Materials and methods

General

All solvents and reagents were used as supplied. *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and *S*-trityl mercaptopropionic acid were purchased from GL Biochem (Shanghai, China). Dimethylformamide (DMF) (AR grade) and acetonitrile (HPLC grade) were purchased from Scharlau (Barcelona, Spain). *N*,*N*′diisopropylethylamine (DIPEA), piperidine, diisopropylcarbodiimide (DIC), triisopropylsilane (TIS), 3,6-dioxa-1,8-ocatanedithiol (DODT) and 2,2,2-trifluoroethanol were purchased from Aldrich (St Louis, MO) and *N*-methylpyrrolidone (NMP) was purchased from Fluka (Buchs, Switzerland). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Alfa Aesar (Wardhill, MA). Guanidine hydrochloride was purchased from MP Biomedicals (Auckland, New Zealand). TFA was purchased from Halocarbon (River Edge, NJ). 1-Hydroxybenzotriazole hydrate (HOBt·H_2O) was purchased from Advanced Chemtech (Louisville, KY). Anhydrous hydrogen fluoride was obtained from Matheson Trigas (Basking Ridge, NJ). Aminomethyl polystyrene (AM-PS) resin was synthesised "in house" as described.[1] 4-[(*R,S*)-α-[1-(9*H*-fluoren-9-yl]methoxycarbonylamino]-2,4-dimethoxy]phenoxy acetic acid (Rink linker) was purchased from GL Biochem. Boc-Ala-PAM (PAM = phenylacetamidomethyl), Boc-Lys(2-Cl-Z)-PAM (Z = benzyloxycarbonyl) and Fmoc-Phe-HMPP (HMPP = hydroxymethylphenoxypropionic acid) linker were purchased from Polypeptides (Strasbourg, France). Fmoc-amino acids were purchased from GL Biochem with the following side chain protection: Fmoc-Arg(Pbf)-OH (Pbf = 2,2,4.6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Asn(Trt)-OH (Trt = triphenylmethyl), Fmoc-Asp(tBu)-OH (tBu = *tert*-butyl), Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Glu-OtBu, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met(O)-OH, Fmoc-Pen(Trt)-OH (Pen = penicillamine), Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH and Fmoc-Trp(Boc)-OH. Boc-amino acids were purchased from Polypeptides with the following side chain protection: Boc-Arg(Tos)-OH (Tos = *p*-toluenesulfonyl), Boc-Asp(cHex)-OH (cHex = cyclohexyl), Boc-Cys(4-MeBn)-OH (Bn = benzyl), Boc-Asn(Xan)-OH (Xan = Xanthyl), Boc-Glu(cHex)-OH, Boc-His(Bom)-OH (Bom = benzyloxymethyl), Boc-His(Tos)-OH.DCHA (DCHA = dicyclohexylamine), Boc-Lys(2-Cl-Z)-OH, Boc-Met(O)-OH, Boc-Ser(Bn)-OH, Boc-Thr(Bn)-OH, Boc-Thz-OH (Thz = 1,3-thiazolidine), Boc-Tyr(2-Br-Z)-OH, Boc-Trp(CHO)-OH.

HPLC and LC-MS

Analytical RP-HPLC was performed with a Dionex (Sunnyvale, CA, USA) Ultimate 3000 system equipped with a four-channel UV detector or a Waters (Milford, MA, USA) 600E system equipped with a two-channel UV detector using a Symmetry C4 (5μ; 2.1 x 150 mm) column and a linear gradient of 5% to 65%B over 31 mins (*ca.* 2%B per minute) at a flow rate of 0.2 ml/min. The solvent system used was A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile). Peptide masses were confirmed by LC-MS using a Dionex Ultimate 3000 equipped with a Thermo (Waltham, MA, USA) Finnegan MSQ mass spectrometer using ESI in the positive mode. Unless otherwise indicated the solvent system, columns, flow rates, and linear gradients were used as above.

Peptides were purified using either a Dionex Ultimate 3000 system equipped with a Foxy Jr fraction collector or a Gilson (Middleton, WI, USA) 214 system using a Gemini C18 (5 μ; 10.0 x 250 mm) column (Phenomenex) at a flow rate of 5 mL/min and eluted using a one-step slow gradient protocol.[2] Unless otherwise indicated the solvent system used was A (0.1% trifluoroacetic acid in H_2O) and B (0.1% trifluoroacetic acid in acetonitrile). Fractions were collected, analysed by either HPLC or LC-MS, pooled and lyophilised.

Boc SPPS

Boc-Lys(2-Cl-Z)-PAM linker (0.2 mmol) was coupled to AM-PS resin (0.1 g, 0.1 mmol, loading 1 mmol/g) with DIC (0.2 mmol) in a mixture of CH_2Cl_2/DMF (5/1, v/v, 1.5 mL) for 1 h, drained and washed with CH_2Cl_2. The Kaiser test was

negative. Solid phase peptide synthesis was performed manually using the Boc *in situ* neutralisation procedure[3] using 100% TFA as deblocking reagent and HBTU as coupling reagent. Following chain assembly the peptidyl resin was cleaved with simultaneous removal of side chain protecting groups with anhydrous HF/*p*-cresol (20:1, v/v) for 1 h at 0 °C. Following evaporation of HF, the peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in 1:1 (v/v) acetonitrile:water containing 0.1% TFA, filtered and lyophilised to afford the crude peptide. Purification by RP-HPLC afforded peptide **13** (81 mg), [$(M+4H)^{4+}$, calc. 1216.90, found 1216.85 Da)] and peptide **14** (65 mg), [$(M+5H)^{5+}$, calc. 669.39, found 669.23 Da)]. Peptide **12** (163 mg crude) was used without any further purification.

Fmoc SPPS

*Using HMBA linker for peptide **15**.* AM-PS resin (0.1 g, 0.1 mmol, loading 1 mmol/g) was reacted with the Rink linker (0.3 mmol), DIC (0.3 mmol) and 6-Cl-HOBt (0.3 mmol) in DMF (1 mL) for 1 hour, drained and washed with DMF. The pent-lysine solubilising tag with base labile HMBA linker was anchored by successive attachment of Fmoc-Lys(Boc)-OH (0.5 mmol) using HBTU (0.475 mmol) and DIPEA (1.2 mmol) in DMF (1mL) followed by double coupling of HMBA (0.3 mmol) with DIC (0.4 mmol) and 6-Cl-HOBt (0.6 mmol) in DMF (1 mL) for 1 hour. The resulting resin was then treated with a 1:1 mixture of 0.2 M NaOH/DMF (4 x 5 mins) followed by H_2O/DMF (4 x 5 mins). The C-terminal amino acid was then attached by double coupling of Fmoc-Phe-OH (0.3 mmol), DIC (0.4 mmol) and DMAP (0.01 mmol) in DMF (1 mL) and the mixture shaken for 50 mins. Solid phase peptide synthesis was performed using a Liberty Microwave Peptide Synthesiser (CEM Corporation, Mathews, NC) as outlined previously[4] using HCTU/DMF and DIPEA/NMP as coupling reagents and 5% (v/v) solution of piperizine + 0.1M 6-Cl-HOBt in DMF for deprotection. The following amino acid were double coupled: Fmoc-Cys(Trt)-OH, Fmoc-Met(O)-OH, Fmoc-Val-OH, Fmoc-Pro-OH and Fmoc-Phe-OH. The peptidyl resin was then treated with TFA/TIS/H_2O/DODT (94/1/2.5/2.5, v/v/v/v) (10 mL) for 2 h and the crude peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed with cold diethyl ether, dissolved in 1:1 (v/v) acetonitrile:water containing 0.1% TFA and lyophilised to afford the crude peptide. Purification by RP-HPLC gave peptide **15** (68 mg), [$(M+6H)^{6+}$, calc. 889.55, found 889.66)].

*Thiotransesterification of peptide **12** thioalkylester to peptide **3** MPAA thioarylester*

To a degassed solution containing 6 M guanidine hydrochloride and 200 mM Na_2HPO_4 (19.9 mL) was added 4-mercaptophenylacetic acid (100 mM) and TCEP (20 mM). The pH of the resulting solution was adjusted to 6.6 using 10 M NaOH prior to the addition of peptide **12** (86 mg, 20 µmol, final peptide conc. 1 mM). The vial was capped under argon, and the reaction mixture was left at r.t. until completion. 2µL aliquot of the reaction mixture was diluted 3.5-fold into a water solution (5% TFA v/v) for LC-MS analysis. Upon completion, the reaction was quenched by addition of 5 M HCl followed by SPE and lyophilisation to afford the crude thioarylester that was purified by RP-HPLC to give peptide **3** (36 mg). [$(M+3H)^{3+}$, calc. 1246.09, found 1245.96 Da)].

*Kinetically controlled ligation (KCL) of peptide **3** and **13.***

To a degassed solution containing 6 M guanidine hydrochloride and 200mM Na_2HPO_4 (8.5 mL) was added TCEP (50 mM). The pH of the resulting solution was adjusted to 6.75 by addition of 10 M NaOH. The two reactants, peptide **3** (31.85 mg, 8.5 µmol, final peptide conc. 1 mM) and peptide **13** (41.4 mg, 8.5 µmol, final peptide conc. 1 mM) were then added, the vial was capped under argon and the reaction mixture was left at r.t. A 2µL aliquot of the reaction mixture was diluted 3.5-fold into a water solution (5% TFA v/v) for LC-MS analysis. Upon completion, the reaction was quenched by addition of 5 M HCl followed by SPE and lyophilisation to afford crude ligation product that was purified by RP-HPLC to give peptide **14** (22 mg). [$(M+7H)^{7+}$, calc. 1205.38, found 1205.64 Da)].

*Native chemical ligation (NCL) of peptide **14** and **15**.*

To a degassed solution containing 6 M guanidine hydrochloride and 200mM Na_2HPO_4 (14.95 mL) was added 4-mercaptophenylacetic acid (100 mM) and TCEP (20 mM). The pH of the resulting solution was adjusted to 7.0 by addition of 10 M NaOH. The two reactants, peptide **14** (50 mg, 15 µmol, final peptide conc. 1 mM) and peptide **16** (78.25 mg, 15 µmol, final peptide conc. 1 mM) were then added, vial was capped under argon and the reaction mixture was left at r.t. A 2µL aliquot of the reaction mixture was diluted 3.5-fold into a water solution (5% TFA v/v) for LC-MS analysis. Upon completion, the reaction was quenched by addition of 5M HCl followed by SPE and lyophilisation to afford crude ligation product that was purified by RP-HPLC to give peptide **17** (11 mg). [$(M+7H)^{7+}$, calc. 1133.18, found 1133.01 Da)].

*Conversion of thiazolidine **17** to cysteinyl peptide **18**.*

To a solution of MeCN:water (1:1, v/v) containing 0.1% TFA (45 mL) was added methoxyamine hydrochloride (1.0 M) followed by crude peptide **17** (25 mg, 3.15 µmol, final peptide conc. 0.07 mmol). The reaction mixture was left at r.t. and open air for 2 days. A 2µL aliquot of the reaction mixture was diluted 3.5-fold into a water solution (0.1% TFA) for LC-MS analysis. The reaction mixture was loaded onto a diphenyl (5 µ; 10.0 x 250 mm) column and eluted with 5%B to remove excess methoxyamine hydrochloride and buffer components. The crude peptide was then purified by RP-HPLC to give peptide **18** (4 mg). [$(M+8H)^{8+}$, calc. 990.15, found 990.21 Da)].

*Native chemical ligation (NCL) of peptide **16** and **18***

To a degassed solution containing 6 M guanidine hydrochloride and 200mM Na_2HPO_4 (356 µL) was added 4-mercaptophenylacetic acid (100 mM) and TCEP (20 mM). The pH of the resulting solution was adjusted to 7.0 by addition of 10 M NaOH. The two reactants, peptide **16** (3 mg, 0.35 µmol, final peptide conc. 1 mM) and peptide **18** (3.38 mg, 0.42 µmol, final peptide conc. 1 mM) were then added, vial was capped under argon and the reaction mixture was left at r.t. A 2µL aliquot of the reaction mixture was diluted 3.5-fold into a water solution (5% TFA v/v) for LC-MS analysis. Upon completion, the reaction was quenched by addition of 5M HCl followed by SPE and lyophilisation to afford crude peptide that was purified by RP-HPLC to give peptide **19** (0.47 mg). [$(M+10H)^{10+}$, calc. 1560.70, found 1561.58 Da)].

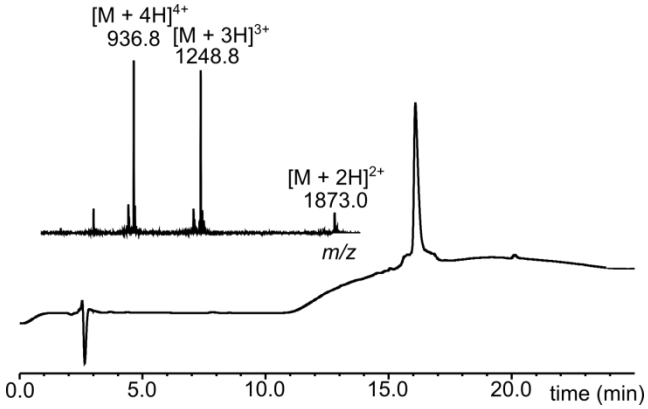

Figure S.1. Crude fragment 1 (Pro^{1}-Gln^{35}-COS-CH_2CH_2-Ala) (**1**); inset: the ESI-MS of the major peak, expected mass $[M + 4H]^{4+}$ 937.08, observed mass 936.8.

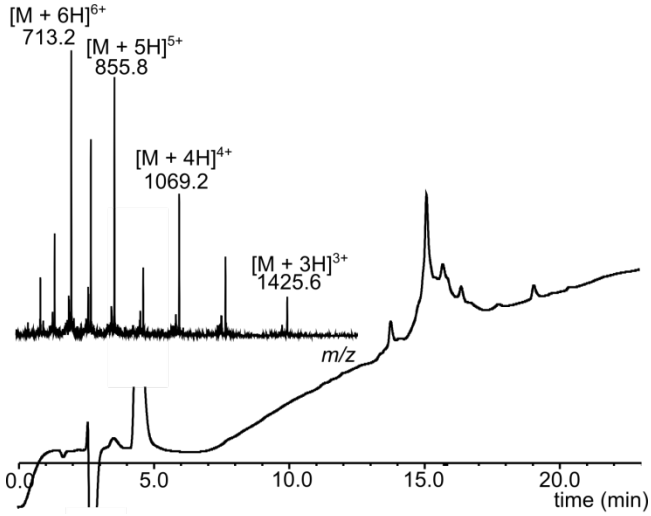

Figure S.2. Crude fragment 2 (Cys^{36}-Thr^{67}-COS-CH_2CH_2-Lys_4) (**2**); inset: the ESI-MS of the major peak, expected mass $[M + 5H]^{5+}$ 856.0, observed mass 855.8.

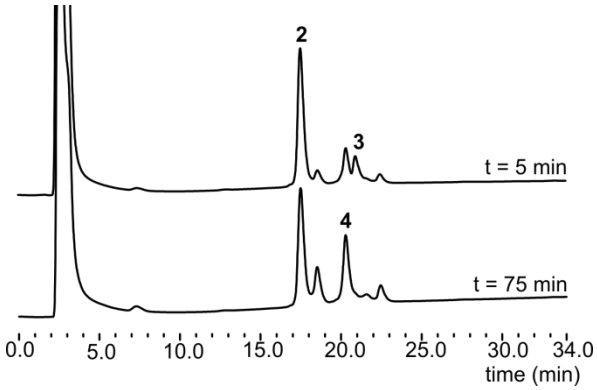

Figure S.3. The KCL reaction between fragment 1 MPAA thioester (Pro^{1}-Gln^{35}-COS-MPAA) (**3**) and fragment 2 Cys^{36}-Thr^{67}-COS-CH_2CH_2-Lys_4), (**2**) to give the N-terminal half of AFPP (Pro^{1}- Thr^{67}-COS-CH_2CH_2-Lys_4) (**4**).

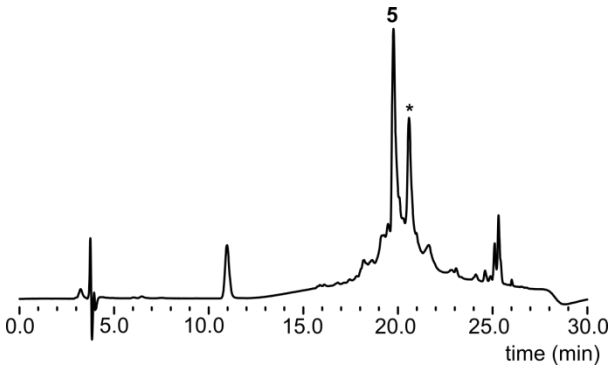

Figure S.4. Crude fragment 3 (Thz^{68}-Asp^{105}-COS-CH_2CH_2-Lys_4) (**5**). (*) denotes hydrolysis product.

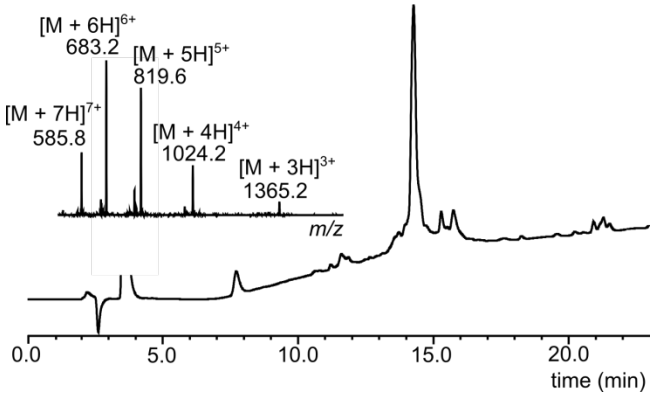

Figure S.5. Crude fragment 3 (Thz^{68}-Met^{97}-COS-CH_2CH_2-Lys$_4$) (**6**); inset: the ESI-MS of the major peak, expected mass [M + 6H]$^{6+}$ 683.30, observed mass 683.2.

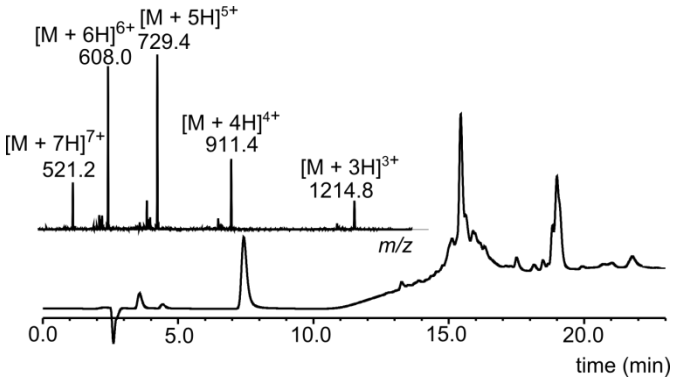

Figure S.6. Crude fragment 3 (Thz^{68}-Ala^{93}-COS-CH_2CH_2-Lys$_5$) (**8**); inset: the ESI-MS of the major peak, expected mass [M + 5H]$^{5+}$ 729.46, observed mass 729.4.

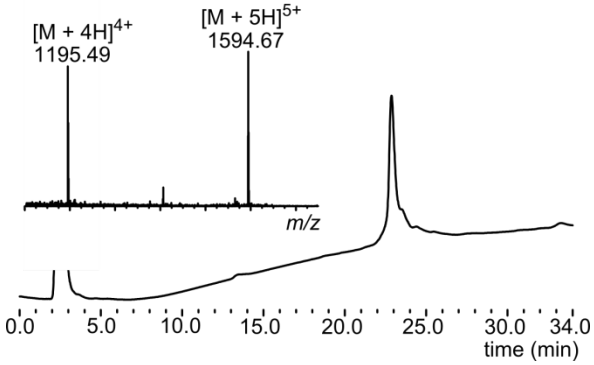

Figure S.7. Purified fragment 4 (Cys^{94}-Gln^{134}COOH) (**9**); inset: the ESI-MS of the major peak, expected mass [M + 5H]$^{5+}$ 1594.86, observed mass 1094.67.

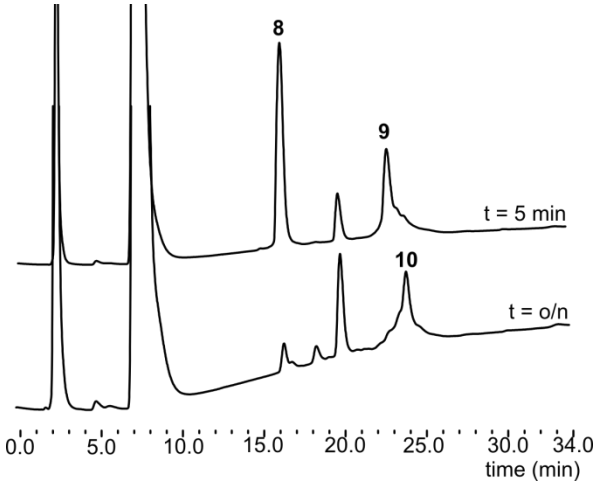

Figure S.8. The NCL reaction between fragment 3 (**8**) and Thz-fragment 4 (**9**) to give the C-terminal half of AFPP (Thz^{68}-Gln^{134}COOH) (**10**).

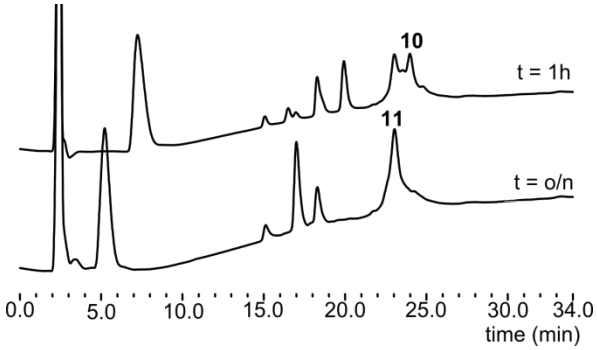

Figure S.9. Conversion of Thz-fragment 3,4-OH (**10**) to Cys-fragment 3,4-OH (**11**).

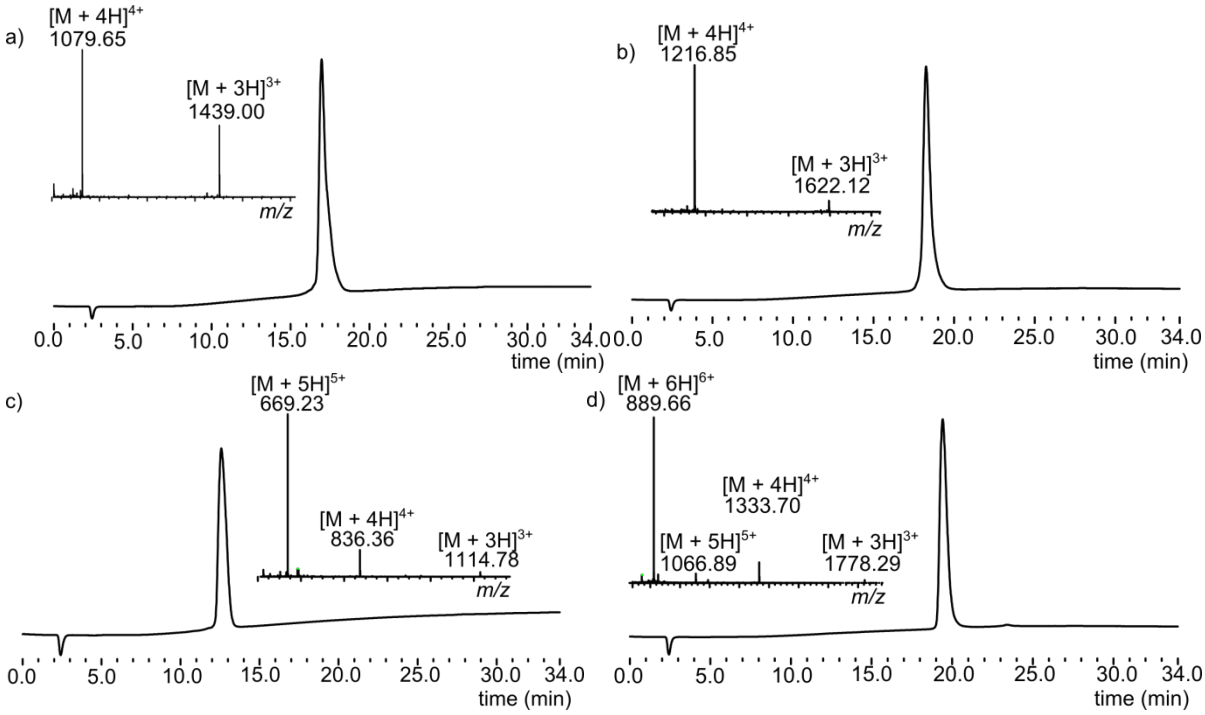

Figure S.10. a) The purified fragment 1 (Pro^{1}-Gln^{35}-COS-CH_2CH_2-Lys$_5$) (**12**); inset: the ESI-MS of the major peak, expected mass $[M + 4H]^{4+}$ 1079.52, observed mass 1079.65. b) Purified fragment 2 (Cys^{36}-Phe^{71}-COS-CH_2CH_2-Lys$_5$) (**13**); inset: the ESI-MS of the major peak, expected mass $[M + 4H]^{4+}$ 1216.90, observed mass 1216.85. c) Purified fragment 3 (Thz^{72}-Ala^{93}-COS-CH_2CH_2-Lys$_5$) (**14**); inset: the ESI-MS of the major peak, expected mass $[M + 5H]^{5+}$ 669.39, observed mass 669.23. d) Purified fragment 4 (Cys^{94}-Phe^{132}-HMBA-Lys$_5$-NH_2) (**15**); inset: the ESI-MS of the major peak, expected mass $[M + 6H]6^{+}$ 889.55, observed mass 889.66.

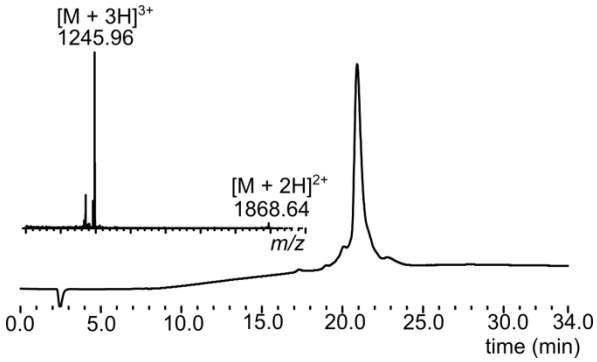

Figure S.11. Purified fragment 1 MPAA thioester (**3**); inset: the ESI-MS of the major peak, expected mass $[M + 3H]^{3+}$ 1246.10, observed mass 1245.96.

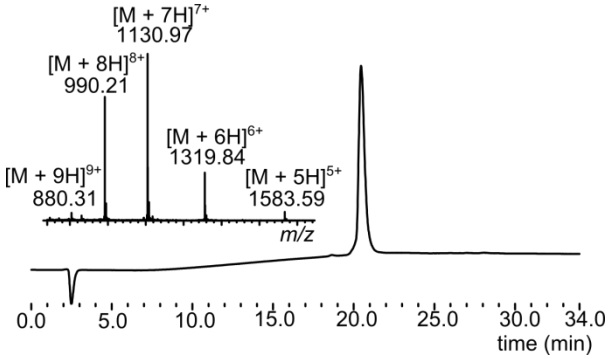

Figure S.12. The purified C-terminal half of AFPP (Cys^{72}-Phe^{132}-HMBA-Lys$_5$-NH_2) (**18**), after unmasking of Thz to Cys; inset: the ESI-MS of the major peak, expected mass $[M + 8H]^{8+}$ 990.28, observed mass 990.21.

References

1. P. W. R. Harris, S. H. Yang and M. A. Brimble, *Tetrahedron Lett.*, 2011, **52**, 6024-6026.
2. P. W. R. Harris, D. J. Lee and M. A. Brimble, *J. Pept. Sci.*, 2012, **18**, 549-555.
3. M. Schnolzer, P. Alewood, A. Jones, D. Alewood and S. B. H. Kent, *Int. J. Pept. Res. Ther.*, 2007, **13**, 31-44.
4. P. W. R. Harris, G. M. Williams, P. Shepherd and M. A. Brimble, *Int. J. Pept. Res. Ther.*, 2008, **14**, 387-392.