Supporting Information for:

Pseudopeptides with a Centrally Positioned Alkene-Based Disulphide Bridge Mimetic Stimulate Kallikrein-Related Peptidase 3 Activity

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1. Peptide synthesis

1.1 General information

L-Allylglycine was Fmoc-protected according to a procedure published previously,[1] and 8-aminooctanoic acid was Fmoc-protected according to the same procedure. All other materials and reagents were acquired from commercial sources and used without further purification. The side-chain protecting groups for the Fmoc-protected amino acids were Trt for His, Boc for Trp, *tert*-Bu for Glu, Thr and Tyr, and Acm for Cys.

1.2 Synthesis of peptides 4-13

Peptides **4-13** were synthesized on Rink Amide AM resin (50 mg, loading 0.575 mmol/g) using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY, USA). Typical procedure for automated peptide synthesis: The Fmoc group was removed using 20% piperidine in DMF twice with 1 min and 5 min deprotection times, followed by washing three times with DMF. The Fmoc-protected amino acids (5.2 eq) were coupled using HBTU (5.2 eq) and DIPEA (7.8 eq) in DMF. Double coupling with 15 min reaction times, both couplings were followed by washing with DMF.

Full-length peptides were cleaved from the resin using 2.0 ml of TFA:H_2O:EDT:TIS 94:2.5:2.5:1. The peptides were isolated from the cleavage solution by precipitation with cold Et_2O followed by centrifugation and removal of the supernatant. The precipitates were washed once with cold Et_2O applying the same procedure. The precipitates were dissolved in 0-50% AcOH in water (depending on solubility) followed by lyophilisation. The peptides were then dissolved in 0-50% AcOH in water and purified by HPLC followed by lyophilisation.

The peptides were dissolved in 50% aqueous AcOH at a concentration of 2 mg/ml, and 1 M aqueous HCl (0.1 ml/mg of peptide) and 0.1 M iodine solution in 50% aqueous AcOH (5 eq/Acm group) were added. The solution was stirred vigorously at room temperature for 40-120 min. The reaction was stopped with 0.1 M sodium thiosulphate. After filtering off (0.45 μm) the peptides, they were immediately purified with HPLC and lyophilized.

1.3 Synthesis of pseudopeptides 24-26, 28a-b and 29

Pseudopeptides **24-26**, **28a-b** and **29** were synthesized on 0.2 mmol scale on Fmoc-protected preloaded Wang resin (Cys(Acm), loading 0.60 mmol/g, or Trp(Boc), loading 0.48 mmol/g) or Rink Amide resin (loading 0.63 mmol/g) in a 10 ml plastic syringe fitted with a filter. Prior to the synthesis of the peptides the resins were swelled in DMF for 1-2 h. The Fmoc-group was removed by shaking the resin 15 min with 5 ml 20 % (v/v) piperidine in DMF. After deprotection the resin was washed five times with 3 ml DMF, the mixture was shaken for one minute per wash-cycle. The Fmoc-protected amino acid (4 eq), TBTU (4 eq) and DIPEA (10 eq) were dissolved in 2 ml DMF, and the solution was allowed to react for one minute. After activation the solution was taken into the syringe and shaken for 45 minutes. After the coupling the resin was washed five times with 3 ml DMF. A new cycle in the peptide synthesis was started by removing the Fmoc protecting group according to the described method above. When the peptide synthesis was completed, the Fmoc protecting group was also removed in the end according to method above. When the peptide synthesis was completed or if the peptide synthesis had to be interrupted, the resin was washed five times with 3 ml DMF, five times with 3 ml DCM and finally twice with 3 ml MeOH, where after the resin was dried in vacuum overnight.

Pseudopeptides **24-26**, **28a** and **29** (on Wang resin) were cleaved using 5 ml Reagent K (TFA:H_2O:phenol:thioanisol:EDT 82.5:5:5:5:2.5) and turning the syringe occasionally for 90 min. The syringe was emptied and washed twice with 1 ml TFA. The pseudopeptides were isolated from the cleavage solution by precipitation with cold Et_2O followed by centrifugation and removal of the supernatant. The crude peptides were washed three times with cold Et_2O by resuspending the peptide in the solvent, centrifuging and removing the supernatant, and finally dried in vacuum overnight. The cleavage of psudopeptide **28b** (on Rink Amide resin) was performed in the same way as above but TFA:H_2O:EDT:TIS 94:2.5:2.5:1 was used as the cleavage solution instead of Reagent K.

The pseudopeptides were dissolved in anhydrous AcOH at a concentration of 11 mg/ml, and 0.06 M aqueous HCl (0.034 ml/mg of peptide) and 0.13 M iodine solution in anhydrous AcOH (10 eq/Acm group) were added. The solution was stirred vigorously at room temperature for 70 min. The peptides were isolated by precipitation with cold Et_2O followed by centrifugation and removal of the supernatant.

The crude peptides were washed three times with cold Et_2O by resuspending the peptide in the solvent, centrifuging and removing the supernatant, and finally dried in vacuum overnight.

2. Ring-closing metathesis reaction

The ring-closing metathesis (RCM) reactions for **21-23** were performed on Fmoc-protected peptides on-resin. Approximately 110-130 mg resin bound peptide (corresponding to 0.05 mmol peptide) and 12 mg (0.02 mmol) Hoveyda-Grubbs 2nd generation catalyst were placed in a microwave vial, followed by the addition of 2.0 ml anhydrous 1,2-dichloroethane and 0.2 ml 0.4 M LiCl in anhydrous DMF (0.08 mmol). The reaction was heated in a microwave cavity at 100 °C for 60 min. The resin was transferred to a plastic syringe fitted with a filter and washed with DCM, DMF, DMF:DMSO 1:1 (overnight to remove catalyst), DMF, five times with DCM and twice with MeOH. The resin was dried in vacuum overnight. All other successful RCM-reactions were performed according to the same method.

3. Peptide/pseudopeptide purification and analysis

Peptides **4-13** and pseudopeptides **24-26**, **28a-b** and **29** were purified by preparative HPLC (Shimadzu, Kyoto, Japan) with a C18 column using 0.1% TFA in water with a 0-90% MeCN gradient for 60 min. The identity was verified by an ABI QSTAR XL hybrid mass spectrometer using the Ionspray interface (Applied Biosystems, Foster City, CA) operating positive ionization mode. Intermediate **27** (the cleaved RCM product of **22**) was purified on a Waters preparative HPLC system, using W 2996 PDA photodiode array detector and Waters micromass ZQ single quadrupole for detection with a C18 column (250 × 10 mm) using 0.1 % formic acid in water with a 0-90 % MeCN:formic acid 99.5:0.5 gradient for 45 min.

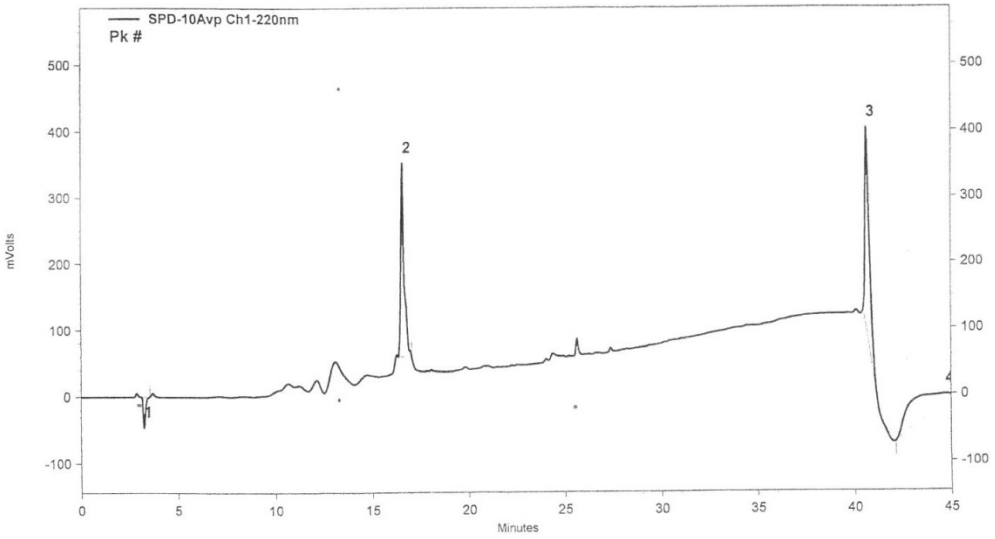
The purity was determined on an analytical HPLC with 240 × 1.4 mm C18 column eluted with 0-90% MeCN for 42 min (A) or 150 × 4.6 mm C18 column eluted with 0-90% MeCN for 26 min (B).

Table S1. Mass spectrometry for peptides **4-13** and pseudopeptides **26-24**, **28a-b** and **29**.

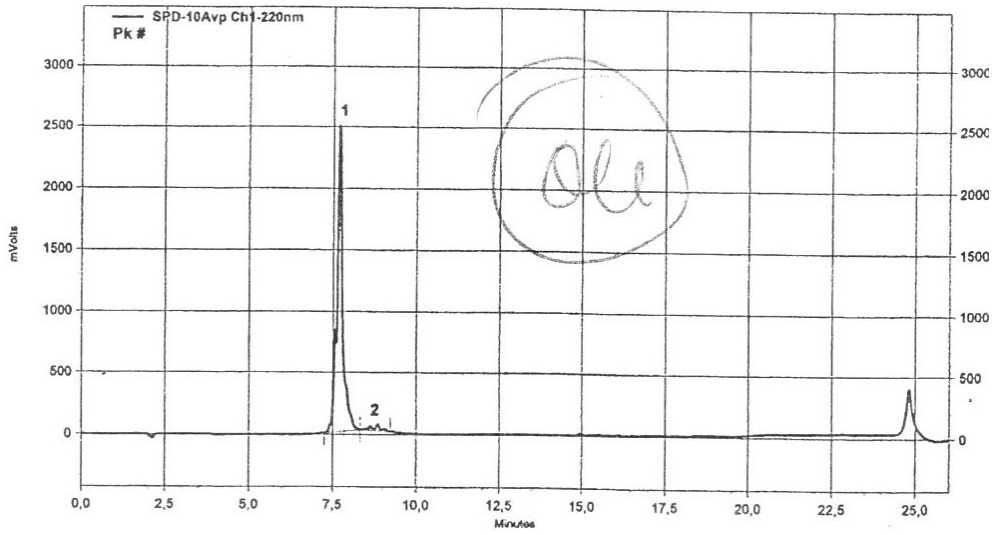
Peptide/ Pseudopeptide	$[M+2H]^{2+}$ calculated	$[M+2H]^{2+}$ observed	Retention time (min)	HPLC Method
4	1430.5*, 715.76	1430.38*, 715.69	16.58	A
5	719.76	719.78	7.69	B
6	719.76	719.79	9.58	B
7	1372.5*, 686.75	1372.37*, 686.69	17.12	A
8	727.74	727.68	14.63	A
9	752.76	752.78	7.39	B
10	798.77	798.84	7.43	B
11	781.78	781.71	14.91	A
12	760.75	760.78	13.09	A
13	753.75	753.77	12.82	A
24	1401.61*, 701.31	1401.57*, 701.28	9.43	B
25	763.32	763.31	9.06	B
26	1559.62*, 780.32	1559.57*, 780.29	9.28	B
28a	1092.49*	1092.50*	9.57	B
28b	1091.51*	1091.39*	9.53	B
29	984.43*	984.45*	10.35	B

*$[M+H]^{+}$ was observed

3.1 HPLC-traces of peptides 4-13 and pseudopeptides 24-26, 28a-b and 29



HPLC trace for peptide **4**



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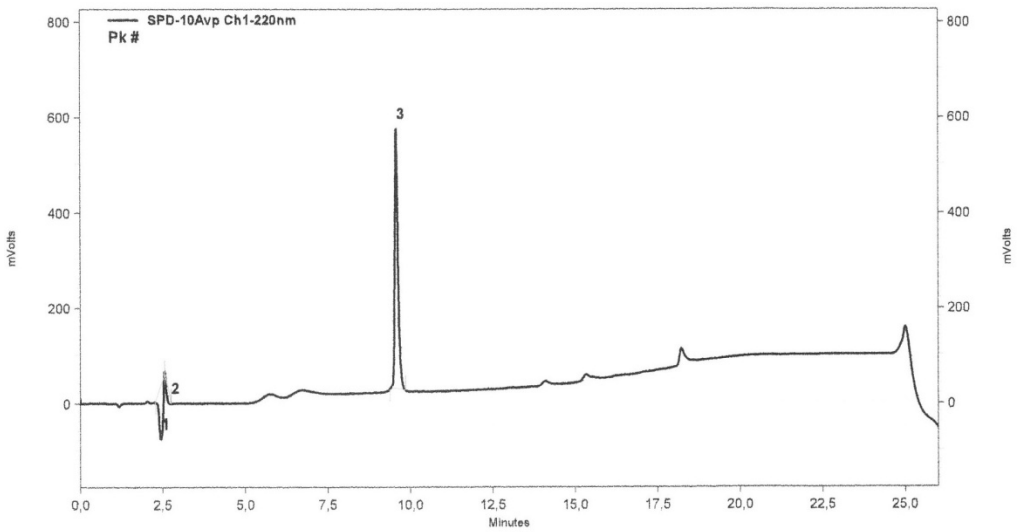

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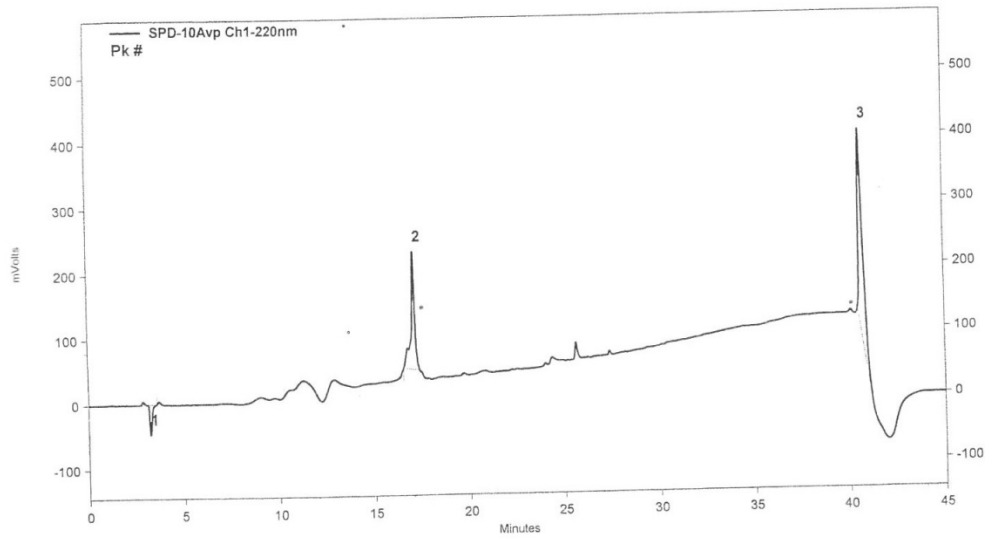

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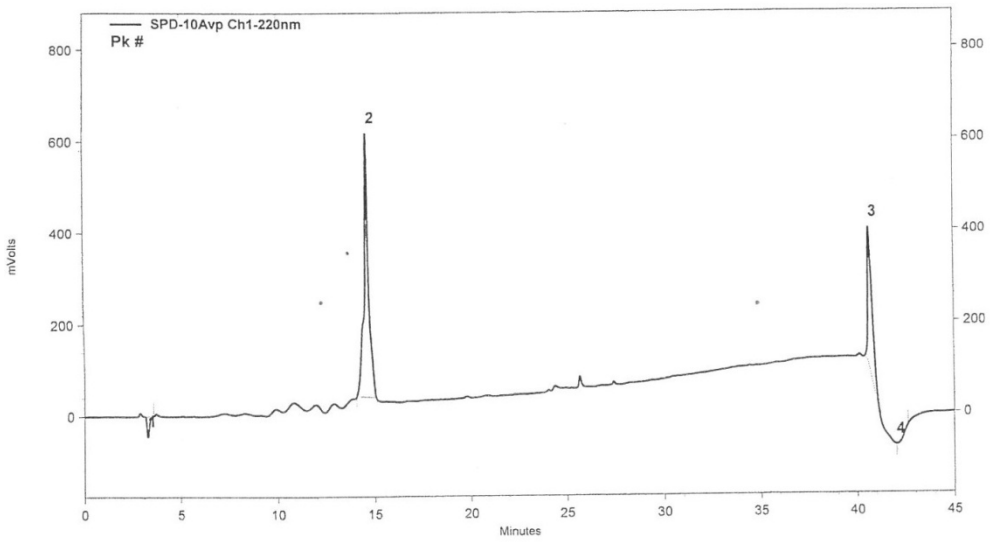

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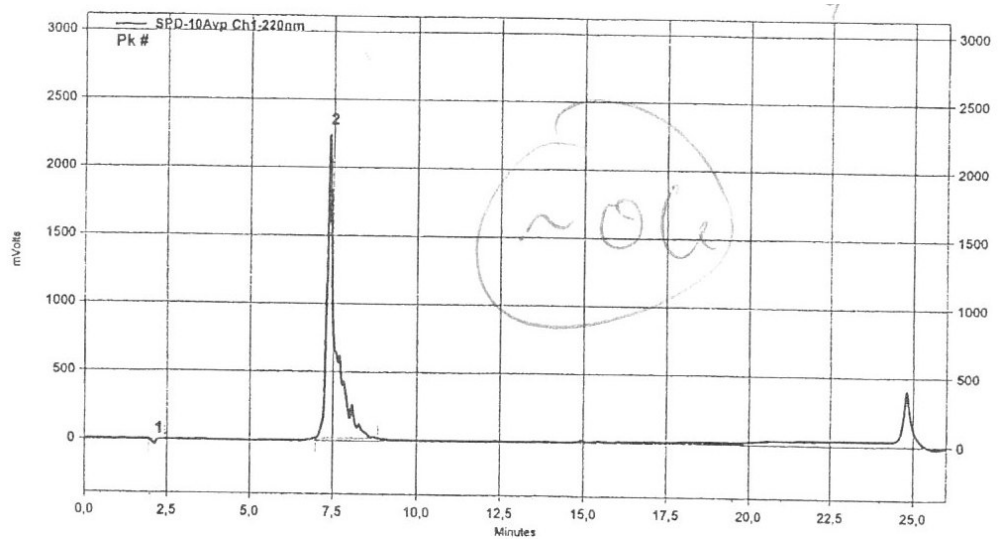
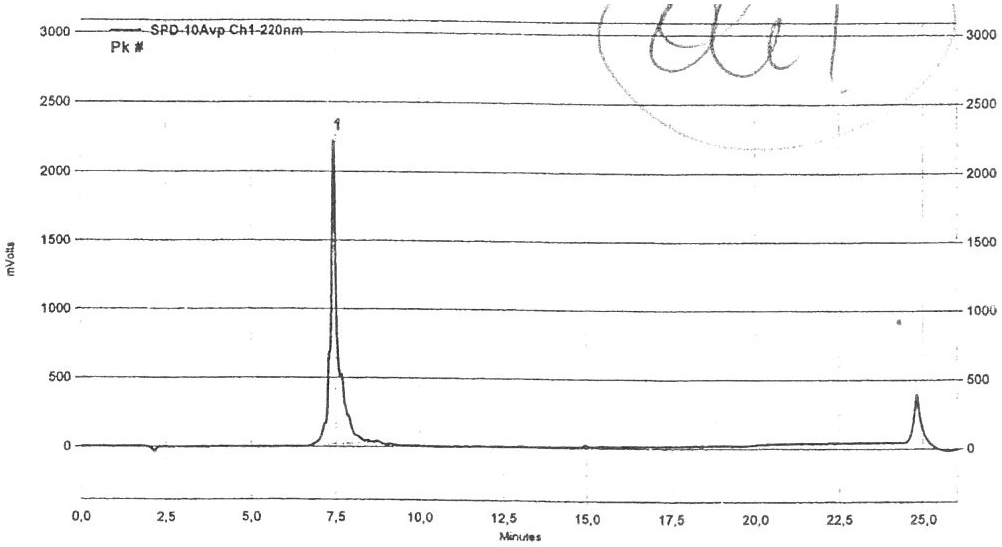


HPLC trace for peptide **9**



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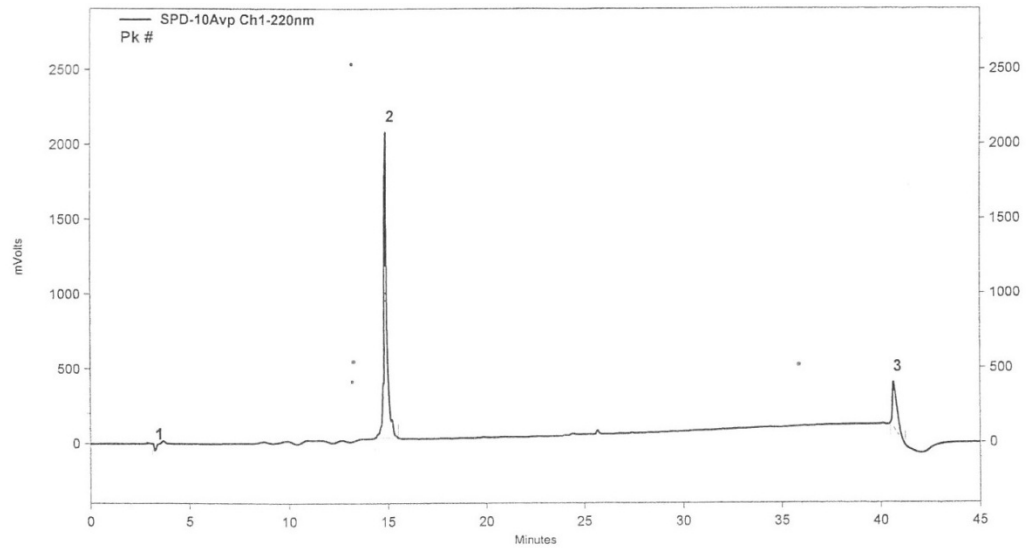

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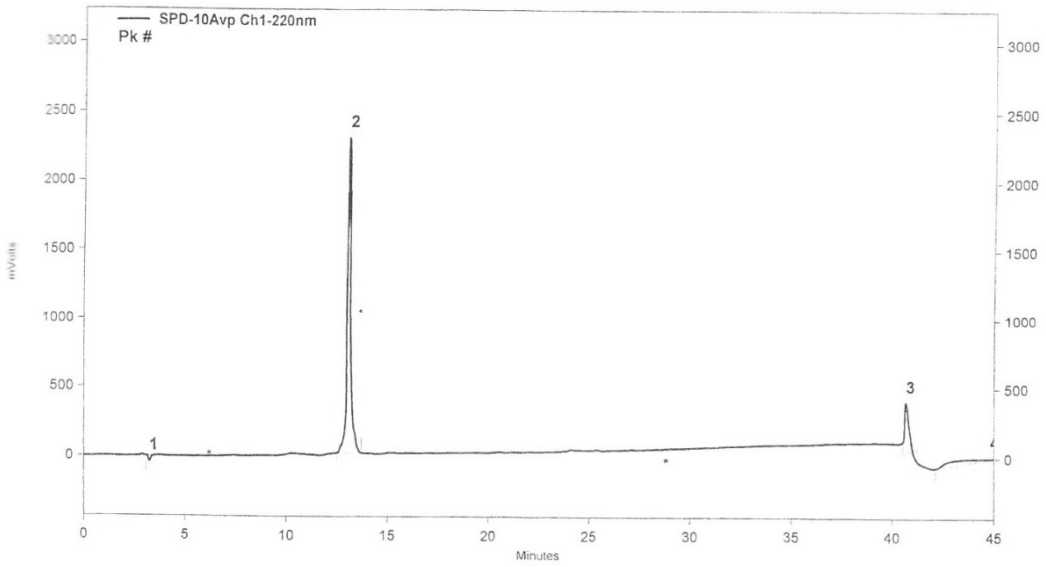
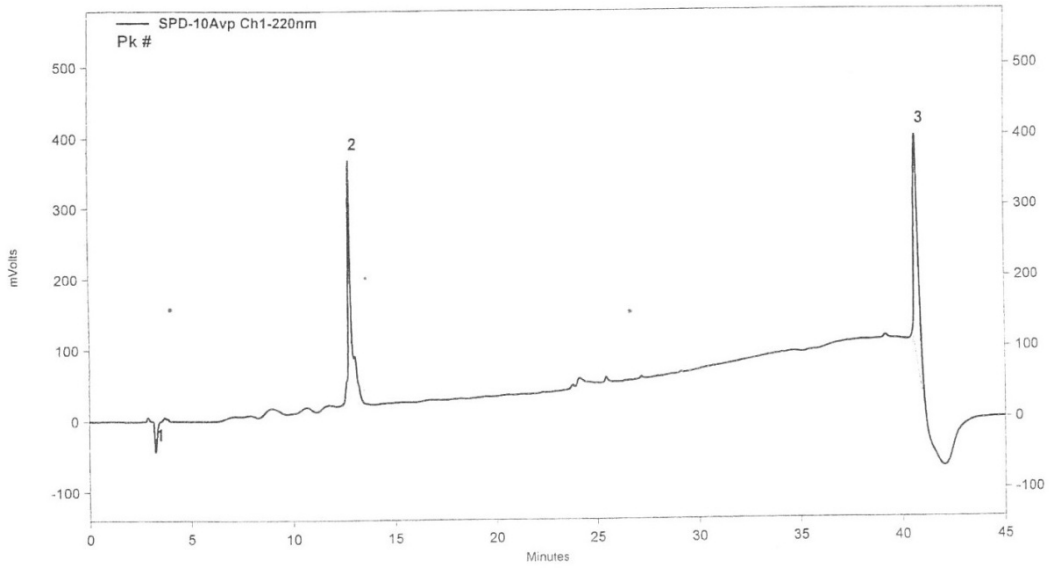
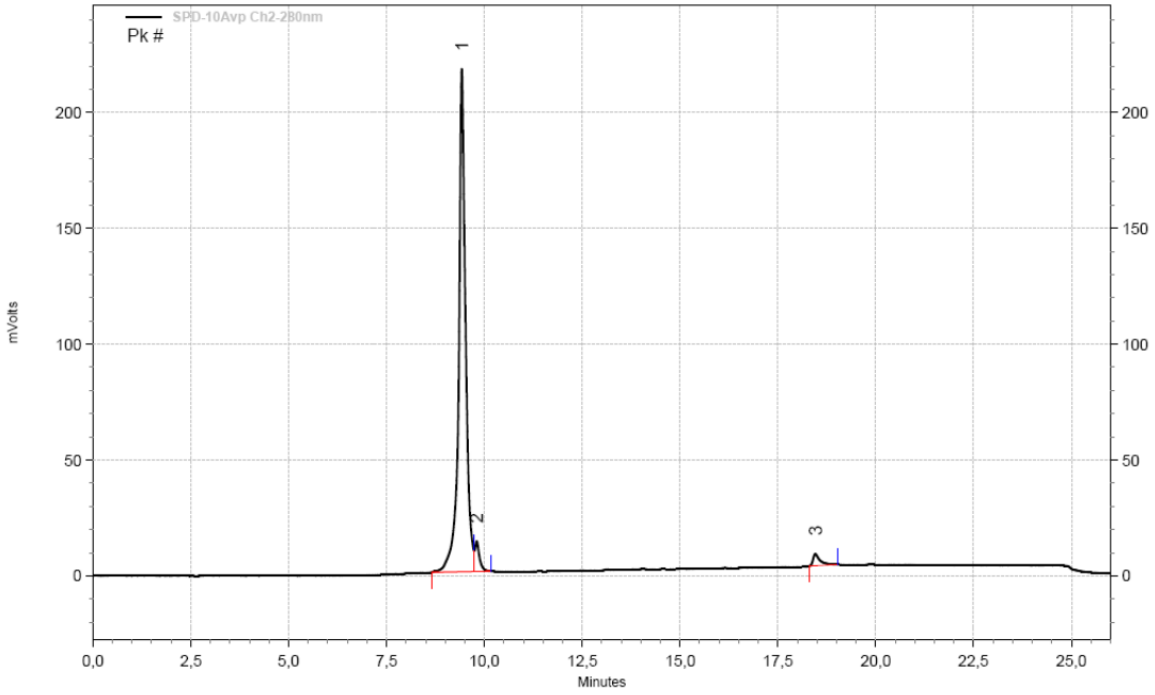


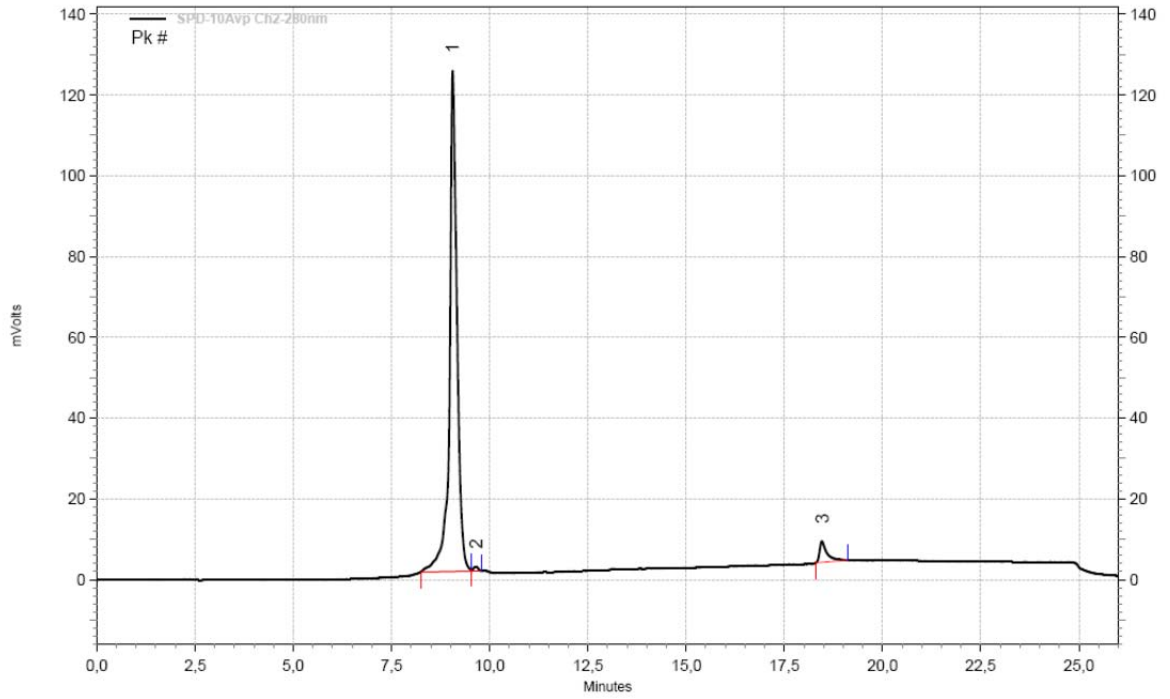
HPLC trace for peptide **12**



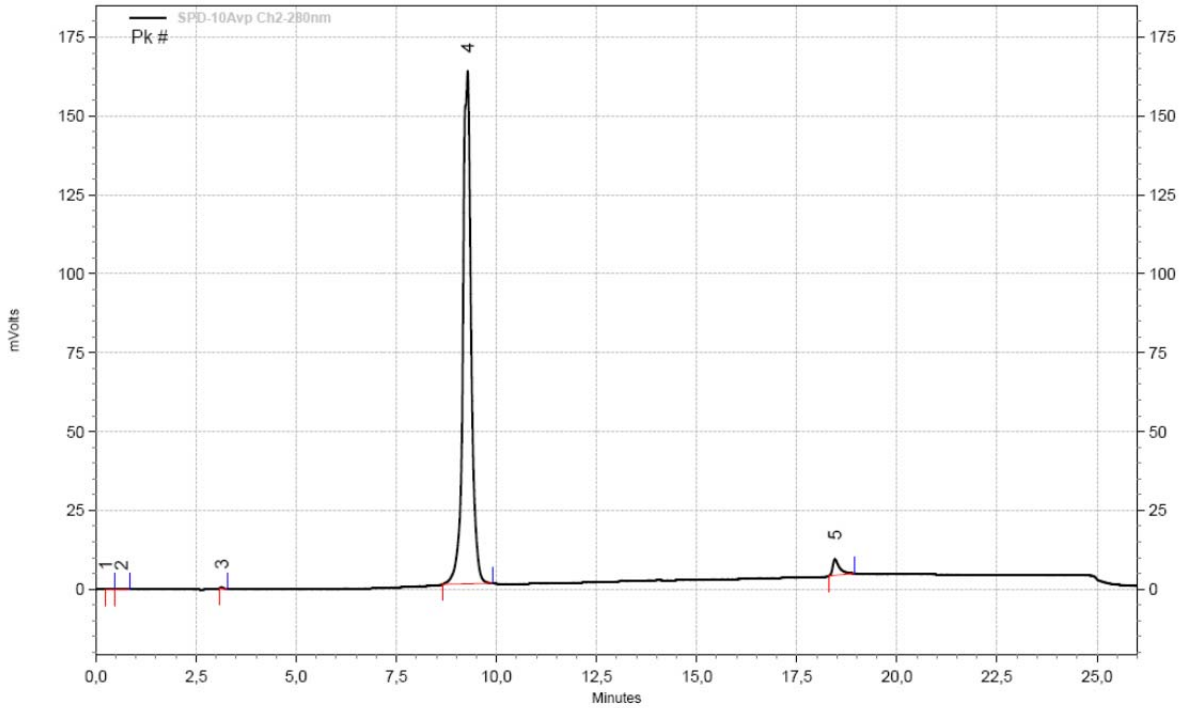
HPLC trace for peptide **13**



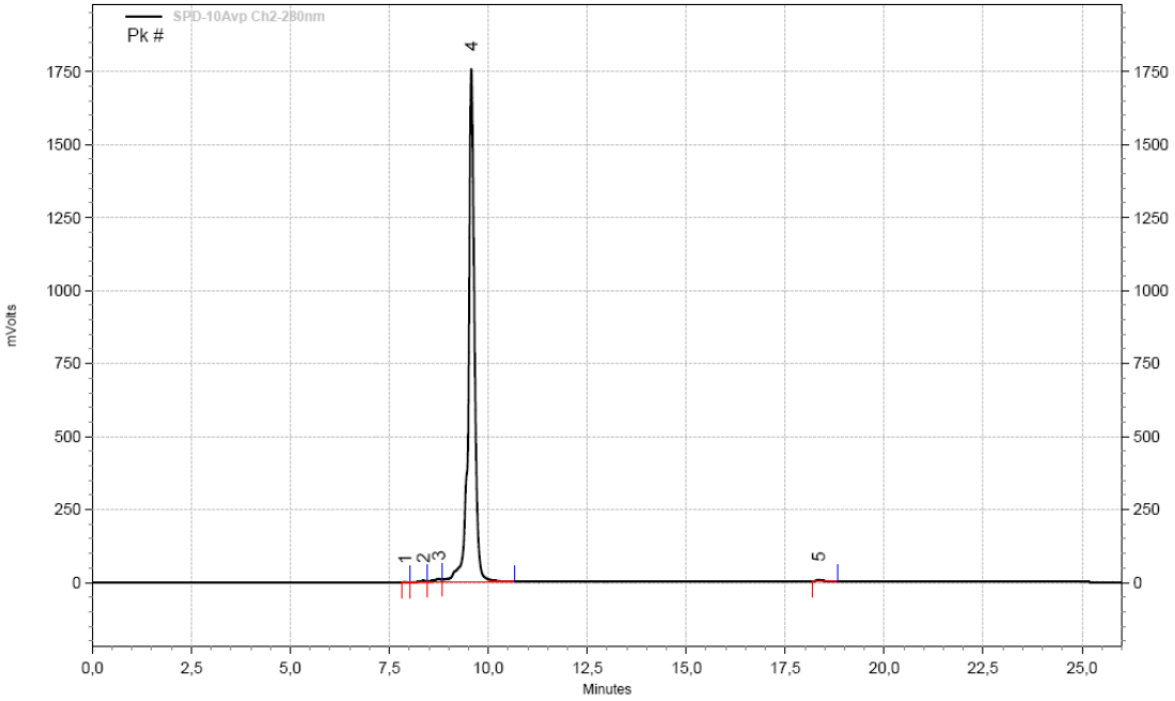
HPLC trace for pseudopeptide **24**



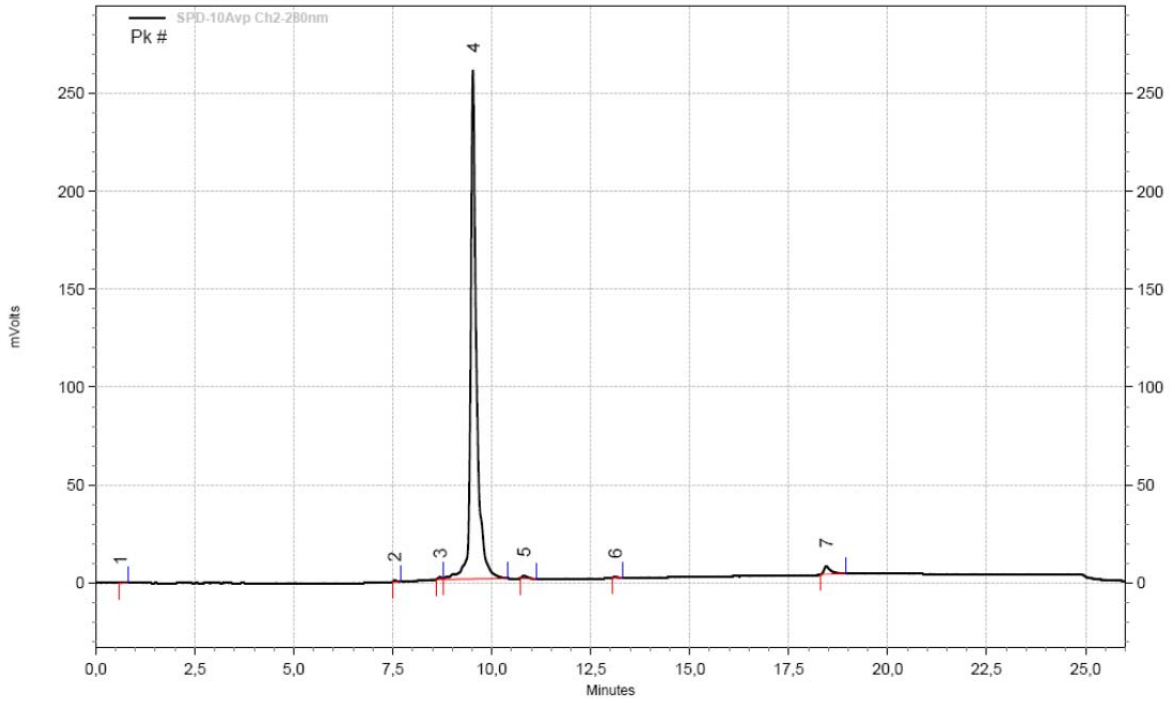
HPLC trace for pseudopeptide **25**



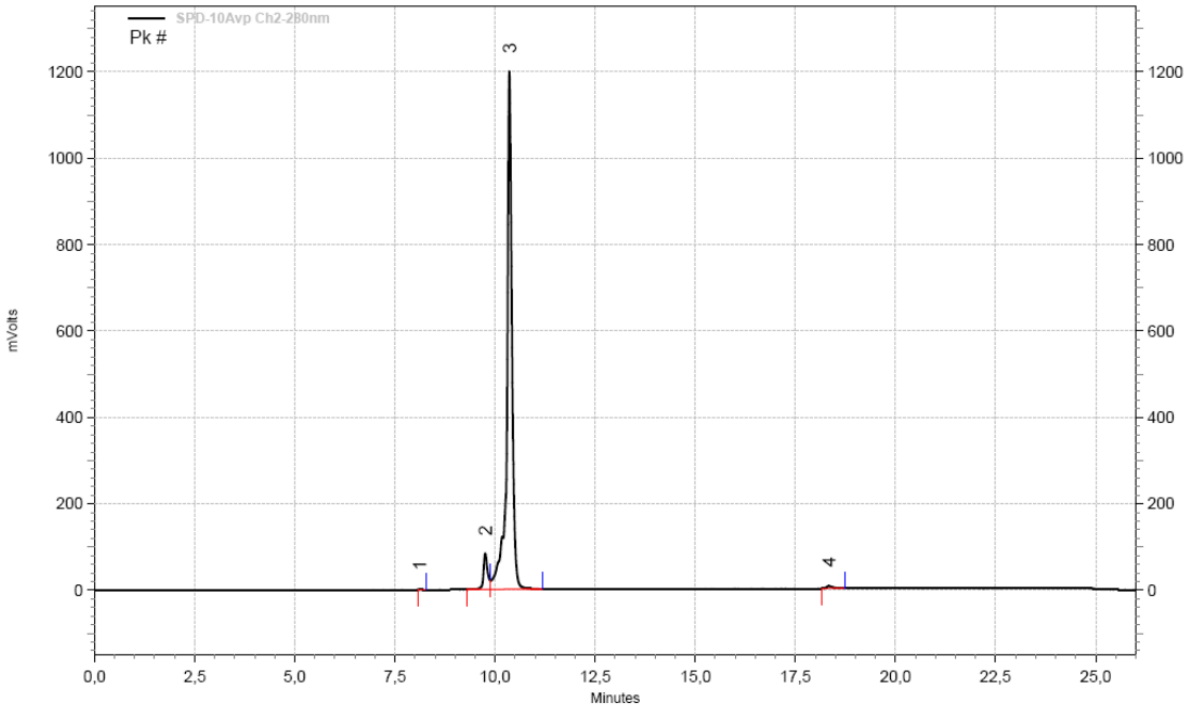
HPLC trace for pseudopeptide **26**



HPLC trace for pseudopeptide **28a**



HPLC trace for pseudopeptide **28b**



HPLC trace for pseudopeptide **29**

4. NMR analysis

Proton NMR spectra of pseudopeptides **27** (the cleaved RCM product of **22**) and **26** were recorded on a Bruker AVANCE DRX spectrometer operating at 500.13 MHz. Spectra of **27** were recorded in a solvent mixture CD_3OD:$CDCl_3$ 1:2 at 300, 310 and 320 K. Spectra of **26** were recorded in CD_3OD at 300 and 310 K. The *E*/*Z*-ratio of the peptides was determined using the method reported by Bhattacharia *et al.*[2]

5. *In vitro* assay for KLK3 activity

The effect of different peptides and pseudopeptides on the enzymatic activity of KLK3 was measured using a chromogenic chymotrypsin substrate (MeO-Suc-Arg-Pro-Tyr-pNA, Peptides International). The peptides and pseudopeptides (0.2-20 µg/ml) were preincubated with KLK3 (10 µg/ml) for 30 min at room temperature in Tris-buffer, pH 7.7, containing 0.154 M NaCl, 8 mM NaN_3 and 0.1 % bovine serum albumin, before the addition of the substrate to a final concentration of 200 µM. The absorbance at 405 nm was measured at 5 min intervals for 30 min with a Victor 1420 Multilabel fluorometer (Perkin-Elmer-Wallac, Turku, Finland).

6. References

1. X. Gu, J. Ying, R. S. Agnes, E. Navratilova, P. Davis, G. Stahl, F. Porreca, H. I. Yamamura, V.J. Hruby, *Org. Lett.*, 2004, **19**, 3285-3288.

2. S. Bhattacharya, H. Zhang, D. Cowburn, A. K. Debnath, *Biopolymers*, 2012, **97**, 253-264.