Rapid Microwave-Assisted CNBr Cleavage of Bead-Bound Peptides

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Supporting materials

Experimental Methods

S-Fig. 1. Edman degradation sequencing of randomly selected beads from a 5-mer bead-based peptide library. The peptides were a) LVGRT, b) FRKIP, c) RDHWS and d) REKEY (not including methionine), and the results illustratec the purity of the library.

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Experimental Methods

General. N-methylpyrrolidone (NMP), diethylether and dichloromethane (DCM) were purchased from Merck. Fmoc-protected amino acids (Fmoc-AA's), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N,N-diisopropylethylamine (DIEA) were purchased from GL Biochem (Shanghai) Ltd. Trifluoroacetic acid (TFA) and triisopropylsilane (TIS) were purchased from Aldrich. α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Bruker. MALDI-MS were obtained with Bruker Autoflex II TOF/TOF. The microwave-assisted CNBr cleavage reaction was done with a CEM Discovery microwave reactor or a household microwave oven (Model: R-248J, 800 W, 2450 MHz) from Sharp Inc. The quantification experiments were done with Waters UPLC/MS (ACQUITY^{TM}, TUV detector, SQ detector). Edman degradation sequence analysis was performed by a Procise cLC 492 protein sequencer.

Synthesis of peptides with known sequence (5mers to 8 mers): Peptides with known sequence was synthesized in a parallel fashion using an automated synthesizer Titan 357 (AAPPTEC). TentaGel S Amino beads (100 mg, 90 μm diameter, the loading of NH_2: 0.24 mmol/g) were swelled in NMP (2 ml) for 120 min in each Reaction Vessel (RV). After draining the solvent, Fmoc-methionine (Fmoc-Met) (3 equiv, 0.1 M solution in NMP) was added to each RV, as well as TBTU (3 equiv, 0.2 M solution in NMP) and DIEA (7.5 equiv, 0.5 M in NMP). Each RV was vortexed for 30 min. After draining the solution, the coupling step was repeated. The resulting beads in each RV were thoroughly washed by NMP (2 ml × 4). Next, 20% piperidine in NMP (2 ml) was added and each RV was vortexed for 15 min. The liquid was drained and a fresh solution of 20% piperidine in NMP (2 ml) was added and each RV was vortexed for another 30 min. The resulting beads in each RV were thoroughly washed by NMP (2 ml x 4) and DCM (2 ml × 4). A fmoc-protected amino acid (3 equiv), TBTU (3 equiv) and DIEA (7.5 equiv) were added to each RV. Each RV was then vortexed for 30 min. After draining the solution, the coupling step was repeated. The resulting beads in each RV were washed by NMP (2 ml × 4). 20% piperidine in NMP (2 ml × 4) was added to each RV, which was vortexed for 15 min. The liquid was drained and a fresh solution of 20% Piperidine in NMP (2 ml) was added with vortexing for another 30 min. The beads in each RV were thoroughly washed by NMP (2 ml × 4) and DCM (2 ml × 4). The above coupling and deprotection steps were repeated until the beads in each RV contained peptides with a designated sequence, including the initial

methionine. The beads in each RV were transferred to an 8 ml reactor with a filter respectively. The protective groups in the residues were removed by shaking in TFA-water-TIS (2 ml, 94:3:3, v/v) for 2 h. The solvent was drained and the resulting beads were thoroughly washed by DCM (3 ml × 3), methanol (3 ml × 3), water (3 ml × 3), methanol (3 ml × 3), DCM (3 ml × 3), and diethylether (3 ml × 1), successively, and then dried under reduced pressure for 24 h. The purity of peptides was demonstrated by HPLC after the cleavage of peptides from beads [HPLC analysis conditions: C18 column (2.1 mm × 100 mm, 1.7 μm particle), A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile; Gradient: 85% A in 0.1 min, 85% – 30% in 5.5 min; 0.3 ml/min, UV detector: 214 nm, 30^{o}C]. All chromatographic peaks from UV detector (214 nm) were identified by a MS which was connected to the LC (see S-Fig. 10 and S-Fig. 11).

Synthesis of peptide library: The synthesis of the peptide library was performed by using an automatic synthesizer Titan 357 (AAPPTEC). TentaGel S Amino beads (1.8 g, 90 μm diameter, the loading of NH_2: 0.24 mmol/g) were swelled in NMP (27 ml) for 120 min in the Collective Vessel (CV). After draining the solvent, Fmoc-methionine (Fmoc-Met) (3 equiv, 0.1 M solution in NMP) was added to the CV, as well as TBTU (3 equiv, 0.2 M solution in NMP) and DIEA (7.5 equiv, 0.5 M in NMP). The CV was vortexed for 30 min. After draining the solution, the coupling step was repeated. The resulting beads were thoroughly washed by NMP (27 ml × 4). Next, 20% piperidine in NMP (27 ml) was added and the CV was vortexed for 15 min. The liquid was drained and a fresh solution of 20% piperidine in NMP (27 ml) was added and the CV was vortexed for another 30 min. The resulting beads were thoroughly washed by NMP (27 ml x 4) and DCM (27 ml × 4), followed by distribution equally into 18 Reaction Vessels (RV). One of the amino acid diversity elements (3 equiv), TBTU (3 equiv) and DIEA (7.5 equiv) were added to each RV. The RV was then vortexed for 30 min. After draining the solution, the coupling step was repeated. The resulting beads in each RV were washed by NMP (2 ml × 4). Again, 20% piperidine in NMP (2 ml) was added to each RV, which was vortexed for 15 min. The liquid was drained and a fresh solution of 20% Piperidine in NMP (2 ml) was added with vortexing for another 30 min. Beads in each RV were thoroughly washed by NMP (2 ml × 4) and DCM (2 ml × 4), which were combined into the CV. The overall split, coupling, deprotection, and mix processes were repeated until the beads appended a hexamer, including the initial methionine. The beads were transferred in a 50 ml reactor, equipped with a filter. The protective groups in the residues were removed by shaking in TFA-water-TIS (27 ml, 94:3:3,

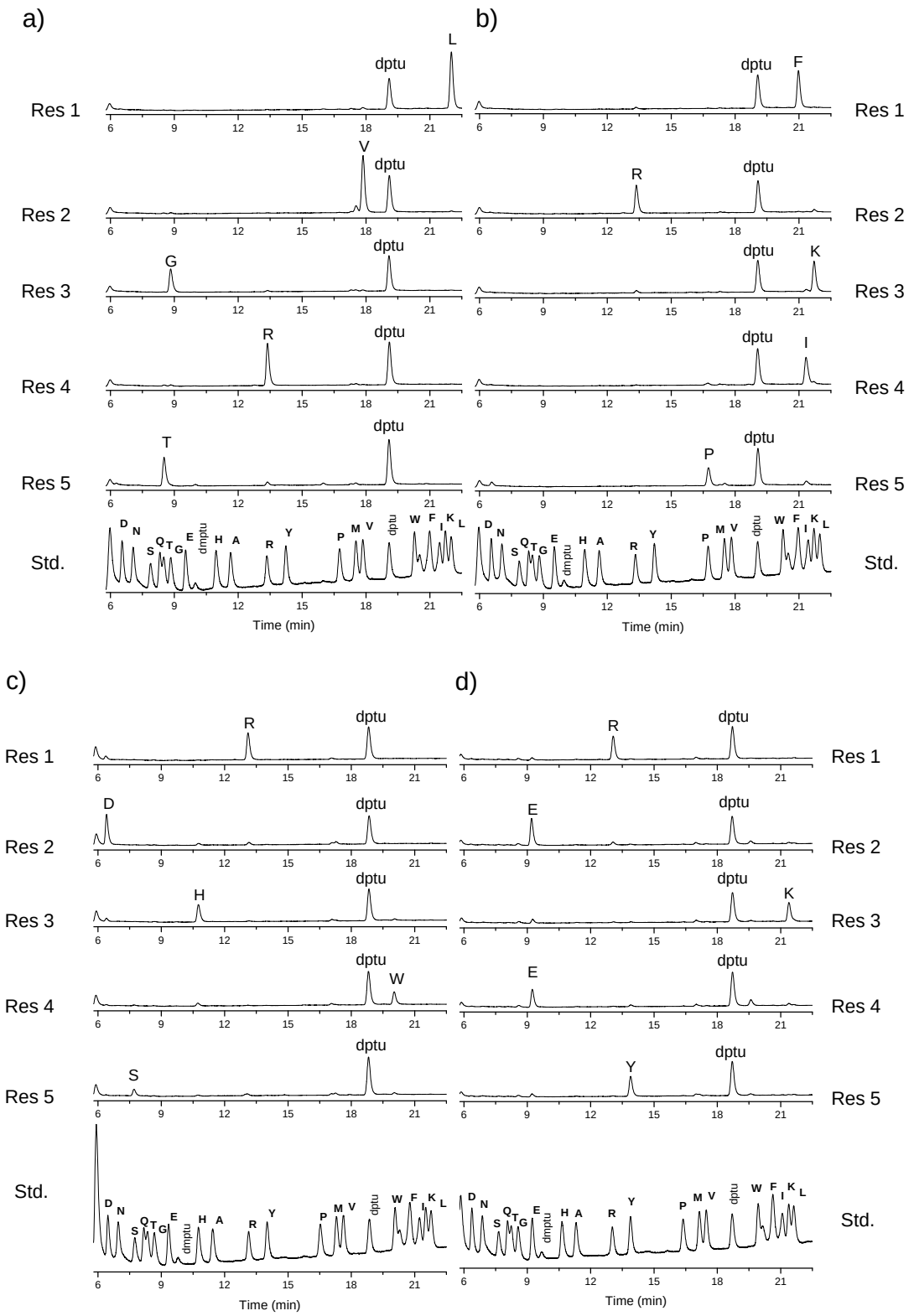
v/v) for 2 h. The solvent was drained and the resulting beads were thoroughly washed by DCM (27 ml × 3), methanol (27 ml × 3), water (27 ml × 3), methanol (27 ml × 3), DCM (27 ml × 3), and diethylether (27 ml × 1), successively, and then dried under reduced pressure for 24 h. The quality of a peptide library was demonstrated by Edman degradation of randomly selected beads (see S-Fig. 1).

CNBr cleavage of peptide from a single bead. A single bead was transferred to a micro-sized vial, containing pure water (10 µl). After addition of CNBr (10 µl, 0.50 M in 0.2 N HCl solution) the reaction vessel was purged with argon and heated to 45^{o}C for 2 – 4 h, or placed in a microwave oven for 1, 2, or 4 min. The resulting solution was dried under centrifugal vacuum for 10 min at 45^{o}C and then for 50 min at 60^{o}C. The temperature during the microwave irradiation was measured, for a few of the single-bead cleavage steps, by an optical fiber thermometer. The thermometer was inserted into the reaction vessel and the vessel was sealed prior to microwave irradation.

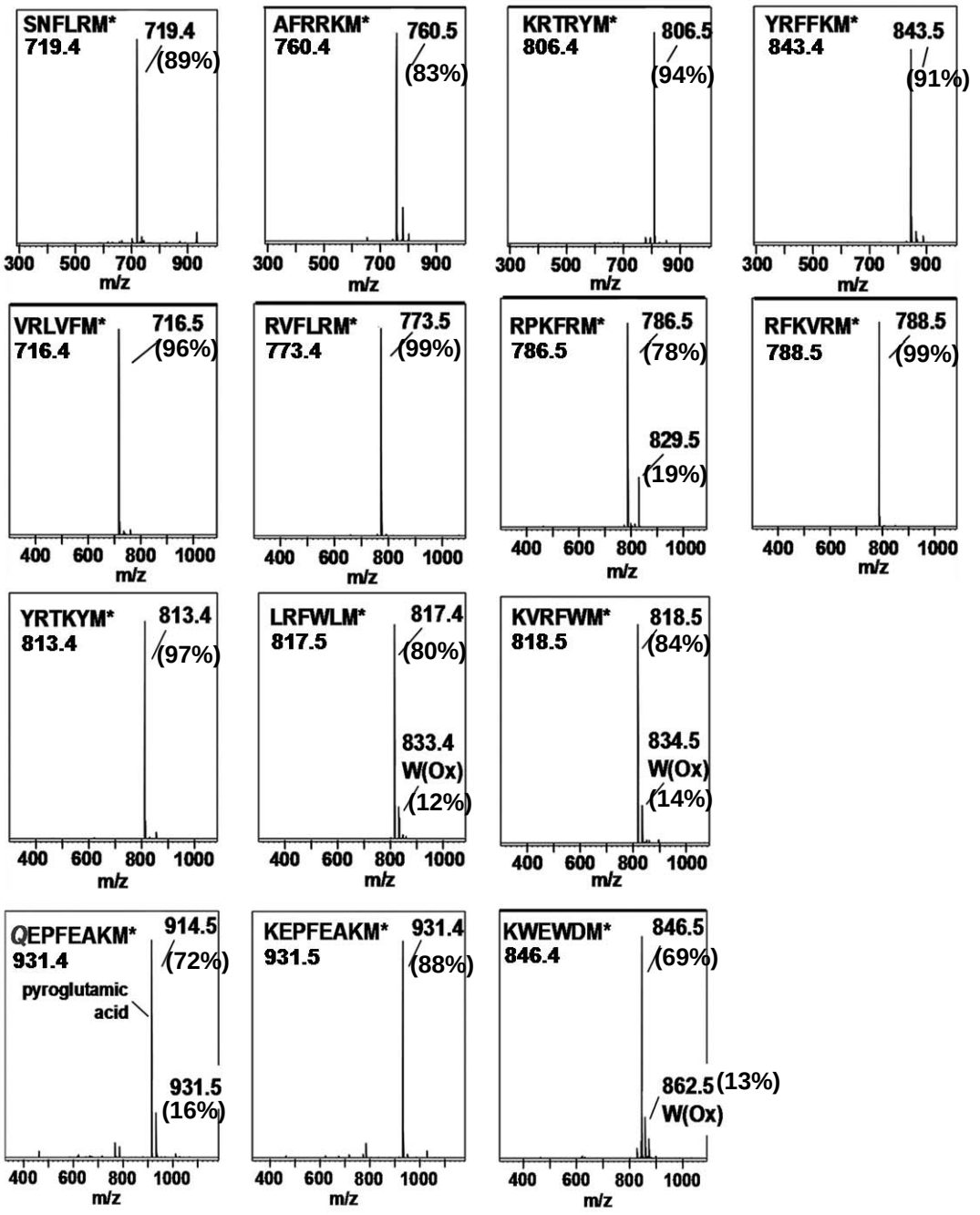
Quantification of cleaved peptides. To a protected peptide of H_2N-HLYFLRM-resin (100 mg, loading: ca. 0.24 mmol/g) in an 8-ml Alltech reactor was added NMP (2 ml), which was appended on 180^{o} shaker for 120 min. Dansyl chloride (3 eq.) and DIEA (7.5 eq.) were added to allow 1 hr on the shaker at dark. The liquid was drained and another portions of Dansyl chloride (3 eq.) and DIEA (7.5 eq.) in NMP (2 ml) were provided for complete coupling. After 1 hr the liquid was drained and the resulting resin was thoroughly washed with NMP (2 ml x 4), DCM (2 ml x 4), methanol (2 ml x 4), water (2 ml x 4), methanol (2 ml x 4), DCM (2 ml x 4), and diethylether (2 ml x 4), followed by drying under reduced pressure. The side-chain protection was removed by incubation with TFA/water/TIS (94/3/3, /v/v/v) at room temperature for 2 h. Finally, the resin was washed serially with DCM, methanol, water, methanol, DCM, and diethylether. The resin was dried in vacuo and stored at 4 ^{o}C after purging with argon. 320 µg of the dansyl-modified beads were weighed in a 200 µl microtube. The beads were swelled for 1 hr in 30 µl of water and then 30 µl of 0.5M CNBr in 0.2 N aq. HCl were added. The microtube was sealed and then placed under microwave irradiation. Upon completion, the microtube was centrifuged at high speed to precipitate the beads. 30 µl of supernatant over the precipitated beads was then collected and dried using a centrifugal concentrator. The obtained products were dissolved in acetonitrile/water (100 µl, 50:50) for quantification by HPLC [HPLC analysis conditions: C18 column (2.1 mm × 100 mm, 1.7 µm particle), A = 0.1% TFA in water, B = 0.1%

TFA in acetonitrile; Gradient: 75% A in 0.1 min, 75% - 30% in 5.5 min; 0.3 ml/min, UV detector: 214 nm, 30^{o}C]. All chromatographic peaks from UV detector (214 nm) were identified by a MS which was connected to the LC.

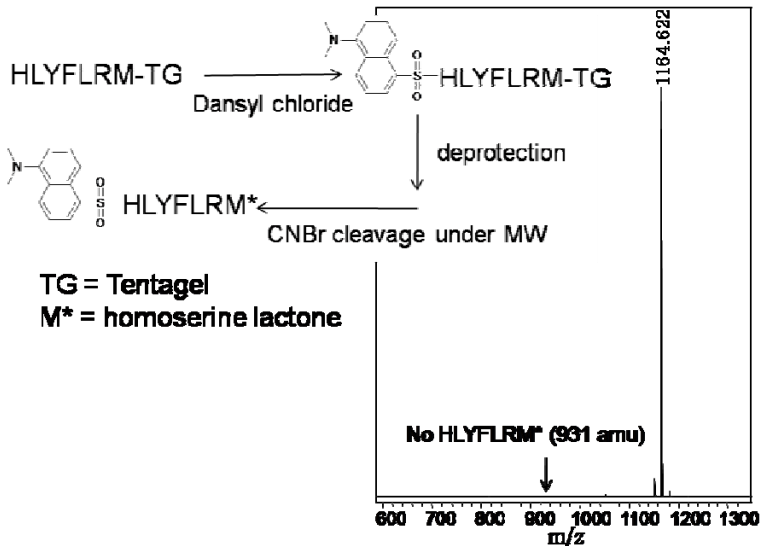
MALDI MS analysis of a single bead. To each micro tube was added CHCA (10 µl, 0.5% solution in acetonitrile/water (70:30)) and then acetonitrile/water (10 µl, 70:30 containing 0.1% trifluoroacetic acid (v/v)). After dissolving the samples, 2µl, corresponding to 10% of the cleaved material, was taken up and spotted onto a 384-well MALDI plate. This plate was dried in air for 15 minutes.



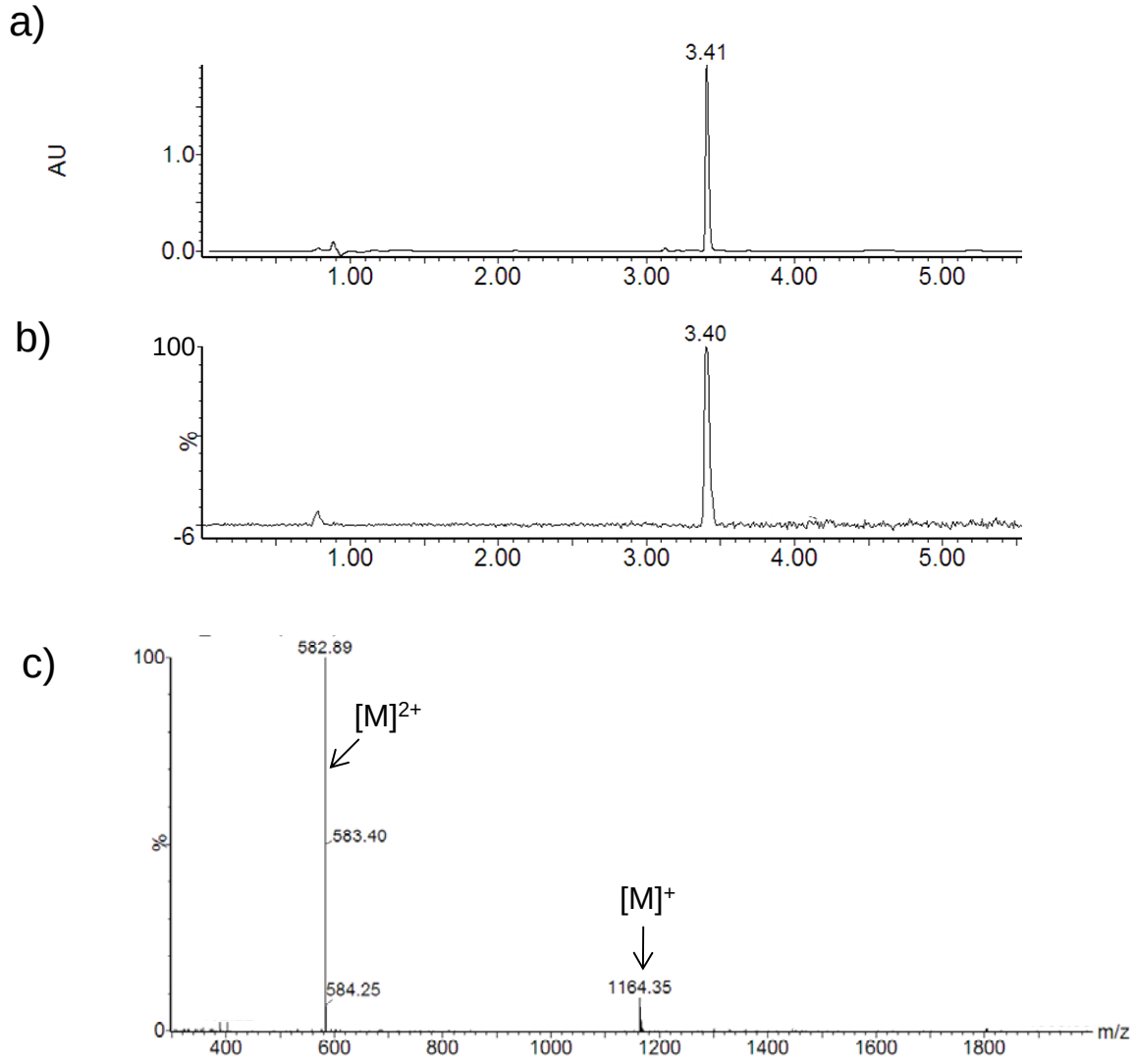
S-Fig. 1. Edman degradation sequencing, from single beads, of a) LVGRT, b) FRKIP, c) RDHWS and d) REKEY from a 5-mer peptide library (not including methionine), illustrating the purity of the library.



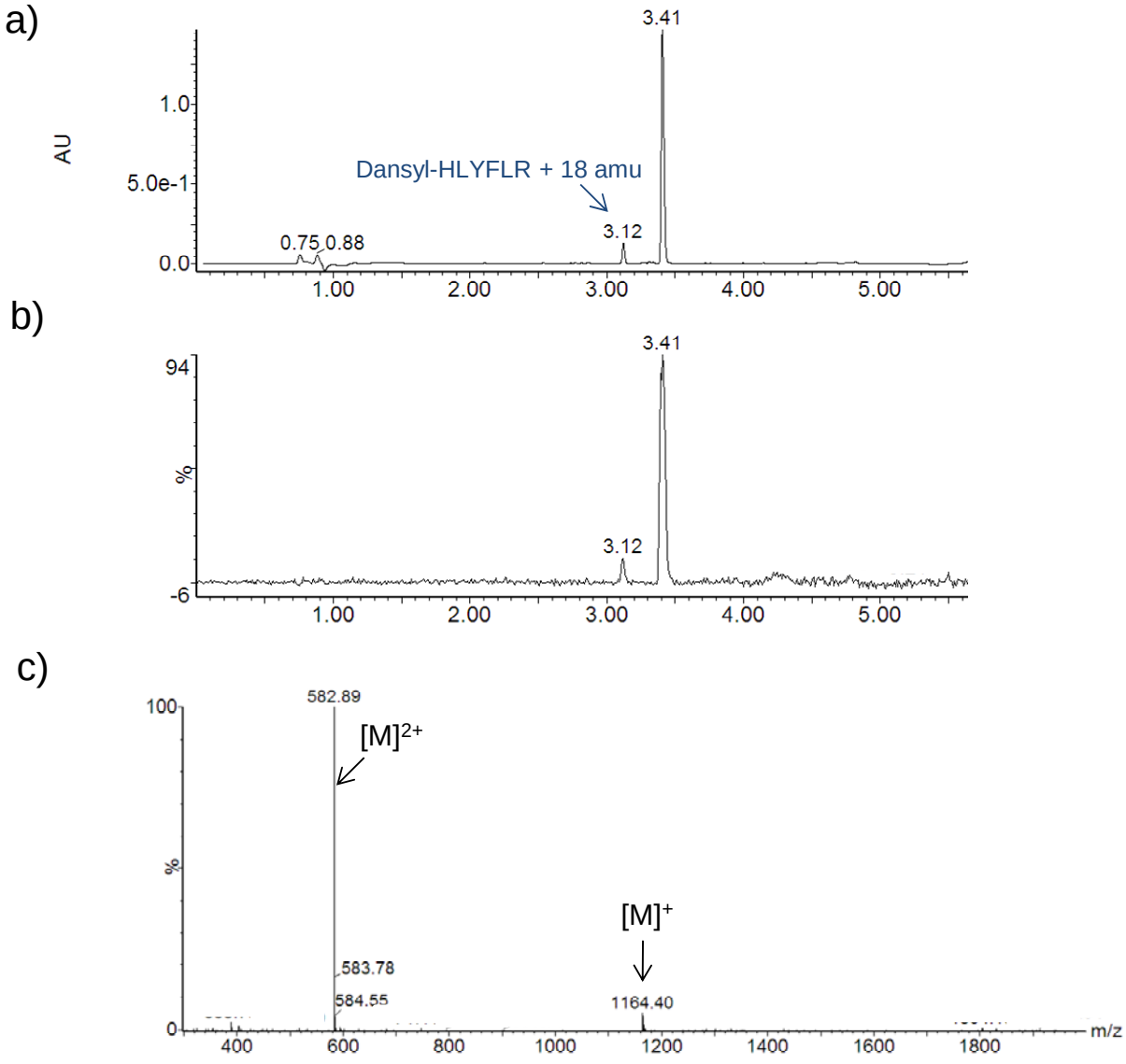
S-Fig. 2. CNBr cleavage of peptides with known sequence from a single bead. Expected molecular weights (in amu) are listed below the peptide sequence.
(Reaction conditions: 10 - 20 µl of 0.25 M CNBr in 0.1 N aq. HCl for 2 – 4 hr at 45^{o}C)
M* = homoserine lactone. ( ) = % of the total intensity



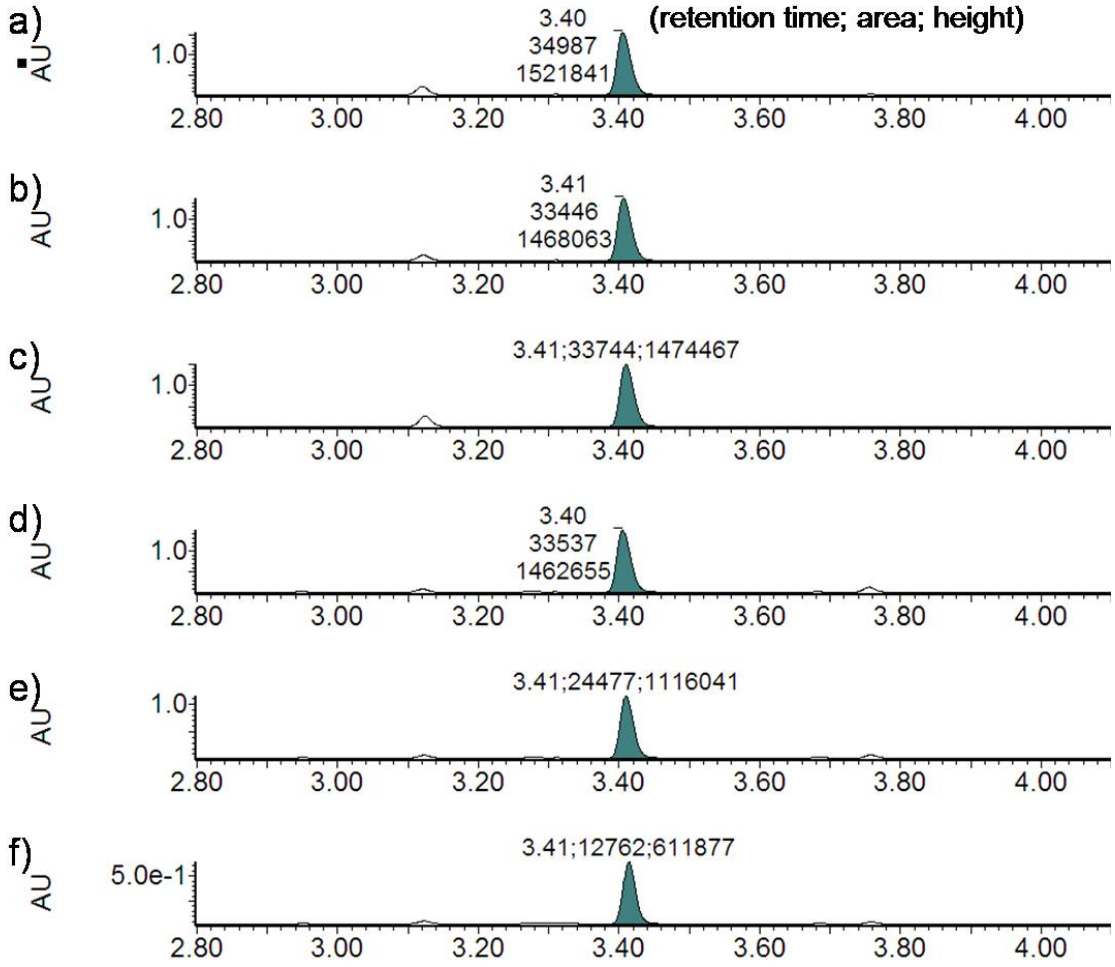
S-Fig. 3. MS spectrum of dansyl-modified HLYFLRM* from a single bead (1164 amu). (Reaction conditions: 20 µl of 0.25 M CNBr in 0.1 N aq. HCl, 1 min, microwave)



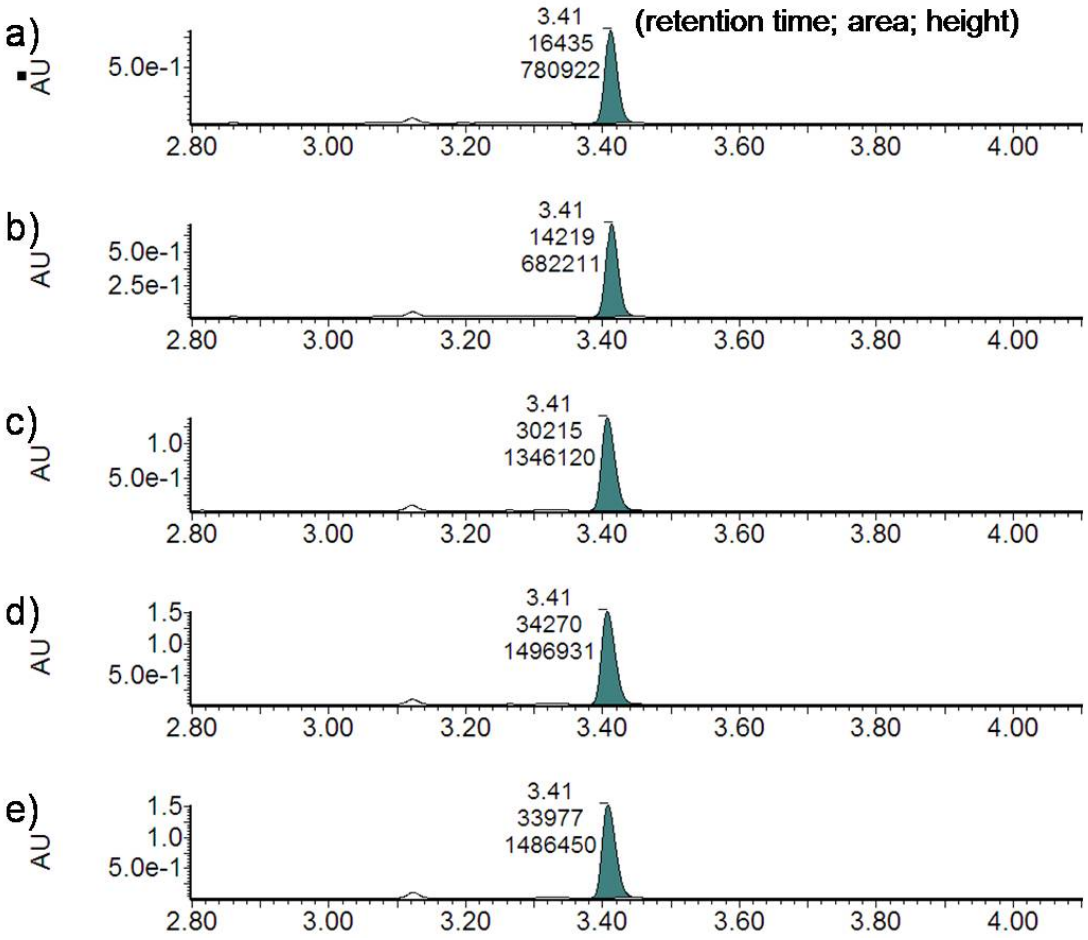
S-Fig. 4. a) HPLC UV spectrum and b) HPLC MS spectrum of dansyl-HLYFLRM* purified by Prep HPLC c) MS spectrum of the peak with the retention time of 3.41 (dansyl-HLYFLRM*, MW = 1163). [HPLC analysis conditions: C18 column (2.1 mm × 100 mm, 1.7 µm particle), A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile; Gradient: 75% A in 0.1 min, 75% - 30% in 5.4 min; 0.3 ml/min, UV detector: 214 nm, 30^{o}C]



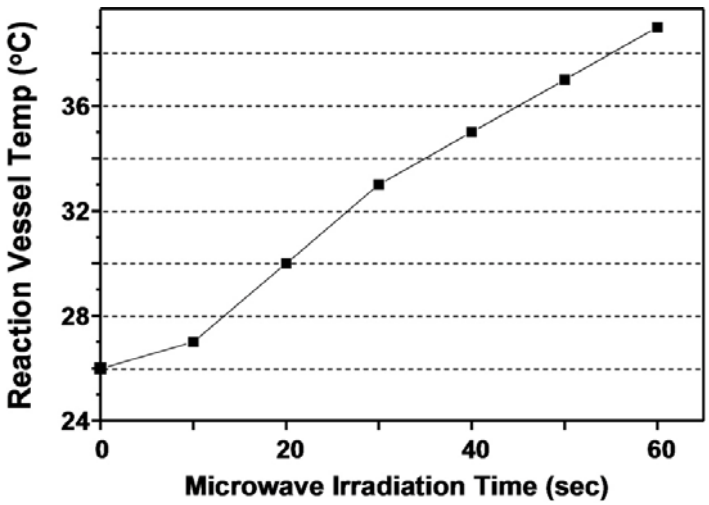
S-Fig. 5. a) HPLC UV spectrum and b) HPLC MS spectrum of the cleaved products from 320 µg of beads for 1 min under microwave irradiation. c) MS spectrum of the peak with the retention time of 3.41 (dansyl-HLYFLRM*, MW = 1163). [HPLC analysis conditions: C18 column (2.1 mm × 100 mm, 1.7 µm particle), A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile; Gradient: 75% A in 0.1 min, 75% - 30% in 5.5 min; 0.3 ml/min, UV detector: 214 nm, 30°C]



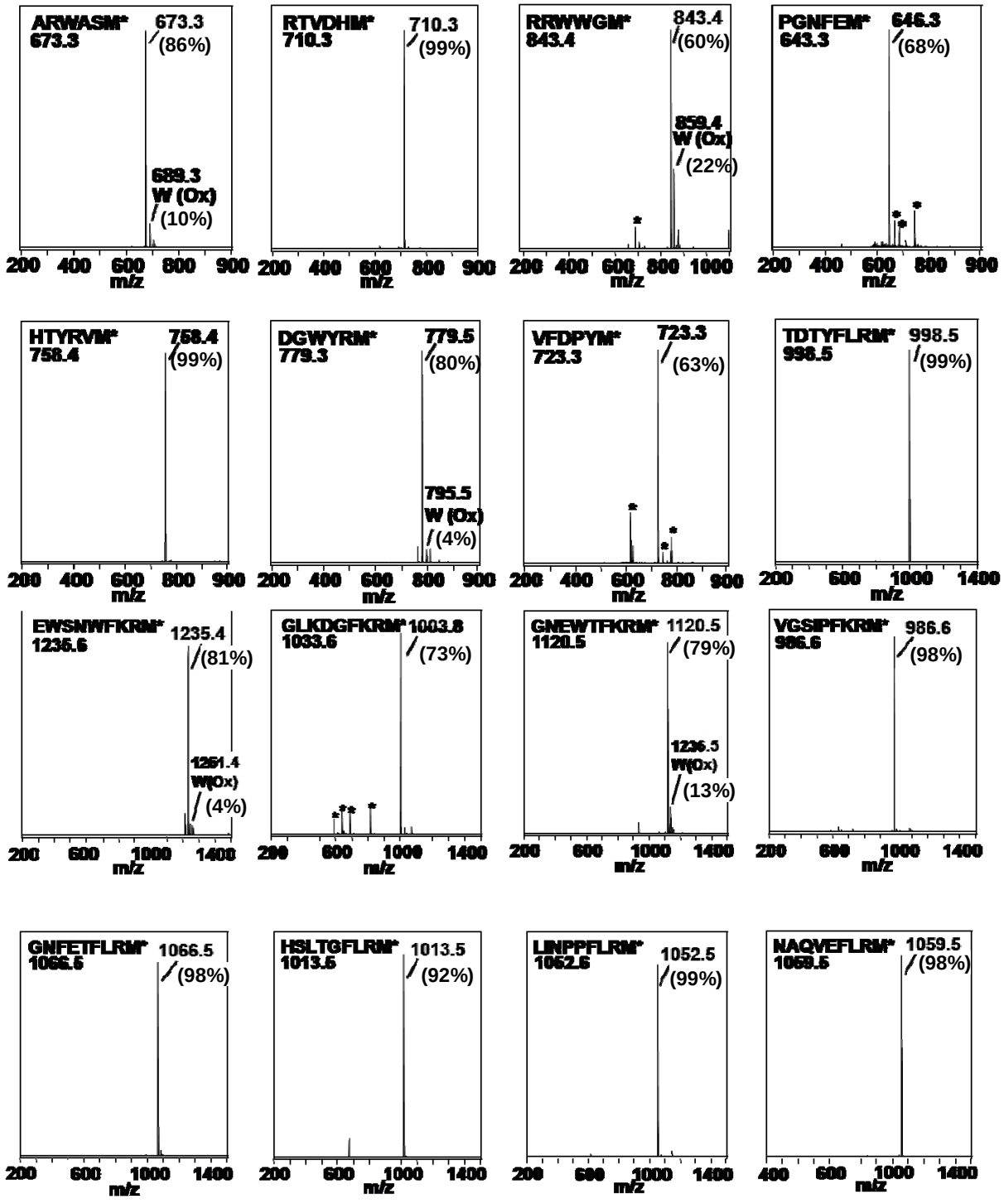
S-Fig. 6. HPLC spectra of **a.** CNBr cleavage (2 min microwave irradiation), **b.** CNBr cleavage (1 min microwave irradiation), **c.** CNBr cleavage for 4 hr at 45^{o}C, **d.** CNBr cleavage for 2 hr at 45^{o}C, **e.** CNBr cleavage for 1 hr at 45^{o}C and **f.** CNBr cleavage for 30 min at 45^{o}C.



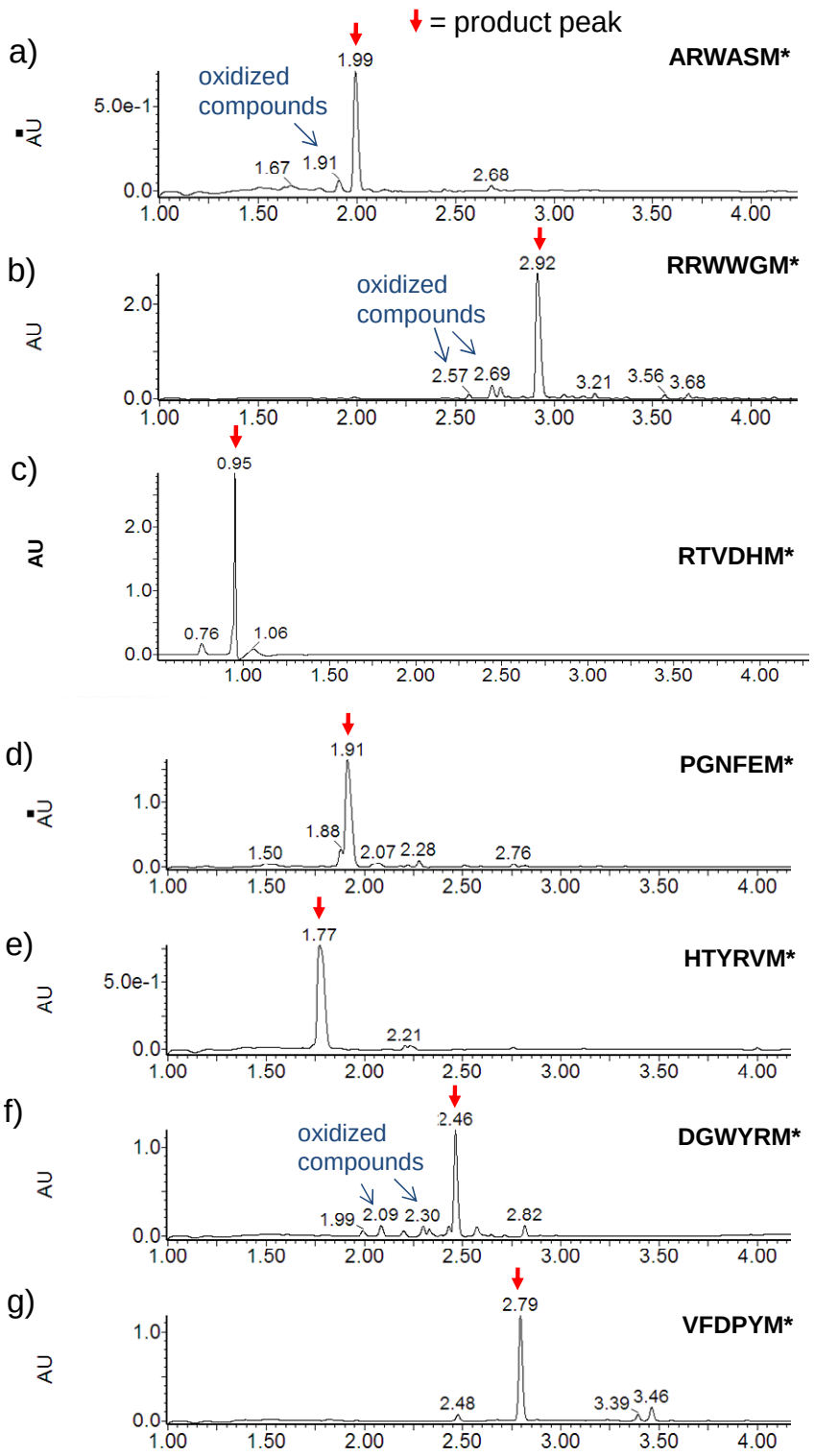
S-Fig. 7. HPLC spectra of **a.** CNBr cleavage for 30 sec under 30 W microwave power, **b.** CNBr cleavage for 1 min under 10W microwave power, **c.** CNBr cleavage for 1 min under 20W microwave power, **d.** CNBr cleavage for 1 min under 30W microwave power and **e.** CNBr cleavage for 1 min under 40W microwave power by a CEM Discovery microwave reactor.



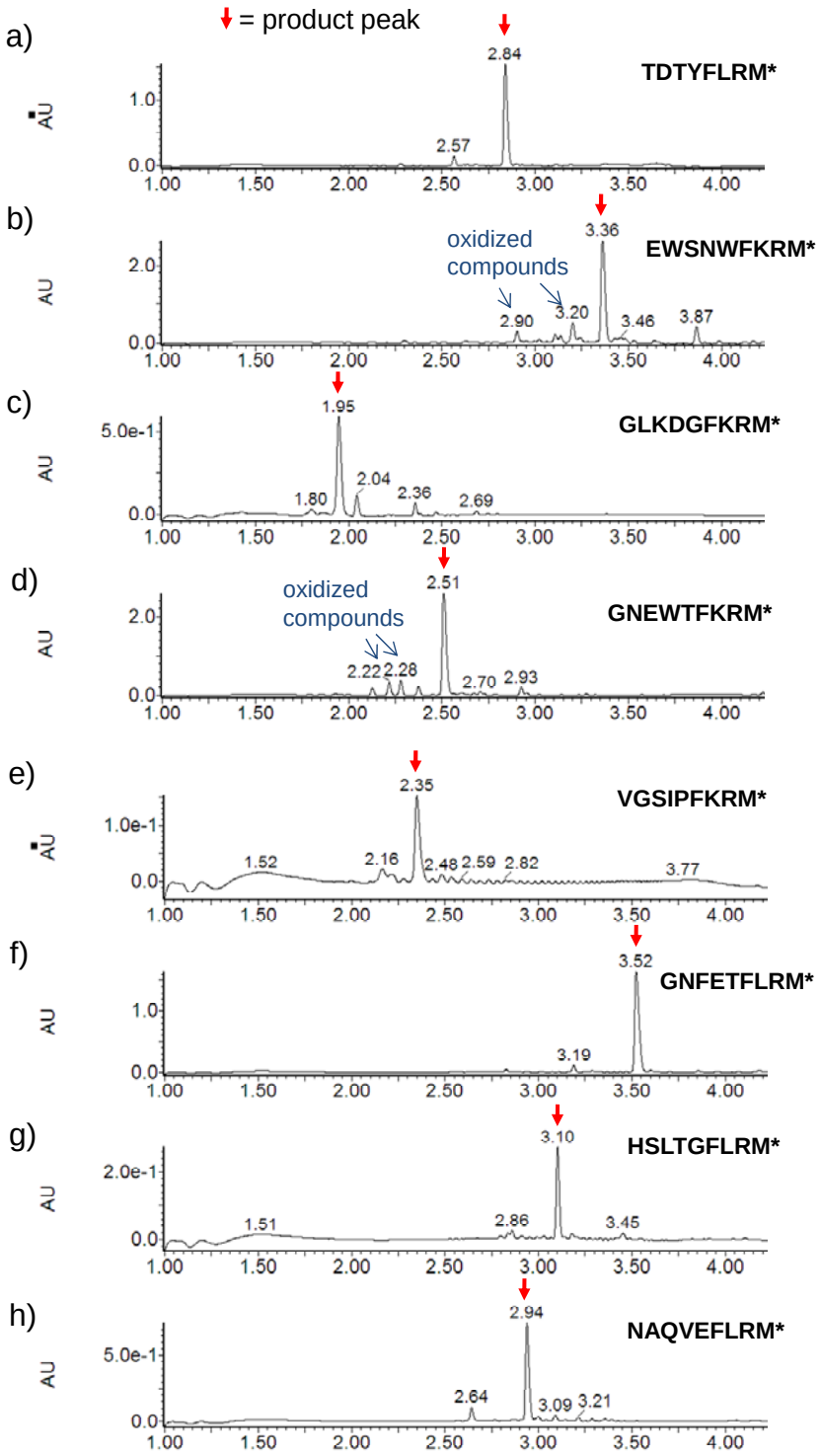
S-Fig. 8. Temperature measurements during the course of a 60 sec microwave irradiation exposure. The CEM microwave reactor (30 Watts) and the household microwave oven showed the same result.



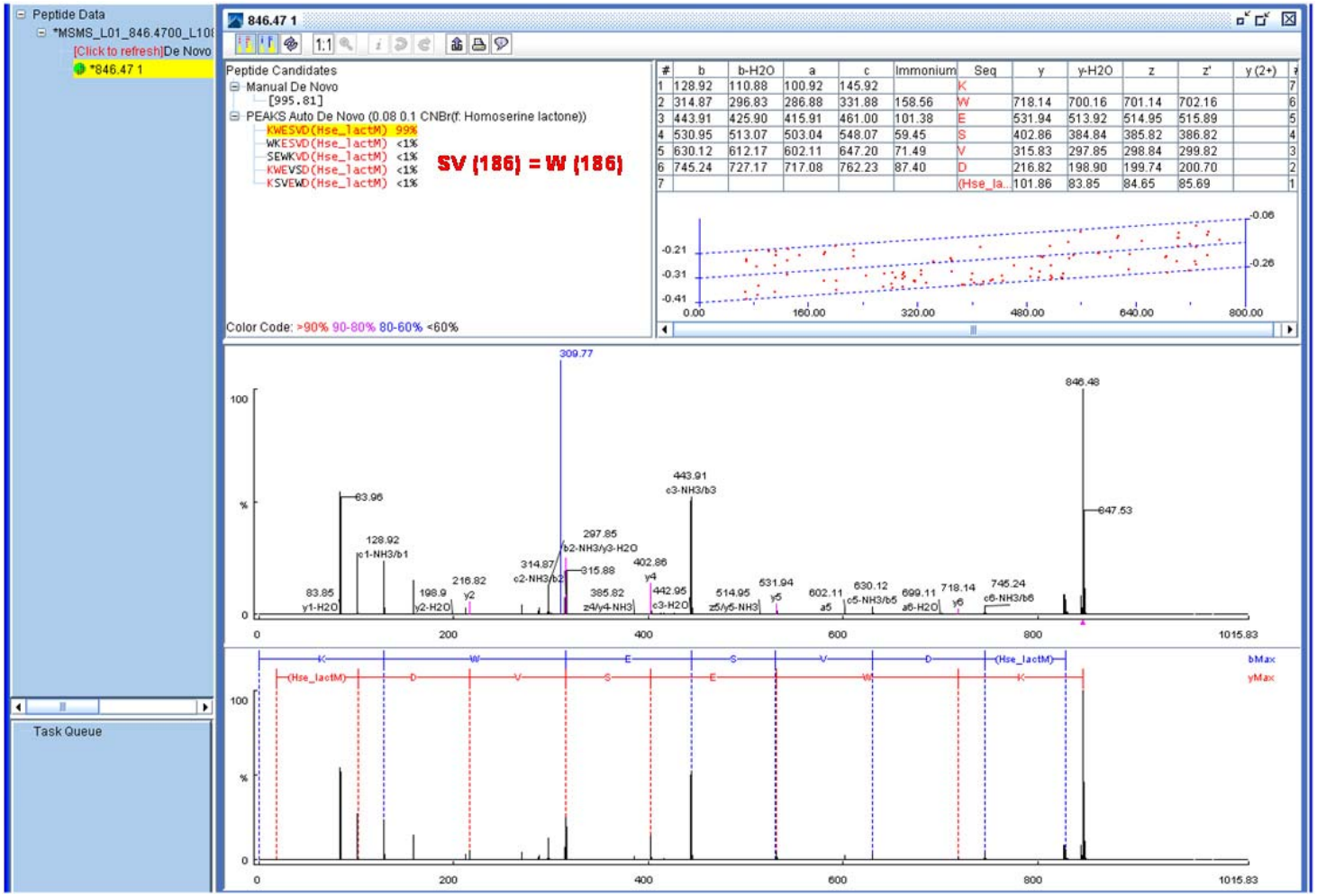
S-Fig. 9. CNBr cleavage of peptides with known sequence from a single bead.
(Reaction conditions: 10 - 20 µl of 0.25 M CNBr in 0.1 N aq. HCl, 1 min, microwave) .
M* = homoserine lactone, * = Peaks from the matrix (CHCA), ( ) = % of the total intensity



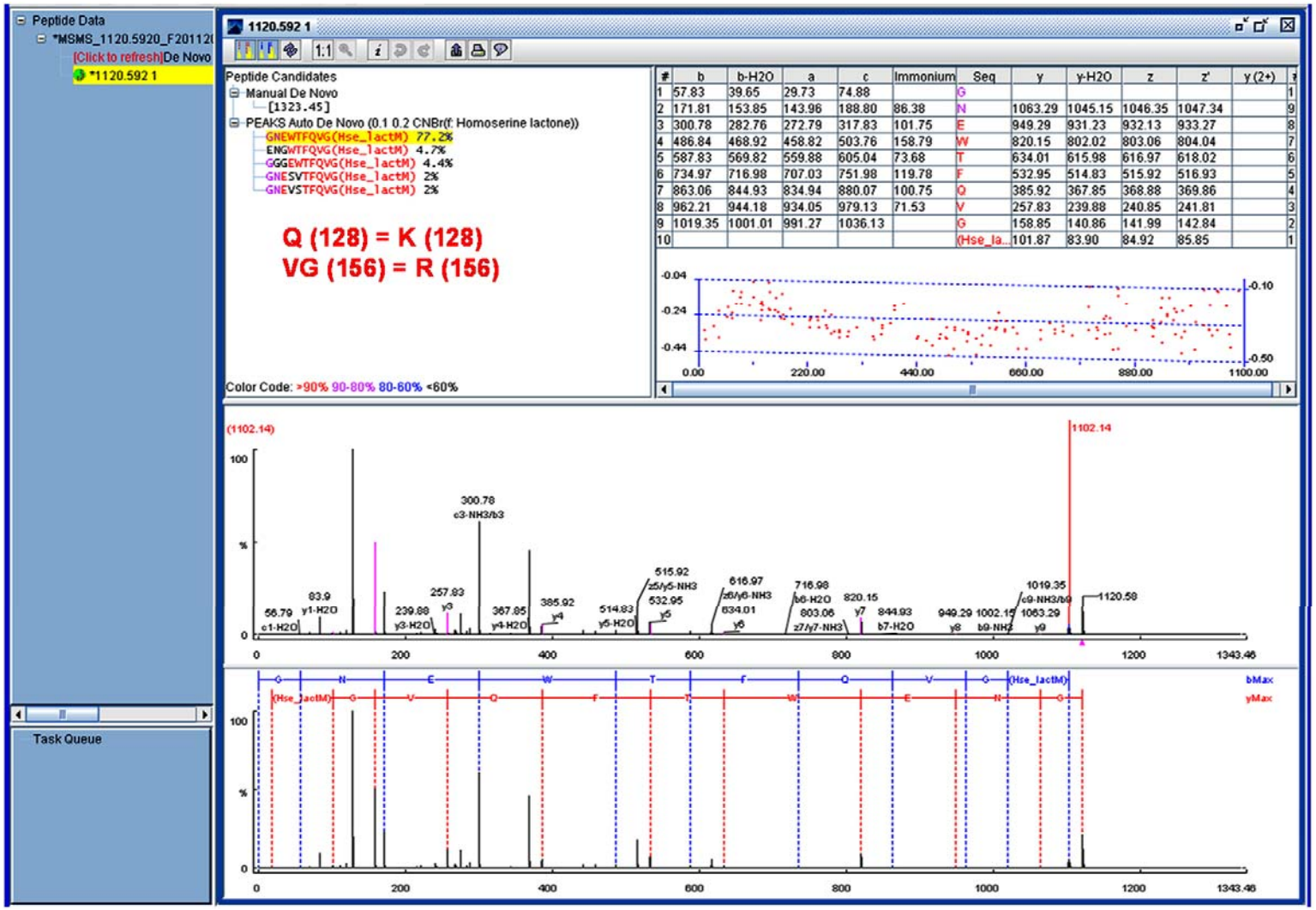
S-Fig. 10. HPLC spectra of cleaved peptides from peptide-bound beads with known sequence. [HPLC analysis conditions: C18 column (2.1 mm × 100 mm, 1.7 µm particle), A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile; Gradient: 85% A in 0.1 min, 85% - 30% in 5.5 min; 0.3 ml/min, UV detector: 214 nm, 30^{o}C]



S-Fig. 11. HPLC spectra of cleaved peptides from peptide-bound beads with known sequence. [HPLC analysis conditions: C18 column (2.1 mm × 100 mm, 1.7 µm particle), A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile; Gradient: 85% A in 0.1 min, 85% – 30% in 5.5 min; 0.3 ml/min, UV detector: 214 nm, 30^{o}C]



S-Fig. 12. Representative MALDI TOF/TOF data for a 5-mer (+ methionine) peptide from a single bead, cleaved using the 1 minute microwave-assisted protocol. The *de novo* peptide sequencing of KWEWDM* (846.470) using PEAKS software. (The sequencing result was manually confirmed. The presence of certain amino acids in the sequence was confirmed by their immonium ion peaks.)



S-Fig. 13. Representative MALDI TOF/TOF sequencing data of an 8-mer peptide (+ M*), cleaved using the 1 min microwave-assisted protocol. The *de novo* peptide sequencing of GNRWTFKRM* (1120.592) using PEAKS software. (The sequencing result was manually confirmed. The presence of certain amino acids in the sequence was confirmed by their immonium ion peaks.)