90 % acetonitrile in 0.1 % trifluoroacetic acid, 30 min, C18). After lyophilization, the reduced form of Hep0 (35mg) was obtained as a white powder in a yield of 11 %. M* = norleucine

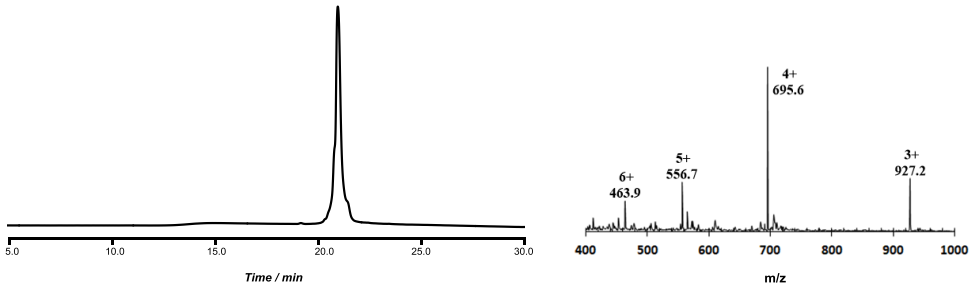


Figure S2. HPLC trace and ESI-MS of the reduced form of **Hep0** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2778.4 (average isotope), observed 2778.2.

The reduced form of **Hep0** (15 mg) was dissolved to a mixture solution of water/acetonitrile (94 mL, 3/7, v/v), with a concentration of 0.16 mg/mL. Then, 7.0 mg glutathione and 13.8 mg glutathione disulfide were added, and pH of the solution was adjusted to 7.5 by 0.1 N NaOH, stirring at 70 rpm. After 16 h, pH of the solution was adjusted to pH 2 with neat TFA, and the acetonitrile was evaporated. After HPLC purification and lyophilization, the folded **Hep0** (2.3 mg) was obtained as a white powder in an isolated yield of 20%.

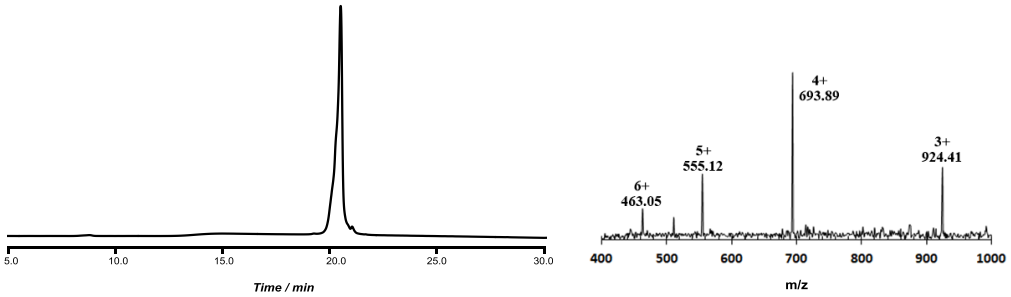
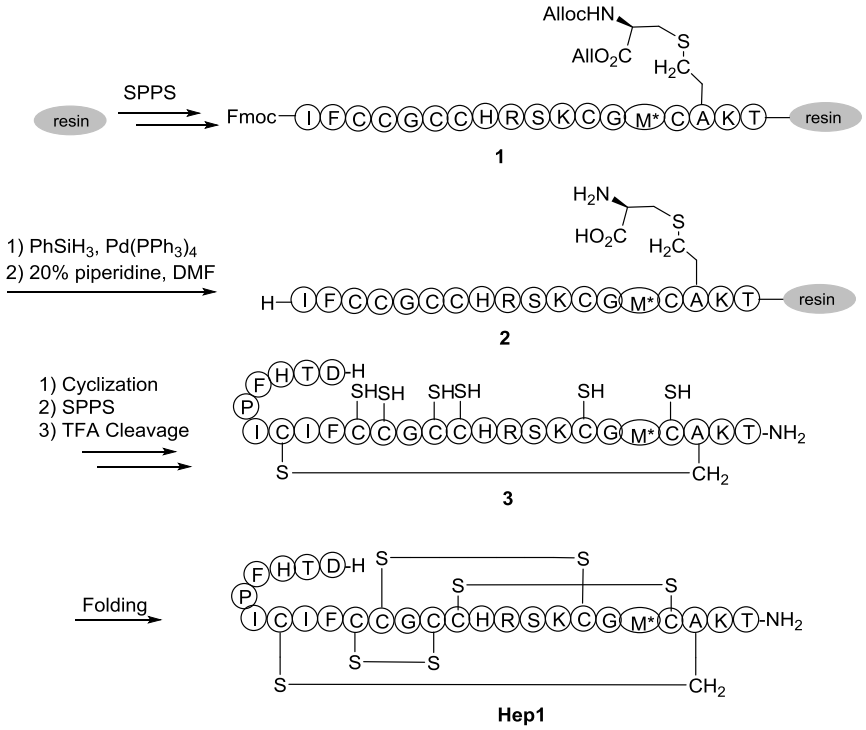


Figure S3. HPLC trace and ESI-MS of the folded **Hep0** after purification. C18 analytical

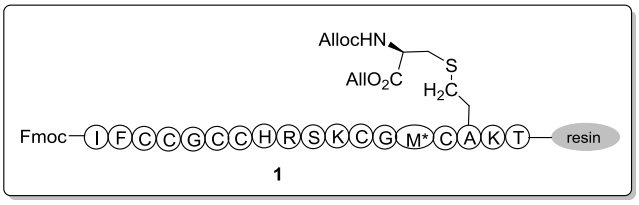
RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2770.4 (average isotope), observed 2771.1.

4. Synthesis of Hepcidin disulfide mimics

4.1. Synthesis of Hep1

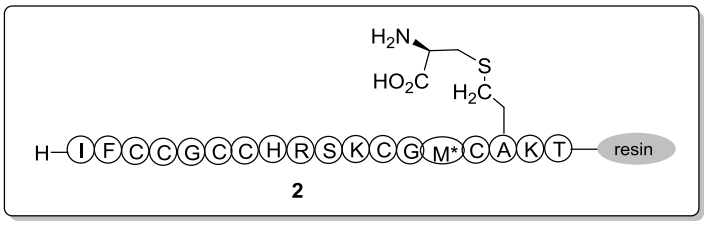


Scheme S3. The route for chemical synthesis of **Hep1**



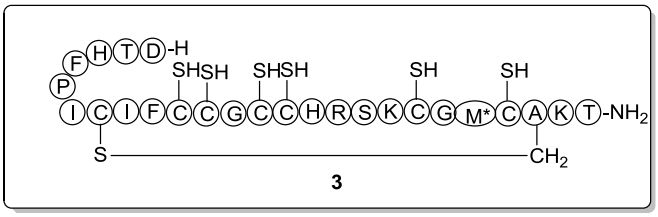
Intermediate 1

Rink amide AM resin (200 mg, 0.32 mmol/g) was used to prepare Hep-1. The resin was swollen in a mixture of DMF/DCM (5 mL/5 mL) for 30 min, followed by Fmoc deprotection with 20% piperidine (twice: 5 min and 10 min). Then, the resin was washed with DMF (5 times), DCM (5 times) and DMF (5 times). Fmoc-Thr(tBu)-OH, Fmoc-Lys (Boc)-OH were coupled by the standard protocol. After the removal of Fmoc group of Lys residue, the resin was treated with a pre-activated solution of **S-C bridged diaminodiacid** (72 mg, 2 equiv.), HATU (35.1 mg, 1.9 equiv.), DIPEA (43.6 uL, 4.0 equiv) in DMF (3 mL), for 2 h. Subsequently, Fmoc-Cys (Trt)-OH, Fmoc-Nle-OH (M*), Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg (Pbf)-OH, Fmoc-His (Trt)-OH, Fmoc-Phe-OH and Fmoc-Ile-OH were coupled to resin-bound peptide by the standard protocol to afford the resin-bound intermediate 1.



Intermediate 2

Deprotection of Alloc and Allyl protecting groups of intermediate 1 was achieved by using a solution of Pd $(PPh_3)_4$ (76 mg, 1.0 equiv) and $PhSiH_3$ (41 uL, 5 equiv) in DMF/CH_2Cl_2 (3 mL/3 mL), rt, for 3 h. After washing the resin with CH_2Cl_2 (15 times) and DMF (15 times), the Fmoc group of Ile residue was removed by 20% piperidine to afford resin-bound intermediate 2.



Intermediate 3 (Reduced form of Hep1)

Intramolecular cyclization between the amino group of the polypeptide chain and the side chain carboxyl group of the diaminodiacid was performed by PyAOP (171.9 mg, 5.0 equiv), HOAt (44.8 mg, 5.0 equiv) and NMM (74 μL, 10.0 equiv) in NMP (4 mL). After 3h, a freshly prepared coupling solution of PyAOP, HOAt and NMM in NMP was added to the resin again. After 3 h, the resin was washed with DMF (5 times), CH_2Cl_2 (5 times) and DMF (5 times). Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-His (Trt)-OH, Fmoc-Thr (tBu)-OH and Fmoc-Asp (OtBu)-OH were coupled by the standard Fmoc-based SPPS. The peptide was cleaved from resin by using cocktail K reagent (TFA/phenol/water/thioanisole/EDT, 82.5:5:5:5:2.5) for 3 h. The combined TFA solution was concentrated by nitrogen blowing. The remaining solution was precipitated with cold Et_2O, and the crude peptide was isolated by centrifugation. Analysis and purification of the crude peptide was carried out by HPLC (a linear gradient from 15% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, C18). After lyophilization, the reduced **Hep1** (13.7mg) was obtained as a white powder in an isolated yield of 5.0%.

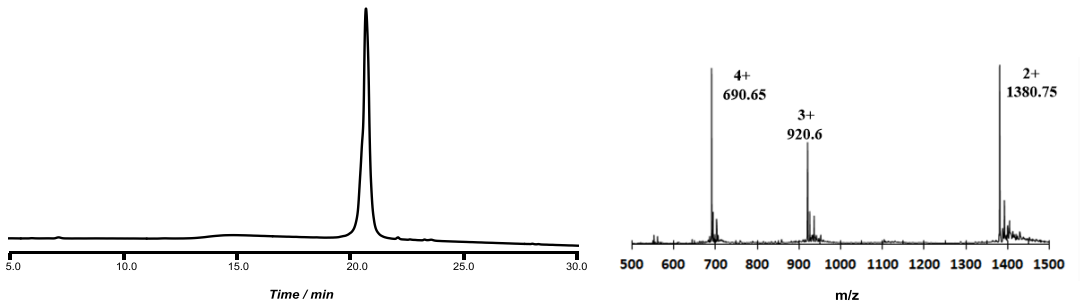
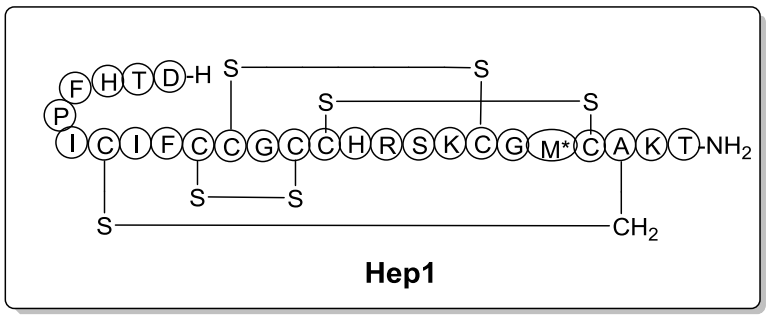


Figure S4. HPLC trace and ESI-MS of the reduced form of **Hep1** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2759.0.



Hep1

Hep1 (Folded Hep-1)
The folding of the reduced form of **Hep1** was carried out by the same procedure as **Hep0**. Analysis and purification of the folded Hep-1 was carried out by HPLC (a linear gradient from 15% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, C18). After lyophilization, the folded **Hep1** (2 mg) was acquired as a white powder in an isolated yield of 15%.

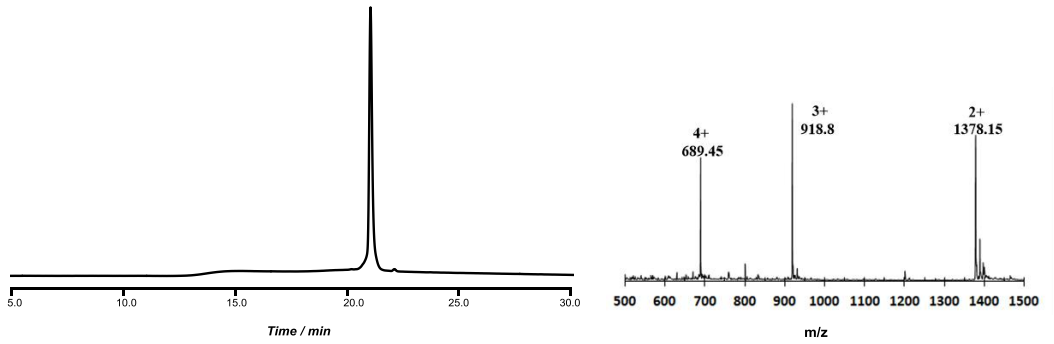


Figure S5. HPLC trace and ESI-MS of the folded **Hep1** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.8.

4.2. Synthesis of Hep2, Hep3 and Hep4

Hep2, **Hep3**, and **Hep4** were prepared according to the same protocol as **Hep1** using 200 mg rink amide AM resin and **S-C bridged diaminodiacid**. Analysis and purification were carried out by HPLC (a linear gradient from 10% to 90% acetonitrile in 0.1% trifluoroacetic acid, 30 min, C18). After lyophilization, the folded **Hep2** (37 mg) was acquired as a white powder in an isolated yield of 14%, the folded **Hep3** (40 mg) was acquired as a white powder in an isolated yield of 15% and the folded **Hep4** (27 mg) was acquired as white powder in an isolated yield of 10%.

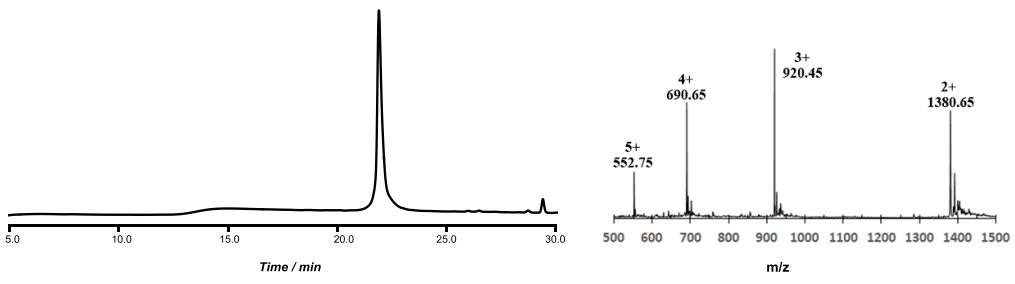


Figure S6. HPLC trace and ESI-MS of the reduced form of **Hep2** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2758.8.

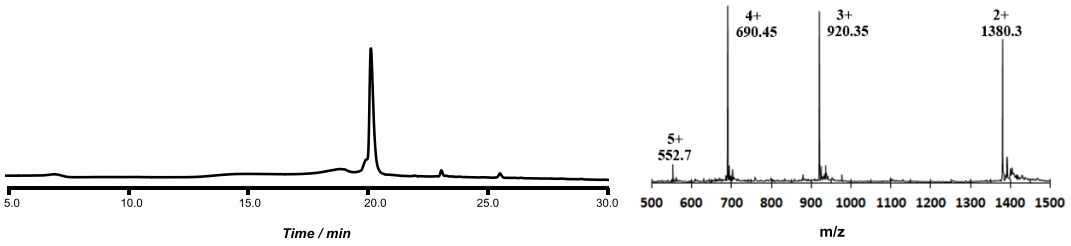


Figure S7. HPLC trace and ESI-MS of the reduced form of **Hep3** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2758.2.

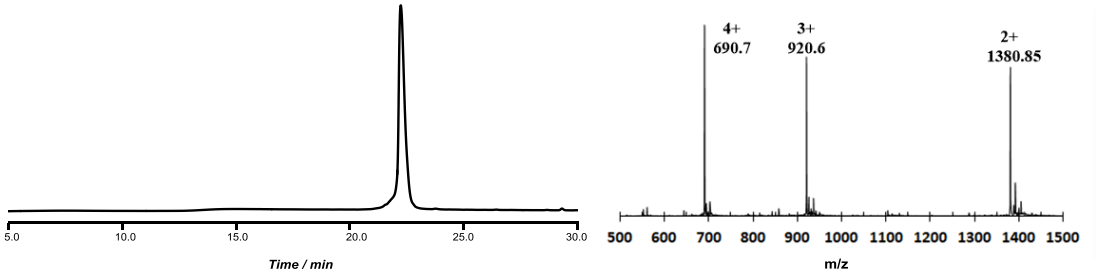


Figure S8. HPLC trace and ESI-MS of the reduced form of **Hep4** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2759.1.

The folding of the reduced form of **Hep2, Hep3** and **Hep4** were carried out according to the procedure of preparation of **Hep0**. Analysis and purification were carried out by HPLC (a linear gradient from 10% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, C18). After lyophilized, the folded **Hep2** (23 mg) was acquired as a white powder in an isolated yield of 62%, the folded **Hep3** (10 mg) was acquired as a white powder in an isolated yield of 25% and the folded **Hep4** (15 mg) was acquired as a white powder in an isolated yield of 67%.

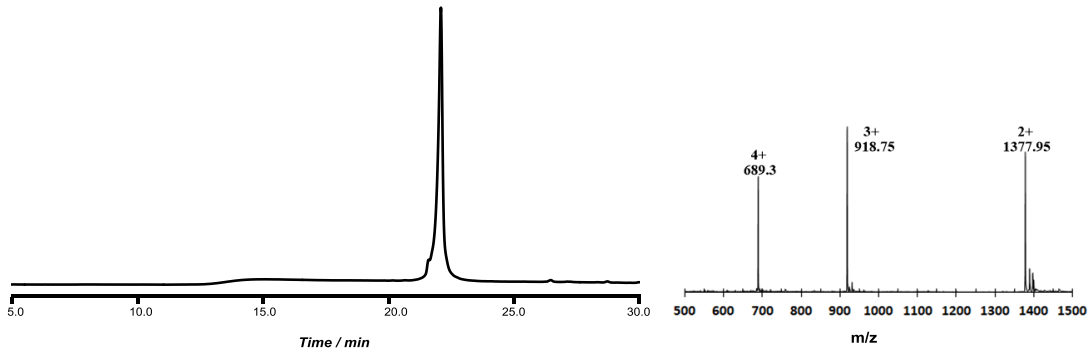


Figure S9. HPLC trace and ESI-MS of the folded **Hep-2** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.4.

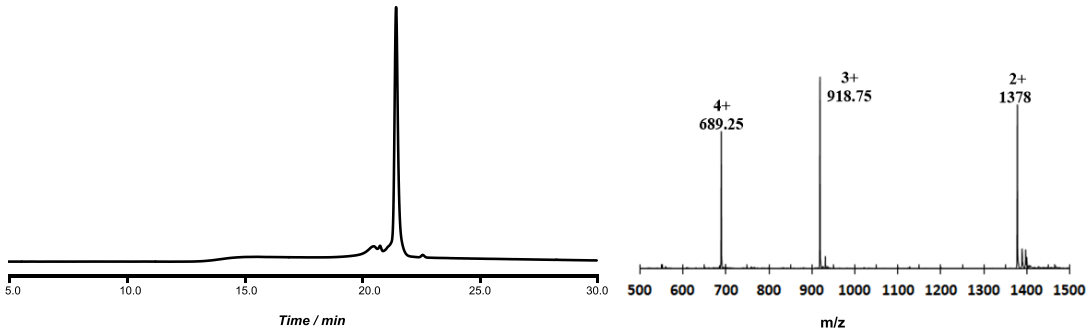


Figure S10. HPLC trace and ESI-MS of the folded **Hep-3** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.4.

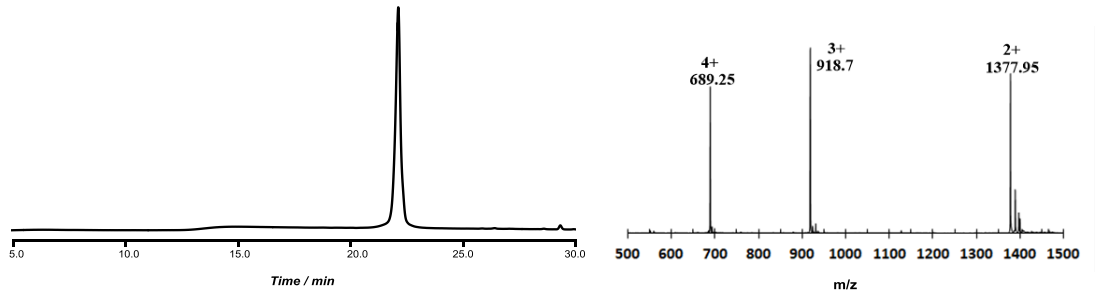


Figure S11. HPLC trace and ESI-MS of the folded **Hep-4** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.3.

4.3. Synthesis of Hep-5, Hep-6, Hep-7 and Hep-8

Hep-5, Hep-6, Hep-7, and **Hep-8** were prepared by the same standard protocol as **Hep-1** using 200 mg rink amide AM resin and **C-S bridged diaminodiacid**. Analysis and purification were carried out by HPLC (a linear gradient from 10% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, C18). After lyophilization, the folded **Hep5** (20 mg) was acquired as a white powder with an isolated yield of 5%, the folded **Hep6** (30 mg) was acquired as a white powder with an isolated yield of 11%, the folded **Hep7** (45 mg) was acquired as a white powder with an isolated yield of 13% and the folded **Hep8** (30 mg) was acquired as a white powder with an isolated yield of 11%.

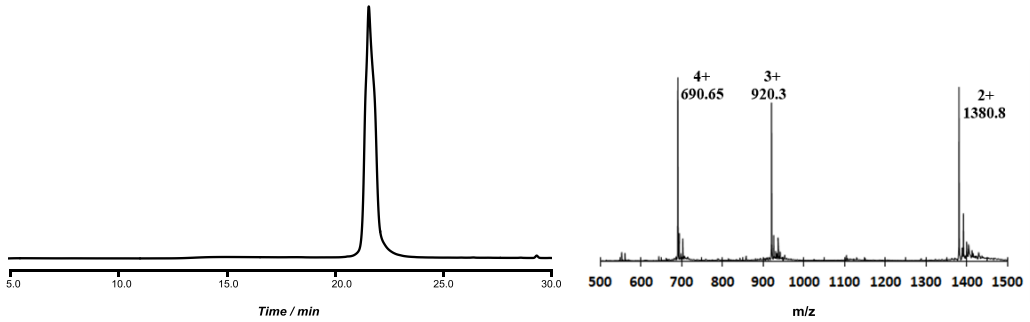


Figure S12. HPLC trace and ESI-MS of the reduced form of **Hep5** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2758.7.

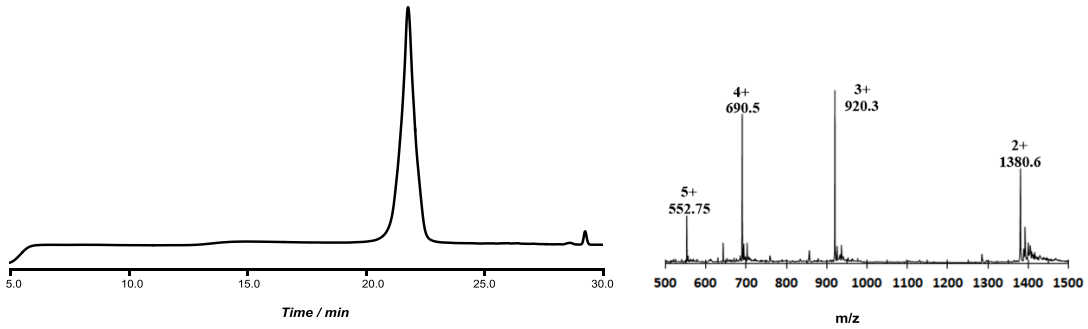


Figure S13. HPLC trace and ESI-MS of the reduced form of **Hep6** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2758.5.

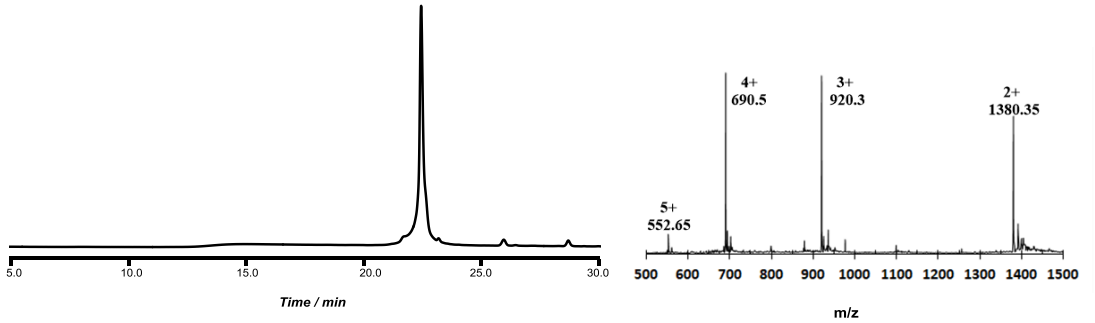


Figure S14. HPLC trace and ESI-MS of the reduced form of **Hep7** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2758.2.

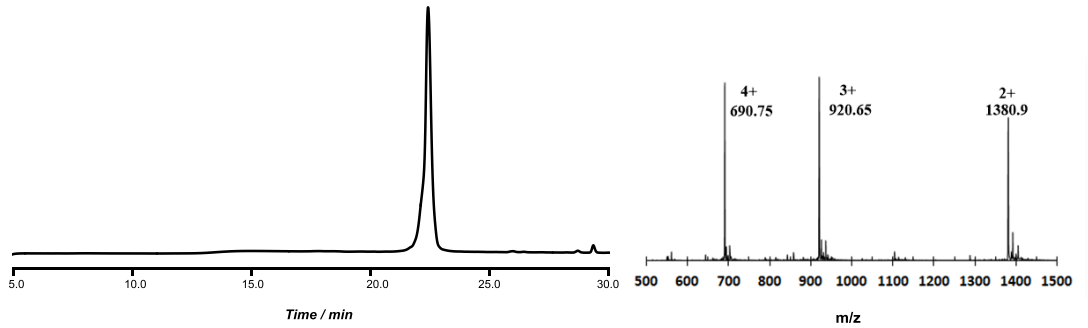


Figure S15. HPLC trace and ESI-MS of the reduced form of **Hep8** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2759.3 (average isotope), observed 2759.3.

The folding of the reduced form of **Hep5, Hep6, Hep7** and **Hep8** were carried out according to the procedure of preparation of **Hep0**. Analysis and purification were carried out by HPLC (a linear gradient from 10% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, C18). After lyophilization, the folded **Hep5** (8 mg) was acquired as a white powder with an isolated yield of 40%, the folded **Hep6** (5 mg) was acquired as a white powder with an isolated yield of 35%, the folded **Hep7** (12.9 mg) was acquired as a white powder with an isolated yield of 29%, and the folded **Hep8** (13 mg) was acquired as a white powder with an isolated yield of 43%.

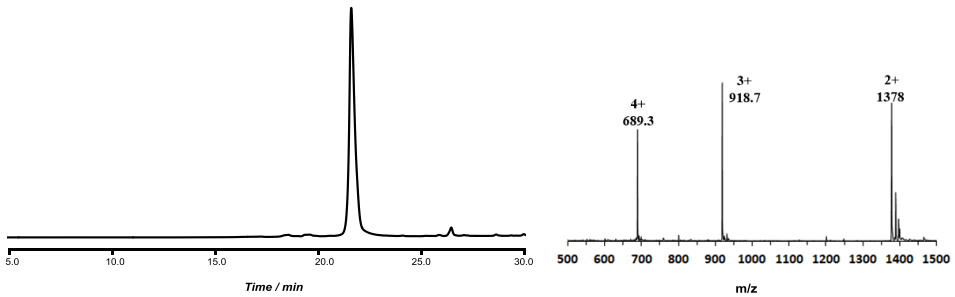


Figure S16. HPLC trace and ESI-MS of the reduced form of **Hep5** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.4.

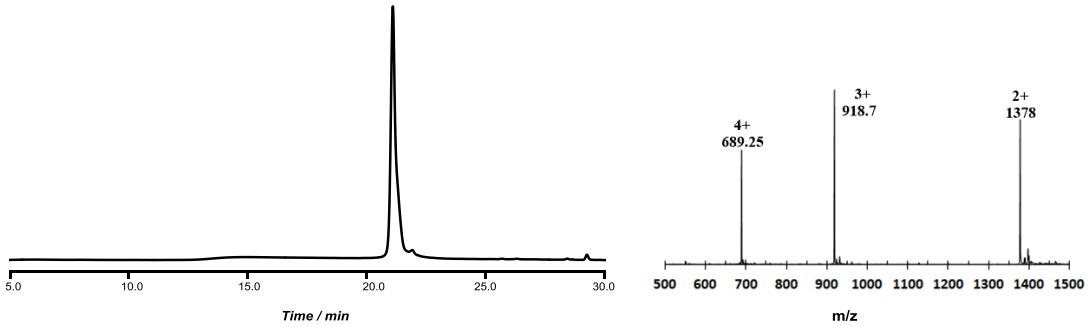


Figure S17. HPLC trace and ESI-MS of the reduced form of **Hep6** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.4.

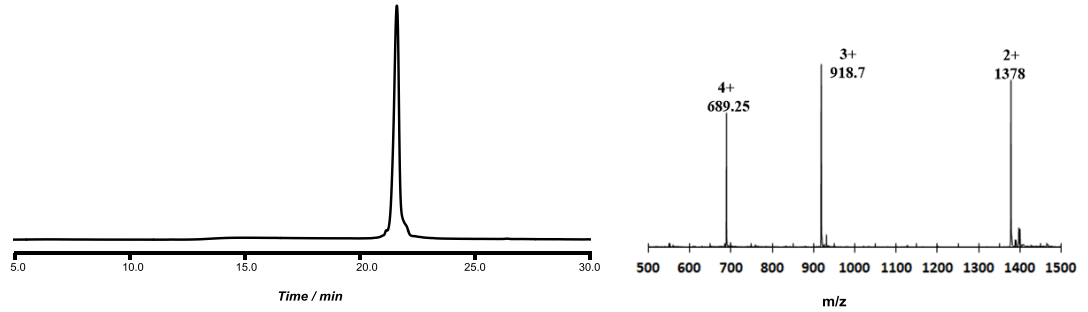


Figure S18. HPLC trace and ESI-MS of the reduced form of **Hep7** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.4.

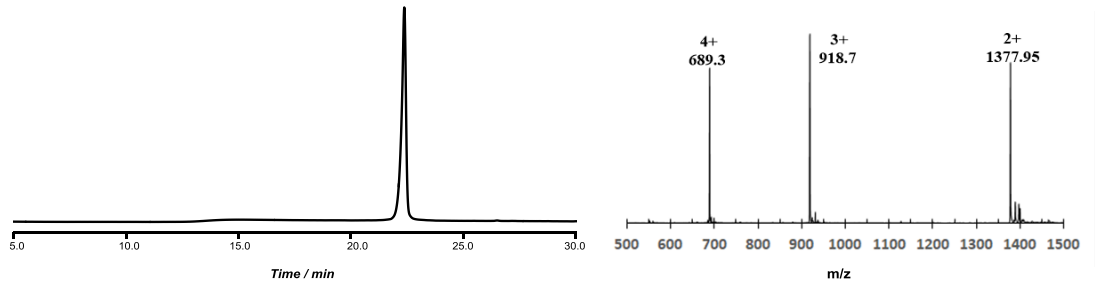


Figure S19. HPLC trace and ESI-MS of the reduced form of **Hep8** after purification. C18 analytical RP-HPLC chromatogram (left). ESI-MS spectrum (right) calcd 2753.3 (average isotope), observed 2753.4.

5. Synthesis of minihepcidins[1]

General Method for Synthesis of Mini-Hepcidins (from Mini1 to Mini25)

According to Method A, B or C as described below, solid phase peptide synthesis (SPPS) was carried out either by hand or using an automatic peptide synthesizer. Peptide synthesis was carried out in the range of 0.1 to 0.25 mmol. Method A was utilized for manual solid-phase synthesis. Automatic peptide synthesis was carried out by Method B (JPT Peptide Technologies GmbH, Volmerstraße 5, 12489 Berlin) or by Method C (Wuxi AppTec Co, Ltd, No1 Building, 288 FuTe ZhongLu, WaiGaoQiao Free Trade Zone, Shanghai, P.R. China).

Method A

Take **Mini5** (Ac-GTHFPRCRF-PEG3-K(Palmitoyl)-NH_2) as an example to specify the procedure of peptide synthesis. Rink amide AM resin (initial substitution 0.74 mmol/g) was used to prepare **Mini5**. The resin was swollen in a mixture of DMF/DCM (5 mL/5 mL) for 30min, followed by Fmoc deprotection with 20% piperidine/DMF solution (5 mL) (twice: 10 min x 2). Then, the resin was washed sequentially with DMF (5 times), DCM (5 times) and DMF (5 times). For Fmoc-Lys(Palmitoyl)-OH , Fmoc-NH-PEG3-COOH or natural amino acid couplings, a solution of the Fmoc-amino acid (3 equivalents), 0.5 M DIC solution (3 equivalents) and 0.5 M Oxyma solution (3 equivalents) in DMF was added to the resin for reacting 2h. Then, the procedure was repeated (a double coupling was carried out) for an additional 2h. This was followed by Fmoc deprotection using a 20% piperidine/DMF solution (5 mL) (twice: 10 min x 2). For N-terminal acylation of the peptide, acetic anhydride (10 equivalents) and diisopropylethyl amine (DIPEA, 2.5 equivalents) were added to the resin for 15 min. The acylation step was repeated (double coupling) for another 15min. After the assembly of **Mini5**, the peptide was cleaved from resin by cocktail TFA reagent (TFA/EDT/thioanisole, 90/3/7), for 3h. The combined TFA solution was concentrated by nitrogen blowing. The remaining solution was precipitated with cold Et_2O, and the peptide was isolated by centrifugation. The crude peptide was dissolved in water/acetonitrile (30% acetonitrile) and purified on an Agilent 1260 Prep reversed-phase HPLC using a Waters X-Bridge column (X-Bridge C18 5μm OBD, 19mm x 250 mm) using a 30% to 85% acetonitrile with a running time of 45 minutes (water and acetonitrile each contain 0.10% TFA). The highest purity fractions were collected and lyophilized. 1 mg of the final peptide was obtained (97.5% pure, 0.56% yield) as a TFA salt and as a white lyophilized powder. Two other fractions of 96% purity were also obtained.

Note: **Mini1, Mini2, Mini3**, **Mini4**, **Mini5, Mini6, Mini7**, **Mini8**, **Mini9**, **Mini10, and Mini13** were prepared by **Method A.**

Method B

The peptide syntheses were performed using the conventional Fmoc/tBu Solid Phase Peptide Synthesis (SPPS) and carried out on a fully automated peptide synthesizer (Syro II, MultiSynTech) equipped with two reactor blocks for the synthesis of 48 peptides in parallel. The peptides with C-terminal amide were synthesized using

TentaGel HL RAM resin (substitution: 0.35 mmol/g). The resin was swollen in DMF (4 mL) for 10 min. Fmoc group was removed using a 20% piperidine/DMF solution (1.9 mL, 10 min + 10 min). Then the resin was washed with DMF (5 x 2 mL). For natural amino acid couplings, a solution of the Fmoc-amino acid (0.675 mL, 0.5 M), HATU (0.675 mL, 0.5 M) and DIEA (0.34 mL, 2 M) in DMF (0.3 mL) was added to the resin for reacting 60 min. Then, double coupling was carried out for another 60 min. In order to prevent truncated sequences, an acetylation step was carried out prior to the Fmoc deprotection step. Specifically, each reaction vessel was filled with 1.425 mL DMF followed by the addition of 0.475 mL of a mixture of acetic acid anhydride/DIPEA/Oxyma dissolved in DMF (7:1.75:0.2 equivalents related to the loading of the resin). After a reaction time of 5 min the reaction mixture was drained and the resin was washed four times with 2.0 mL DMF. The Fmoc group was removed by 20% piperidine/DMF solution (1.9 mL, 10 min + 10 min). Then the resin was washed with DMF (5 x 2 mL). After assembly of the peptide, the resin was washed with DMF (4 × 2 mL) and diethyl ether (4 x 2 mL). The peptides were cleaved off manually by addition of TFA/EDT/H_2O (94/3/3, 8 mL, 3 h) to the resin. Afterwards, the peptide containing cleavage solution was separated and the peptide was precipitated by addition of cold diethyl ether. The precipitated peptide was washed twice with cold diethyl ether, dried overnight and analyzed.

The crude peptide was dissolved in DMSO or TFA and purified with an appropriate eluent mixture (eluent A: H_2O with 0.1% TFA; Eluent B: acetonitrile with 0.1% TFA; linear gradient) using the following columns: Waters SunFire C18 OBD 5 µm 150 x 50 mm, Agilent PLRP-S 100 A 8 µm 150 x 25 mm or Agilent PLRP-S 100 A 8 µm 300 x 50 mm. The selected ratio of A to B depended on the quality and physicochemical properties of the crude peptides. The highest purity fractions were collected and lyophilized.

Note: **Mini11**, **Mini12**, **Mini14**, **Mini15**, **Mini16**, **Mini17**, **Mini18**, **Mini19**, **Mini20**, **Mini21**, **Mini22**, **Mini23**, and **Mini24** were prepared by **Method B.**

Method C

The peptide syntheses were performed using the conventional Fmoc/tBu Solid Phase Peptide Synthesis (SPPS) by manual synthesis. The resin was swollen in a mixture of DMF for 30min, followed by Fmoc deprotection with 20% piperidine/DMF solution (twice: 10 min x 2). Then, the resin was washed with DMF (3 times). The amino acid was anchored by treating the resin with a pre-activated solution of Fmoc-L-XXa-OH (3.0 equiv), HBTU (2.95 equiv) and DIPEA (6.0 equiv) in DMF for 2h. Then, double coupling was carried out for another 2h. For N-terminal acylation of peptide, the cocktail of 5% Ac_2O/10% NMM/85% DMF was allowed to react with the resin for about 0.5 h. Finally, the resin was drained and washed with DMF (3 times) and with MeOH (3 times). After assembly of the peptide, the peptide was cleaved from resin by cocktail TFA reagent (TFA/TIPS/H_2O/EDT, 90/5/2.5/2.5, 10 mL) for 3 h. The combined TFA solution was concentrated by nitrogen blowing. The remaining solution was precipitated with cold Et_2O, and the peptide was isolated by centrifugation.

Note: **Mini25** was prepared by **Method C**.

For optional cyclization (disulfide bond formation), The crude peptide was dissolved to a mixture solution of water/acetonitrile (1:1), with a concentration of 1 mM. The pH of the solution was adjusted to about 8-9 with 1 M NH_4HCO_3. The solution was allowed to react for about 8 h at room temperature. The reaction was monitored by LC-MS. After the reaction was completed, the pH of the solution was adjusted to pH 6 with AcOH. The reaction mixture was lyophilized and the resulting solid was purified by reversed-phase HPLC.

Analytical Methods for Mini hepcidin (1-25)

LC-MS Method A

Equipment type: Thermo Fisher Scientific LTQ-Orbitrap-XL; Equipment type HPLC: Agilent 1200SL; Column: Agilent, POROSHELL 120; 3 x 150 mm, SB – C18 2.7 µm; Eluent A: 1 L Water + 0.1% Trifluoroacetic acid; Eluent B: 1 L Acetonitrile + 0.1% Trifluoroacetic acid; Gradient: 0.0 min 5% B→0.3 min 5% B→7.0 min 98% B→10 min 98% B; Oven: 40°C; Flow rate: 0.75 ml/min; UV-Detection: 210 nm.

LC-MS Method B

Equipment type MS: Thermo Fisher Scientific LTQ-Orbitrap-XL; Equipment type HPLC: Agilent 1200SL; Column: Agilent, POROSHELL 120, 3 x 150 mm, SB – C18 2.7 µm; Eluent A: 1 L Water + 0.1% Trifluoroacetic acid; Eluent B: 1 L Acetonitrile + 0.1% Trifluoroacetic acid; Gradient: 0.0 min 2% B→1.5 min 2% B→15.5 min 95% B→18.0 min 95% B; Oven: 40°C; Flow rate: 0.75 ml/min; UV-Detection: 210 nm.

Table 1. HPLC data and Mass spectrometric analysis of minihepcidins

No.	Sequence	R_T (min)	LC-MS Method	m/z (+) calc.	m/z (+) found[g]	Purity (%)
Mini1	DTHFPRCRF-NH_2	3.92	Method A	1176.56	1176.57	>99
Mini2	^{Ac}CDTHFPRCRF-NH_2[a]	3.95	Method A	1319.56	1319.58	>99
Mini3	DTHFPRCRF-*PEG3*-R-K(Palmitoyl)-NH_2[c]	5.27	Method A	1946.13	1946.14	>99
Mini4	^{Ac}CDTHFPRCRF-*PEG2*-K(Palmitoyl)-NH_2[a]	5.68	Method A	1889.01	1889.02	>99
Mini5	^{Ac}GTHFPRCRF-*PEG3*-K(Palmitoyl)-NH_2	5.71	Method A	1774.03	1774.04	97.42
Mini6	^{Ac}DTHFPRCRF-*PEG3*-K(Octanoyl)-NH_2	4.54	Method A	1719.91	1719.92	>99
Mini7	THFPRCRF-*PEG3*-K(Palmitoyl)-NH_2	5.62	Method A	1675.00	1675.01	>99
Mini8	DTHFPRSRF-*PEG2*-K(Palmitoyl)-NH_2	5.51	Method A	1730.024	1730.035	>99
Mini9	DTHFPRMRF-*PEG2*-K(Palmitoyl)-NH_2	5.59	Method A	1774.03	1774.04	>99

No.	Sequence	R_T (min)	LC-MS Method	m/z (+) calc.	m/z (+) found[g]	Purity (%)
Mini10	DTHFPR**M***RF-*PEG2*-K(Palmitoyl)-NH_2[b]	5.62	Method A	1756.08	1756.11	>99
Mini11	Sar-THFPRCRF-*PEG3*-K(Palmitoyl)-NH_2[d]	12.12	Method B	1746.04	1746.06	91
Mini12	Hoo-THFPRCRF-*PEG3*-K(Palmitoyl)-NH_2[d]	12.28	Method B	1815.02	1815.04	98
Mini13	Iso-DTHFPCRF-PEG3-K(Palmitoyl)-NH_2[d]	6.08	Method A	1717.98	1717.99	91
Mini14	Ida-THFPrCrF-*PEG3*-K-NH_2[d, e]	6.93	Method B	1551.8	1551.82	>99
Mini15	Ida-THFPrCrF-*PEG3*-K(Ac)-NH_2[f]	7.19	Method B	1593.81	1593.83	>99
Mini16	Ida-THFPRCrF-*PEG3*-K(Palmitoyl)-NH_2	11.84	Method B	1790.03	1790.05	98
Mini17	Ida-THFPrCRF-*PEG3*-K(Palmitoyl)-NH_2	12	Method B	1790.03	1790.05	98
Mini18	Ida-THFPrCrF-*PEG3*-K(Palmitoyl)-NH_2	5.54	Method A	1790.03	1790.05	96
Mini19	Ida-THFPrCrF-*PEG3*-K(Dodecanoyl)-NH_2	10.2	Method B	1733.96	1733.98	95
Mini20	Ida-THFPrCrf-*PEG3*-K(Palmitoyl)-NH_2[e]	11.92	Method B	1790.03	1790.05	96
Mini21	Ida-THFPrCrF-*PEG3*-k(Palmitoyl)-NH_2	11.95	Method B	1790.03	1790.04[h]	97
Mini22	Ida-THFPrCrY-*PEG3*-K(Palmitoyl)-NH_2	11.76	Method B	1806.02	1806.04	98
Mini23	Ida-THFPrCrY-*PEG3*-K(Palmitoyl)-NH_2	11.37	Method B	1771.05	1771.07	98
Mini24	Ida-THFPrMrF-*PEG3*-K(Palmitoyl)-NH_2	11.97	Method B	1818.06	1818.08	98
Mini25	Ida-THFPr**C(Me)**rF-*PEG3*-K(Palmitoyl)-NH_2[f]	12.24	Method B	1804,04	1804.06	>99

Note: [a] Disulfide bond between ^{1}Cys and ^{8}Cys. [b] M*: norleucine. [c] *PEG2*: $NH_2(CH_2CH_2O)_3CH_2CH_2COOH$; *PEG3*: $NH_2(CH_2CH_2O)_4CH_2CH_2COOH$. [d] Sar: sarcosine; Hoo: L-dihydroorotic acid; Iso: Isovaleric acid; Ida: Iminodiacetic acid. [e] r, k, and f represent D-form of Arg, Lys, and Phe, respectively. [f] K(Ac): side chain of Lys is acetylated; C(Me): side chain of Cys is methylated. [g] unless otherwise noted, [M+2H+] was observed. [h] [M+3H+] observed.

Reference:

[1] Bierer, D.; Pook, E.; Stampfuss, J.; Feldman, R. I.; Alt, C. WO 2018/128828 AI.

6. Ferroportin internalization assay

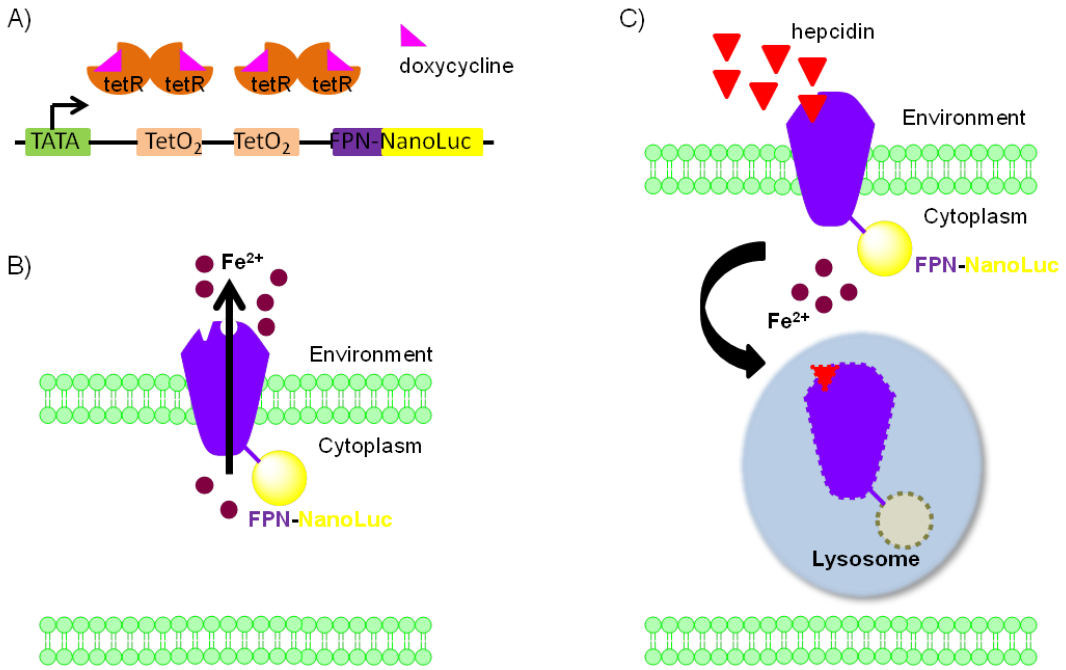


Figure S20. Ferroportin internalization assay. A) CHO T-REx cells were engineered to express a fusion of ferroportin (FPN) and NanoLuc under the control of a doxycycline responsive promoter. B) After the addition of doxycycline, FPN-NanoLuc is expressed and located on the cell membrane of cells. In the absence of hepcidin or its mimics, ferroportin transports iron from the interior of a cell to the outside of the cell. C) Binding of hepcidin or its mimic to FPN induces to internalization of FPN-NanoLuc and its degradation in lysosomes, accompanying by a decrease in luminescence from NanoLuc.

CHO T-Rex cells (Invitrogen) were stably transefcted with a vector expressing the ferroportin/NanoLuc fusion under control of the doxycycline dependent promoter (pCDNA5TO_hSLC40A1-NanoLuc). Functional internalization of ferroportin protein was determined by measuring the NanoLuc activity which is proportional to active ferroportin. 2500 Cells per well were plated in 384 MTP and incubated for 24 h in the presence of ferric ammonium citrate (FAC). Ferroportin protein expression was induced with doxycycline for 24 h. The next day medium was removed and cells were in incubated with test compounds in OptiMEM for 6 h, Subsequently luciferase activity was measured using the Nano-Glo Luciferase Assay System and in a FLIPR for 60 seconds.