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Visually distinguishing between tumor tissue and healthy tissue within ten minutes using proteolytic probes

Debora Reinhardt,[1#] Björn ter Mors,[1#] Marc D. Driessen,[1,2#] Marcus Gutmann,[1] Julian Faber,[3] Lukas Haug,[4] Anna-Maria Faber,[4] Anna Herrmann,[1] Prisca Hamm,[1] Tessa Lühmann,[1] Christian Linz,[2,6*] Lorenz Meinel[1,7*]

[1] Institute for Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, DE-97074 Würzburg, Germany

[2] Institute for Molecular Medicine I, Heinrich-Heine-University Duesseldorf, DE-40225 Duesseldorf, Germany

[3] Department of Oral and Maxillofacial Plastic Surgery, University Hospital Würzburg, D-97070 Würzburg, Germany

[4] Department of Pathology, University of Würzburg, Germany, DE-97080 Würzburg, Germany

[5] Department of Dermatology and Venerology, University of Cologne, DE-50937, Cologne, Germany

[6] Department for Oral and Craniomaxillofacial and Plastic Surgery, University Hospital Cologne and Faculty of Medicine, University of Cologne, Germany

[7] Helmholtz Institute for RNA-Based Infection Research (HIRI), Helmholtz Center for Infection Research (HZI), DE-97080 Würzburg, Germany

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[#] contributed equally

[*] corresponding authors

Please direct correspondence to:

Prof. Dr. Dr. Christian Linz, Faculty of Medicine, University of Cologne, DE-50937, Cologne, Germany, christian.linz@uk-koeln.de

Prof. Dr. Dr. Lorenz Meinel, Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany, lorenz.meinel@uni-wuerzburg.de

SUPPORTING INFORMATION

Safety Statement

No unexpected or unusually high safety hazards were encountered.

Abbreviations

DABCYL, (dimethylamino)phenyl)azo)benzoic acid; EDANS, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid; FPLC, fast protein liquid chromatography; FRET, fluorescence resonance energy transfer; 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, HEPES; HNSCC, head and neck squamous cell carcinomas; HPLC, high pressure liquid chromatography; HPN, hepsin; KLK13, kallikrein-13; LC–MS, liquid chromatography–mass spectrometry; METAP2, methionine aminopeptidase 2; NNC, non-cleaving control; norm score, normalized score; PICS, proteomic identification of cleavage sites; PCSK6, proprotein convertase Subtilisin/Kexin Type 6; ST14, matriptase; TMPRSS11E, transmembrane protease serine 11E; USP4, ubiquitin-specific peptidase 4.

Materials

Spectra 9 medium was purchased from Eurisotop (Saint-Aubin, France). Cyanoborohydride coupling buffer, 10 x phosphate-buffered saline (PBS), Furin Inhibitor I (34493), and Protease Inhibitor Cocktail I (I3911) were purchased from Merck (Darmstadt, Germany). The proteases for PICS library generation, GluC (sequencing grade; V1651), and trypsin (sequencing grade; V5117) were obtained from Promega (Madison, USA). Recombinant Matrix Metalloprotease 8 (MMP-8) protein (10254-HNAH) was purchased from Sinobiological (Beijing, China). Recombinant furin protein and anti-furin antibody (rabbit-derived; ab183495) were purchased from Abcam (Cambridge, United Kingdom). Anti-rabbit HRP-linked antibody (goat-derived; 7074P2) was purchased from Cell Signaling (Leiden, Netherland). Streptavidin sepharose high performance (17511301) was purchased from Cytiva Sweden AB (Uppsala, Sweden). Both ^{14}N and ^{15}N *Escherichia coli (E. coli),* formic acid LC–MS grade, HEPES buffer 1M, the Pierce BCA Protein Assay Kit, Pierce Quantitative Colorimetric Peptide Assay, EZ-Link Sulfo-NHS-SS-Biotin, and SuperSignalTM West Pico PLUS Chemiluminescent Substrate were purchased from Thermo Scientific (Waltham, USA). BioTrace NT nitrocellulose membrane was obtained from Pall Corporation (Port Washington, USA). Strata-X reversed-phase columns (10 mg, 500 mg) were obtained from Phenomenex (Aschaffenburg, Germany). Two-chlorotrityl chloride resin was obtained from Chem-Impex Wood (Dale, IL). All amino acid analogues used for Fmoc acid coupling strategy (solid-phase peptide synthesis) were purchased from VWR (Ismaning, Germany). All other chemicals used were purchased from Sigma-Aldrich or VWR.

Proteome-derived PICS library generation

Protease cleavage sites were identified according to PICS as described by Schilling et al.[1] The ^{14}N *E. coli* cells were cultured in a lysogeny broth medium for 16 h at 37° C. The ^{15}N-labeled cells were kept in Spectra 9 medium under the same conditions. The cell suspension was centrifuged with 4,000 g at 4 °C for 20 min, and the supernatant was discarded. The cell pellet was washed two times with PBS following centrifugation and decantation of the supernatant. The cell pellet was kept.

Lysis of the cell pellets was carried out via suspension of the pellets in lysis buffer (125 mM tris, 5% sodium dodecyl sulphate, 100 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride) following three cycles of sonication (Sonopuls HD3100, Bandelin, Berlin, Germany) with 25% amplitude, 30 s sonication, and a 90-s pause for 10 min on ice. After sonication, the solution was adjusted to 1x radio immunoprecipitation assay buffer (RIPA buffer; 150 mM NaCl, 50 mM tris-HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate) with 6 M urea and 5% sodium dodecyl sulphate and was incubated for 90 min at 4° C under shaking. The lysate was centrifuged at 20,000 g for 30 min, the supernatant was collected, and the concentration was determined via BCA assay.

Free sulfhydryl groups were protected by adjusting the lysate to 100 mM of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) with a pH of 7.5, and 20 mM iodoacetamide. The sample was incubated for 1 h under light exclusion. After incubation, 5 mM dithiothreitol final concentration was added to quench excess iodoacetamide. The samples were adjusted to 15% trichloroacetic acid (v/v), incubated on ice for 1h, and centrifuged at 8,500 g at 4 °C for 1 h. The pellets were washed with cold methanol and were air dried. The dry pellets were solubilized in ice-cold 20-mM NaOH via an ultrasonic bath for 1 h, followed by sonication (Sonopuls HD3100, Bandelin, Berlin, Germany) with 25% amplitude, 30 s of sonication, and a 90-s pause for 10 min on ice to reach a 2 mg/mL concentration. The samples were adjusted to 200 mM HEPES with a pH of 7.5 and were centrifuged for 30 min at 8,500 g. The protein concentration in the supernatant was determined via BCA assay.

Specific endoprotease digestion was performed with trypsin or GluC at a protease-to-proteome ratio of 1:100 (w/w). The reaction buffer was adjusted to pH 7–9, and the reaction was incubated for 16 h at 37 °C. Digestion was verified via sodium dodecyl sulphate gel, with no major bands > 10 kDa. After inactivation of the protease by heating at 70 °C for 20 min, 1 mM PMSF was added, followed by 1 M of guanidine hydrochloride. The samples were centrifuged at 4,500 rpm at 4 °C for 1h to remove undigested proteins. The supernatant was separated and incubated with 5 mM dithiothreitol for 1 h at 37 °C. For a second round of cysteine protection, 40 mM iodoacetamide was added and incubated for 1.5 h at 37 °C under light exclusion. Another 15 mM dithiothreitol was added to quench excess iodoacetamide at 37 °C for 10 min. Primary amines of the new N-termini and lysine side-chains were

blocked by addition 30 mM of formaldehyde, 30 mM sodium cyanoborohydride and incubation at 25 °C for 2 h. Another 30 mM formaldehyde and 30 mM sodium cyanoborohydride were added and incubated at 25 °C for 16 h. Then, 100 mM glycine was added and incubated at 25 °C for 30 min. The reaction was stopped via acidification with 0.5% trifluoroacetic acid. The peptide libraries were purified by solid-phase extraction (Strata-X, 500 mg) and were lyophilized. The lyophilized peptides were resolved in 200 mM HEPES with a pH of 7.5 and were quantified (Quantitative Peptide Assay). Two mg/ml of aliquots was prepared and stored at –80 °C until further usage.

Each batch of peptide libraries was tested for performance with MMP-8, and the resulting cleavage patterns, the number of cleavages observed, and the number of unmodified residues were assessed (data not shown). The libraries were then used to analyze furin cleavage sites.

Purification of PICS cleavage fragments

After cleavage, prime-side peptide fragments were isolated via biotin-streptavidin-based affinity chromatography. First, the free N-termini of the furin cleavage sites were modified with 0.5 mM sulfo-NHS-SS-biotin at 25 °C for 2 h. Next, high-capacity streptavidin sepharose was added to the biotinylated samples (1.5:1 (v/v)) and was incubated for 0.5 h at 22 °C. The slurry was transferred to a spin column and centrifuged. The eluate was applied one more time to the resin and was centrifuged again. After washing with wash buffer (50 mM HEPES with a pH of 7.5 and 150 mM NaCl), 500 µL elution buffer (50 mM HEPES and 10 mM dithiothreitol with a pH of 7.5) was added to the slurry and incubated for 2 h at 25 °C. The column was centrifuged, and the eluate was kept. Another 500-µl elution buffer was added, and the column was centrifuged again. Combined eluates were purified via solid-phase extraction (Strata-X 10 mg). Then, 0.4% formic acid in water was used for washing steps (threefold loading volume), and 0.4% formic acid in 80% acetonitrile (1.8 ml) was used for elution.

PICS data analysis

After library digestion and purification, the samples were analyzed via LC–MS/MS. Results from the LC–MS/MS were processed via PMi-Byos software by Protein Metrics Inc. (United States). Data files were searched with the following settings: protease set as trypsin (C-terminal to R, K), semi-specific digest (ragged peptide N-terminus), up to 3 missed cleavages; precursor tolerance of 7.5 ppm, fragment mass tolerance of 15 ppm; carbamidomethyl @ C as fixed modification, dimethyl/methyl/thioacyl @ K as common, and thioacyl @ peptide N-term as common (residue from the cleavable enrichment probe), with max allowed common modifications at 3 and rare max set to 1. For ^{15}N samples, a fixed modification for each amino acid was used to enable the search for heavy labeled peptides (see list below). (We tested X to add modifications for potentially unlabeled or not

fully labeled amino acids (e.g., ^{15}N-remove_Gly / –0,997035 @ G); however, due to the needed number of additional modifications (i.e., at least 21 and up to 30 in order to account for partial labeling), we found no improvement in the generated results; data not shown.) Data were searched against *E. coli* reference proteome (downloaded from UniProt).[2] Search results have been filtered at the peptide level to exclude inverted proteins (from decoys) and to contain only peptides that contained 'N-terminal thioacyl with a PEP 2D score of < 0.1 and a total score of > 100.

The resulting peptide lists of the replicates were merged and submitted to the WEB-PICS application (http://clipserve.clip.ubc.ca/pics), and the results were used to enable the design of custom peptides.[1] Both ^{14}N and ^{15}N tryptic peptide libraries were tested with furin in three independent replicates, resulting in a total of 566 unique analyzed cleavage sites. GluC-derived peptide libraries (^{14}N) were tested once with furin and resulted in 164 analyzed cleavage sites.

^{15}N – Custom modifications

15N_Gly / +0.997035 @ G | fixed
15N_Ala / +0.997035 @ A | fixed
15N_Ser / +0.997035 @ S | fixed
15N_Thr / +0.997035 @ T | fixed
15N_Cys / +0.997035 @ C | fixed
15N_Val / +0.997035 @ V | fixed
15N_Leu / +0.997035 @ L | fixed
15N_Ile / +0.997035 @ I | fixed
15N_Met / +0.997035 @ M | fixed
15N_Pro / +0.997035 @ P | fixed
15N_Phe / +0.997035 @ F | fixed
15N_Tyr / +0.997035 @ Y | fixed
15N_Asp / +0.997035 @ D | fixed
15N_Glu / +0.997035 @ E | fixed
15N_Trp / + 1.994070 @ W | fixed
15N_Asn / +1.994070 @ N | fixed
15N_Gln / +1.994070 @ Q | fixed

Peptide and probe synthesis

All peptides were synthesized in-house using Fmoc-based solid-phase peptide synthesis. One-hundred-µmol-loaded 2-chlorotrityl chloride resin was loaded to a polypropylene reactor

(MultiSynTech, Witten, Germany), and the first amino acid was coupled with a 5-molar excess relative to the loading of the resin. Then, 2 mL dichloromethane and 285 µL N,N-diisopropylethylamine were added, and the mixture was incubated at 25 °C under rigorous shaking for at least 3 h. To block free-resin-binding sites, 100 µL methanol was added to the mixture and incubated again for 30 min at 25 °C under rigorous shaking. The resin was washed 3 times with dichloromethane and 3 times with dimethylformamide.

The subsequent amino acids were coupled either via semi-automatic Biotage® Initiator+ SP Wave peptide synthesizer or Liberty Blue™ automatic peptide synthesizer. Coupling via Biotage® Initiator+ SP Wave peptide synthesizer was performed via solubilization of 500 µmol of the respective Fmoc-protected amino acid in 2 mL dimethylformamide containing 0.5 M hydroxybenzotriazole, 0.5 M diisopropylcarbodiimide, and 0.2 M N,N-diisopropylethylamine at 50 °C for 10 min, followed by three wash steps with dimethylformamide. Fmoc deprotection was carried out via incubation in 20% piperidine in dimethylformamide for 3 min at 75 °C, followed by four wash steps with dimethylformamide. The coupling of subsequent amino acids via Liberty Blue™ was performed with 0.2 M of the respective amino acid in dimethylformamide containing 1 M ethyl cyanohydroxyiminoacetate and 0.5 M of diisopropylcarbodiimide for 15 s at 75 °C, followed by 110 s at 90 °C. Fmoc deprotection was carried out in dimethylformamide with 20% piperidine at 75 °C for 15 s, followed by 50 s at 90 °C.

After the coupling of the last amino acid, the peptide was washed three times with dichloromethane and three times with diethyl ether before being dried. Cleavage of the peptide was performed via incubation in cleavage solution (trifluoroacetic acid, 2.5% water, 2.5% triisopropylsilane, 2.5% 2,2’ (ethylenedioxy)diethanethiol) for 1 h at room temperature under rigorous shaking. The peptide was precipitated in ice-cold diethyl ether, centrifuged, and decanted, and the procedure was repeated two more times.

To acetylate the N-terminus of the peptides, 2 mL dimethylformamide was added to the resin-bound peptides. Additionally, 90 µL DIPEA and 50 µL acetic anhydrite were added following incubation of the mixture for 30 min at 25 °C under rigorous shaking. The resin was washed 3 times with dimethylformamide, 3 times with dichloromethane, and 3 times with diethyl ether, and it was then dried and stored at 4 °C until further usage.

Preparative peptide purification was carried out via fast protein liquid chromatography (FPLC) on a GE ÄKTA pure (GE Healthcare, Chalfont St Giles, UK) system with a Luna 15 µm C18 100 Å column with an internal diameter of 21.2 mm and a length of 250 mm (Phenomenex Inc., Torrance, CA) with Eluant A (0.1% trifluoroacetic acid in water (v/v)) and Eluent B (0.1% trifluoroacetic acid in ACN (v/v)) with varying gradients starting from 10–50% (depending on the peptide solubility) and up to 100% B in 60

min. UV absorption was read at λ = 214 nm, 254 nm, and 280 nm. The samples were frozen in liquid nitrogen, freeze-dried via CoolSafe 110-4 Pro (LaboGene A/S, Allerød, Denmark), and stored at –80 °C until further usage.

Preparation of standard curve

A dilution series (10 – 0.078 µM) of synthesized **Probe a** cleavage fragments (**Probe a1** and **Probe a2**) was prepared in triplicate, and fluorescence was measured using Infinite M Plex (Tecan trading AG, Männedorf, Switzerland). The excitation and emission wavelengths were set to 340 nm and 490 nm, respectively.

Probe cleavage in the presence of a broad-range inhibitor

Tissue specimens of healthy tissue (control) and tumor tissue (6–9 mg) were washed with reaction buffer (100 mM HEPES with a pH of 7.5 and 1 mM CaCl2). The progress of the probe cleavage over time was monitored with a fluorescence plate reader (Infinite M Plex, Tecan Trading AG, Männedorf, Switzerland) with an excitation wavelength of λ = 340 nm and an emission wavelength of λ = 490 nm. The tissue specimens were incubated in 200 µL of reaction buffer containing 10 µM of C1S or Z6S. Then, 100 µM protease-inhibitor cocktail was added to inhibit protease activity. Incubation was performed at 37 °C. For analysis, 100 µl of reaction buffer was taken at t = 0, 5, 10, 15, 20, 25, and 30 min. Experiments were performed in three replicates.

Comparison of Probe a and Probe 1 cleavage

Tissue specimens (4–5 mg) were first incubated with **Probe a** for 30 min in reaction buffer, followed by a second incubation with **Probe 1** for 30 min and a third incubation with **Probe a** for 30 min. Between the incubation steps, the specimens were washed with reaction buffer. Each probe was used in a concentration of 10 µM. Sample preparation, reaction conditions, and analysis were performed as described above.

Stability of probe-cleavage activity

Stability of proteolytic activity was monitored over time by incubating two tissue samples (6.1 mg and 6.9 mg) four times with 10 µM **Probe A** in reaction buffer. Each cleavage step was performed for 30 min. Between the incubation steps, the specimens were washed with reaction buffer. Sample preparation, reaction conditions, and analysis were performed as described above.

Western blot

For sample preparation, two healthy tissue samples and four tumor tissue samples were lysed in RIPA buffer using glass beats and a Mini-Beadbeater (Biospec, USA), followed by centrifugation at 12,000 g at 4 °C for 20 min. The protein concentration was determined via BCA assay. A 10% SDS-Page was performed with 10 ug protein per sample. After protein transfer onto a nitrocellulose membrane, the membrane was blocked in 5% (w/v) skim milk powder in tris-buffered saline with Tween20 (TBS-T) for 1 h at 24 °C. Ponceau S staining was used as the loading and transferring control. The membrane was washed with TBS-T and incubated with the primary antibody (anti-furin, 1:1,000 dilution in TBS-T) overnight at 4 °C. The secondary antibody (anti-rabbit, 1:1,000 dilution in TBS-T) was incubated for 1 h at 24 °C. Signals were detected with SuperSignalTM West Pico PLUS Chemiluminescent Substrate and a chemiluminescence imager (Odyssey FC, Leicor, Bad Homburg vor der Höhe, Germany).

Liquid chromatography–mass spectrometry (LC–MS)

LC–MS spectra were acquired via a Shimadzu LC–MS system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a DGU-20A3R degassing unit, an LC20AB liquid chromatograph using a Synergi 4-µm fusion-RP column (150 x 4.6 mm) (Phenomenex Inc., Torrance, CA), and an SPD-20A UV/Vis detector coupled with a single quadrupole LCMS-2020. Mobile Phase A was 0.1% (v/v) formic acid (FA) in Millipore water, and Mobile Phase B was methanol with 0.1% (v/v) FA. Flow was set to 1 ml/min, the wavelength of the detector was set to λ = 214 nm, and a volume of 20 µL was injected into the sample. The gradient was increased in 8 min from 5–90% B and was then held at 90% B for 5 min before being reduced to 5% B within 1 min and held at 5% B for 4 min. The detection range was set to 60–1,000 (m/z), and the spectra were measured at a voltage of 3.5 kV and a capillary temperature of 210 °C using N2 as a carrier gas.

Liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS)

Samples were re-suspended in the loading buffer (0.2% FA / 2% can / 98% H_2O) prior to analysis. NanoLC–MS/MS analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Scientific) equipped with a PicoView Ion Source (New Objective) and coupled to an EASY-nLC 1,000 (Thermo Scientific). Peptides were loaded on capillary columns (PicoFrit, 30 cm x 150 µm ID, New Objective) that were self-packed with ReproSil-Pur 120 C18-AQ 1.9 µm (Dr Maisch) and separated with a 120-min linear gradient from 3–30% acetonitrile and 0.1% formic acid with a flow rate of 500 nl/min.

MS scans were acquired in the Orbitrap analyzer with a resolution of 30,000 at m/z 400, and MS/MS scans were acquired in the Orbitrap analyzer with a resolution of 7,500 at m/z 400 using HCD fragmentation with 30% normalized collision energy. A TOP5 data-dependent MS/MS method was

used; dynamic exclusion was applied with a repeat count of 1 and an exclusion duration of 15 seconds; singly charged precursors were excluded from the selection. The minimum signal threshold for precursor selection was set to 50,000. Predictive AGC was used with an AGC target value of 1e6 for MS scans and 5e4 for MS/MS scans. A lock mass option was applied for internal calibration in all runs using background ions from protonated decamethylcyclopentasiloxane (m/z 371.10124).

High-performance liquid chromatography (HPLC)

Analytical HPLC was carried out via an Agilent 1260 Infinity II HPLC (Agilent Technologies Inc., Waldbronn, Germany) using an Eclipse XDB-C18 5-µM analytical 4.6-x-1,500-mm LC column (Agilent Technologies Inc., Waldbronn, Germany). The device was equipped with a diode array detector (G7115A, Agilent), a fluorescence detector (G7121A, Agilent), an automatic vial sampler (G7129C, Agilent), a flexible pump (G7104C, Agilent), and a multi-column oven (G7116A, Agilent). Mobile Phase A was 0.1% trifluoroacetic acid in Millipore water. Mobile Phase B was ACN with 0.1% trifluoroacetic acid, the flow was set to 1 ml/min, the injection volume was 15 µl, and the wavelength of the detector was set to λ = 214 nm for PICS-derived peptides. Peptide purity was analyzed with the gradient increased from 5% B to 100% B in 15 min.

For analysis of peptide cleavage, the gradient was held at 15% B for 1 min and was then increased to 40% within 7 min, increased again for 0.5 min to 95% B, held for 0.5 min, returned to 15% B within 0.5 min, and held for 5.5 min.

SUPPORTING DATA

Table S1: Relative occurrence of amino acids for GluC-digested proteome, in %.

	P6 [%]	P5 [%]	P4 [%]	P3 [%]	P2 [%]	P1 [%]	P1‘ [%]	P2‘ [%]	P3‘ [%]	P4‘ [%]	P5‘ [%]	P6‘ [%]
A	3.6	5.5	7.3	10.3	12.7	8.5	9.7	15.8	14.5	7.9	11.5	11.5
C	0.0	0.6	0.6	0.0	1.2	1.2	0.0	1.2	0.6	0.0	0.6	0.0
D	1.2	4.8	4.8	3.0	3.6	0.6	1.8	1.2	4.8	3.6	3.6	4.8
E	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	4.8	6.1	3.0	5.5
F	1.2	0.6	3.0	4.8	4.2	7.3	0.0	1.2	1.2	6.7	2.4	2.4
G	4.2	2.4	3.6	4.2	3.6	3.6	9.7	10.3	11.5	10.3	10.3	10.9
H	1.2	1.2	0.6	1.2	0.6	3.0	3.6	1.8	4.2	4.8	1.8	0.6
I	3.6	3.6	3.6	6.7	4.2	3.0	1.8	4.2	5.5	4.8	9.7	6.1
K	4.8	6.7	8.5	7.3	9.1	12.7	4.8	10.3	10.9	10.9	4.8	8.5
L	6.7	4.8	6.1	8.5	11.5	10.3	1.2	4.8	2.4	6.7	4.2	1.8
M	1.8	1.8	3.6	3.6	6.1	6.1	0.0	1.2	1.8	0.6	0.6	0.6
N	0.6	1.8	3.0	1.8	3.6	4.2	1.2	3.0	4.8	4.2	1.8	6.1
P	4.8	2.4	1.2	1.2	3.6	3.0	0.0	9.1	4.8	1.2	6.1	5.5
Q	3.6	1.2	2.4	3.0	1.2	4.8	4.2	3.6	3.6	3.0	4.8	1.2
R	7.9	12.7	7.9	13.9	12.7	21.8	49.1	7.9	8.5	8.5	10.9	6.1
S	3.6	3.6	4.8	4.8	5.5	3.0	6.7	4.8	5.5	7.9	8.5	7.9
T	1.8	2.4	5.5	3.0	2.4	0.6	0.6	6.1	4.2	3.0	4.8	6.7
V	2.4	7.9	5.5	8.5	6.1	1.2	4.2	10.9	4.2	7.9	8.5	10.3
W	0.6	1.2	1.8	1.2	0.6	0.6	0.0	0.0	0.0	0.0	0.6	0.0
Y	1.8	0.6	1.2	0.6	4.2	3.0	1.2	0.6	1.8	1.8	1.2	2.4

Table S2: Relative occurrence of amino acids for trypsin-digested proteome, in %.

	P6 [%]	P5 [%]	P4 [%]	P3 [%]	P2 [%]	P1 [%]	P1‘ [%]	P2‘ [%]	P3‘ [%]	P4‘ [%]	P5‘ [%]	P6‘ [%]
A	2.7	0.9	2.7	4.8	7.8	13.3	12.0	12.0	9.2	10.6	8.1	10.6
C	0.5	0.4	0.5	0.5	0.2	1.1	0.2	0.5	0.7	1.6	1.2	0.5
D	1.2	1.1	1.1	1.2	1.1	1.4	3.4	3.2	5.3	4.8	4.8	6.2
E	1.1	0.9	1.2	1.1	0.9	0.5	2.8	4.6	6.9	6.0	6.4	4.8
F	1.4	1.2	1.1	1.8	2.8	10.6	0.5	2.5	4.6	4.2	1.9	2.8
G	2.7	3.9	4.4	0.9	4.1	3.2	11.8	8.8	7.2	9.0	6.5	7.6
H	0.4	0.7	0.2	1.8	0.9	3.4	10.1	3.7	5.1	4.2	4.2	4.6
I	1.6	1.2	2.5	3.0	2.3	3.7	7.2	7.6	6.7	8.5	7.6	5.8
K	0.0	0.0	0.0	0.0	0.2	0.0	1.1	1.2	0.5	0.2	0.7	2.1
L	1.4	3.0	1.8	2.8	6.9	11.0	3.9	11.3	10.1	9.2	10.2	10.6
M	1.1	1.2	1.9	1.2	3.5	18.6	0.9	1.6	1.4	0.5	2.1	1.4
N	0.5	1.4	1.1	1.9	1.6	3.4	4.1	3.7	4.4	4.2	5.1	4.2
P	2.5	1.2	1.8	0.9	3.0	1.2	0.5	10.6	8.3	7.2	9.9	8.5
Q	0.4	1.1	1.4	2.1	2.8	1.4	3.5	4.6	3.9	5.3	4.6	4.9
R	0.2	0.2	0.2	0.2	0.0	0.0	2.5	1.4	1.8	1.8	1.9	5.3
S	1.8	0.9	2.7	2.3	4.1	3.2	12.4	3.7	5.1	4.1	4.9	4.1
T	0.7	1.4	2.3	4.8	6.4	6.2	13.8	5.1	6.7	6.7	6.4	6.5
V	1.4	3.4	1.6	4.6	3.2	5.5	6.4	11.8	9.4	9.5	8.5	6.4
W	0.2	0.2	0.4	0.5	0.4	0.9	0.2	0.7	0.5	0.2	0.9	0.0
Y	0.7	0.4	1.4	1.6	2.3	11.3	2.8	1.2	2.1	2.1	3.9	1.9

Table S3: Overview of peptides and probes detailed in this manuscript. Sequence abbreviations: The one-letter amino acid code was used, except for N-terminal acetylation (Ac) and for FRET labels on the respective amino acid: 4-((4-(dimethylamino)phenyl)azo)benzoic acid (DABCYL) and 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS).

Abbreviation	Sequence	Characterization	Cleavage [%]	Standard deviation [%]
a	RRARSVAS	Figure S2	77.07	2.06
b	SSARSVAS	Figure S2	4.62	2.21
1	RTAMTAGA	Figure S3	26.16	2.19
2	RTAMSAGA	Figure S3	26.79	2.71
3	RTAMRAGA	Figure S3	7.40	0.74
4	RTAGSAGA	Figure S3	7.61	1.08
5	RTAMGAGA	Figure S3	3.39	1.58
6	GTAMTAGA	Figure S3	–2.36	0.86
7	GTAMGAGA	Figure S3	3.43	1.15
Probe a	Ac-K(DABCYL)-RRARSVAS-E(EDANS)	Figure S4	-	-
Probe 1	Ac-EG-K(DABCYL)-RTAMTAGA-E(EDANS)-GE	Figure S4	-	-
Probe a1	K-(DABCYL)-RRAR	Figure S4	-	-
Probe a2	SVAS-E(EDANS)	Figure S4	-	-

Table S4: *Overview of tissue samples from HNSCC patients. The corresponding microscopy images are shown in Figure S5.*

Patient	Cleavage experiment	Western blot	Comment
1	Figure S9 (right)	Lanes 3 & 7	
2	Figure S12		
3	Figure 3F	Lanes 2 & 5	
4	Figure 3G		
5	Figure S10 (left)		
6	Figure S10 (right) & Figure S13		
7	Data not shown	Lane 4	
8	Figure S9 (left)		
9	Figures 3E & S8		
10	Figure S11		

Table S5: In-silico determination of protease activity against RRARSVAS (sequence a) via Prot-Agonist.[3, 4] The norm score is defined as the sum of amino acid occurrences at each position divided by the number of known cleavage sites per position. The 18 proteases with the highest norm scores are displayed. The cleavage pattern shows the preferred amino acids in positions P4–P4' according to the MEROPS database.[3, 4] The ↓ arrow marks the cleavage site. X represents any amino acid without preferences. Parentheses indicate that different amino acids may occur in that position. Strong preferences are indicated by uppercase letters, weak preferences by lowercase letters. HNSCC: head and neck squamous cell carcinoma; OSCC: oral squamous cell carcinoma.

MEROPS ID[3, 4]	**Gene name**	**Norm score (cleavage position)**	**Cleavage pattern[3, 4]**	**Potential relevance (predicted location[5, 6])**
S08.075	PCSK6	2.7 (P1↓P1')	RX(KR)R↓s(val)XX	Overexpressed in OSCC[7] (intracellular and secreted)
S08.074	PCSK4	2.6 (P1↓P1')	RX(KR)R↓s(val)XX	-
S08.071	Furin	2.6 (P1↓P1') 1.6 (P3↓P2)	RX(Kr)R↓svXX	Overexpressed in OSCC[8] (plasma membrane)
S08.077	PCSK7	2.5 (P1↓P1')	RX(KR)R↓s(val)XX	-
S08.076	PCSK5	2.5 (P1↓P1')	RX(KR)R↓s(val)XX	-
M01.010	NPEPPS	2.0 (P1'↓P2')	XXX(qkl)↓qQQQ	Overexpressed in HNSCC[9] (intracellular)
S08.073	PCSK2	2.0 (P1↓P1')	rX(Kr)R↓XXXX	-
S08.063	MBTPS1	1.9 (P1↓P1')	R(rsk)LL↓ (ags)(ft)(fe)(st)	-
S01.302	ST14	1.8 (P1↓P1')	rXXR↓kvXg	Overexpressed in OSCC[10] (nucleoplasm and plasma membrane)
S01.021	TMPRSS11E	1.8 (P1↓P1')	XXXR↓XvXX	Expressed in normal head and neck

				epithelium; decreased expression in HNSCC[11] (plasma membrane and secreted)
C14.026	MALT1	1.8 (P1↓P1')	L(rvi)(SP)R↓GtdX	-
S01.224	HPN	1.8 (P1↓P1')	XXXR↓kgXX	Expressed in normal oral mucosa[5, 6] (intracellular and plasma membrane)
S01.214	F9 (FIX)	1.8 (P1↓P1')	XXgR↓XXXX	-
C19.022	USP15	1.7 (P1↓P1')	LRGG↓Mhgs	Expressed in normal oral mucosa[5, 6] (intracellular)
S01.213	F11 (FXI)	1.7 (P1↓P1')	(kd)(fl)tR↓(va)(ve)(gt)(gv)	-
S01.047	TMPRSS11D	1.7 (P1↓P1')	XXXR↓gXXX	-
S01.306	KLK13	1.6 (P1↓P1')	vr(sl)R↓(il)Xgg	Expressed in normal oral mucosa[5, 6]; decreased expression in OSCC[12] (secreted)
S01.217	F2	1.6 (P1↓P1')	XX(palg)R↓(sag)XXX	-

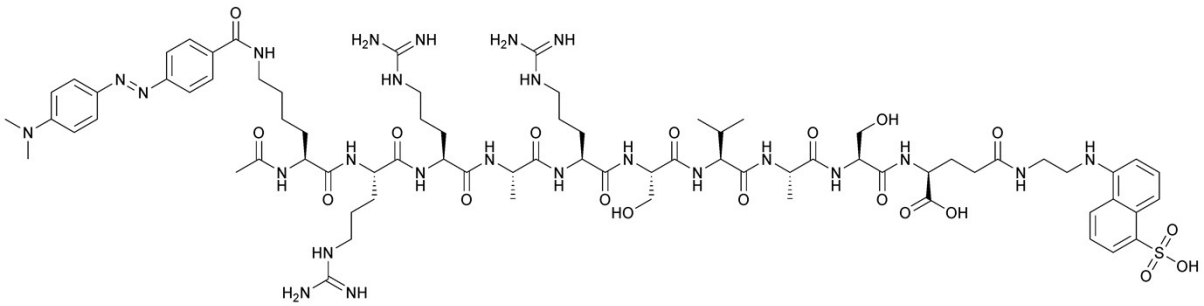
Table S6: In-silico determination of protease activity against RTAMTAGA (sequence 1) via Prot-Agonist.[3, 4] The norm score is defined as the sum of amino acid occurrences at each position divided by the number of known cleavage sites per position. The 18 proteases with the highest norm scores are displayed. The cleavage pattern shows the preferred amino acids in positions P4–P4' according to the MEROPS database[2,3]. The ↓ arrow marks the cleavage site. X represents any amino acid without preferences. Parentheses indicate that different amino acids may occur in that position. Strong preferences are indicated by uppercase letters, weak preferences by lowercase letters. HNSCC: head and neck squamous cell carcinoma; OSCC: oral squamous cell carcinoma.

MEROPS ID[3, 4]	**Gene name**	**Norm score (cleavage**	**Cleavage pattern[3, 4]**	**Potential relevance (predicted location[5, 6])**

		site)		
C19.022	USP15	1.6 (P2↓P1)	LRGG↓Mhgs	Expressed in oral mucosa[5, 6] (intracellular)
M24.028	METAP1D	1.4 (P1↓P1')	XXXM↓(Gastv)dv(ea)	RNA expressed in esophagus and tongue[5, 6] (intracellular)
S08.075	PCSK6	1.3 (P1↓P1')	RX(KR)R↓s(val)XX	Overexpressed in OSCC[7] (intracellular and secreted)
C19.001	USP5	1.3 (P2↓P1)	LRGG↓kXXX	Expressed in oral mucosa[5, 6] (intracellular)
S01.357	TMPRSS9	1.3 (P2↓P1)	XXX(Rk)↓XXXX	-
S08.074	PCSK4	1.3 (P1↓P1')	RX(KR)R↓s(val)XX	-
S08.077	PCSK7	1.3 (P1↓P1')	RX(KR)R↓s(val)XX	-
S08.076	PCSK5	1.3 (P1↓P1')	RX(KR)R↓s(val)XX	-
S08.071	Furin	1.3 (P1↓P1')	RX(Kr)R↓svXX	Overexpressed in OSCC[8] (plasma membrane)
C19.010	USP4	1.2 (P2↓P1)	LRGG↓XXXX	Overexpressed in HNSCC[13] (intracellular and plasma membrane)

M24.002	METAP2	1.2 (P1↓P1')	XXXM↓(asvpt)XXX	Potential role in OSCC due to interaction with overexpressed S1004[14, 15] (intracellular and plasma membrane)
C48.008	SENP5	1.2 (P1↓P1')	QTGG↓KXeX	Overexpressed in OSCC[16] (intracellular)
S01.357	polyserase-IA unit 1	1.2 (P1↓P1')	XXX(Rk)↓XXXX	-
S08.063	MBTPS1	1.2 (P1↓P1')	R(rsk)LL↓ (ags)(ft)(fe)(st)	-
M01.010	NPEPPS	1.1 (P1'↓P2')	XXX(qkl)↓qQQQ	Overexpressed in HNSCC[9] (intracellular)
S01.214	F9 (FIX)	1.1 (P2↓P1)	XXgR↓XXXX	-
C19.016	USP7. HAUSP	1.1 (P2↓P1)	LRGG↓KX(eg)X	Overexpressed in HNSCC[17] (intracellular)
S01.072	TMPRSS7	1.1 (P1↓P1')	XXXR↓kXXX	-

Ac-Lys(Dabcyl)-Arg-Arg-Ala-Arg-Ser-Val-Ala-Ser-Glu(Edans)



Ac-Glu-Gly-Lys(Dabcyl)-Arg-Thr-Ala-Met-Thr-Ala-Gly-Ala-Glu(Edans)-Gly-Glu

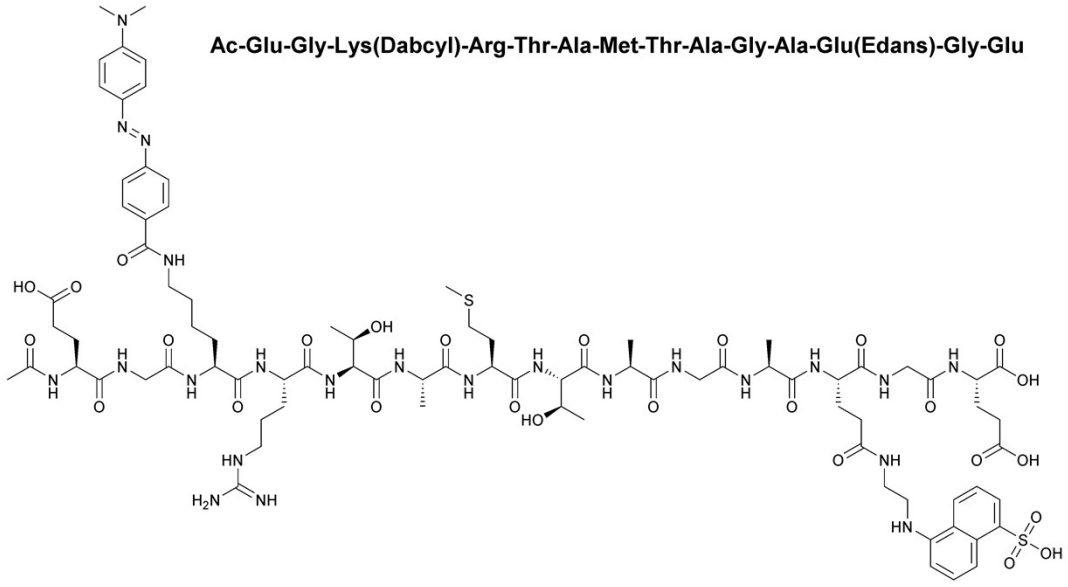


Figure S1: Chemical structure of Probe a and Probe 1.

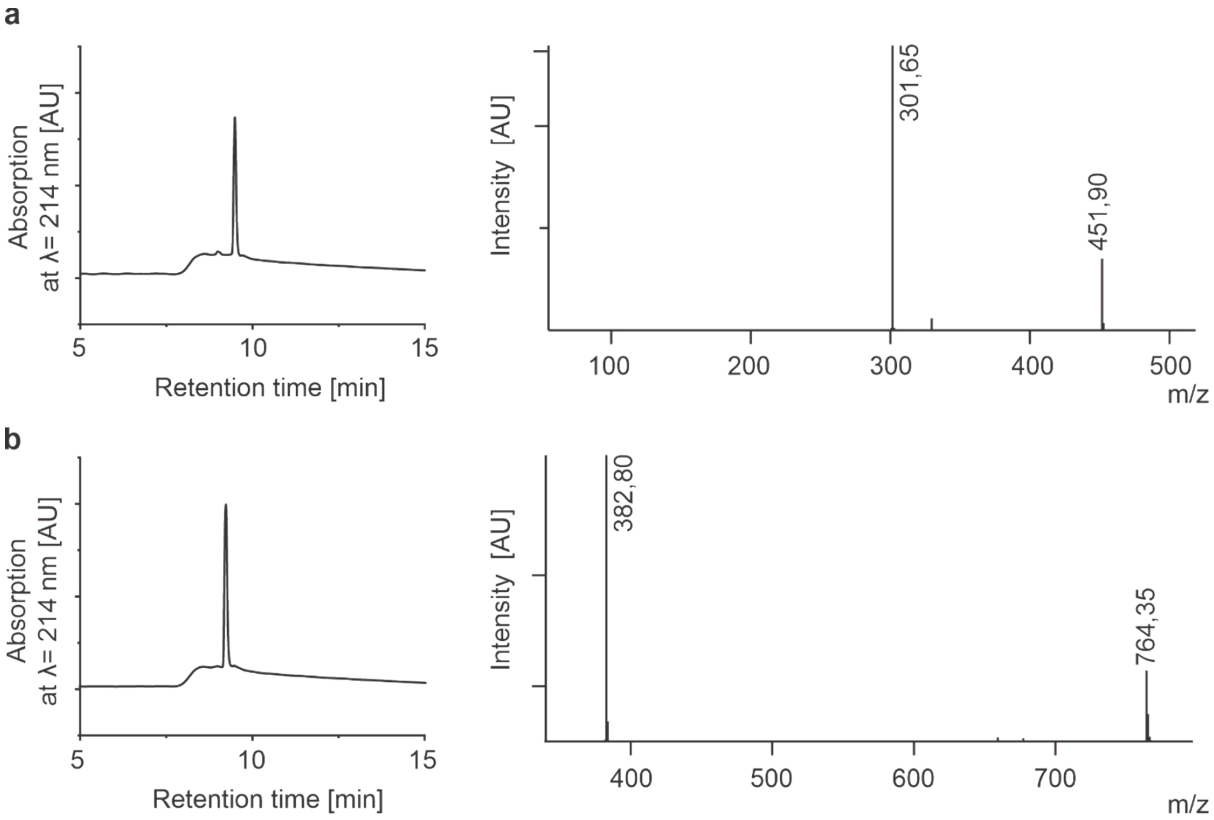
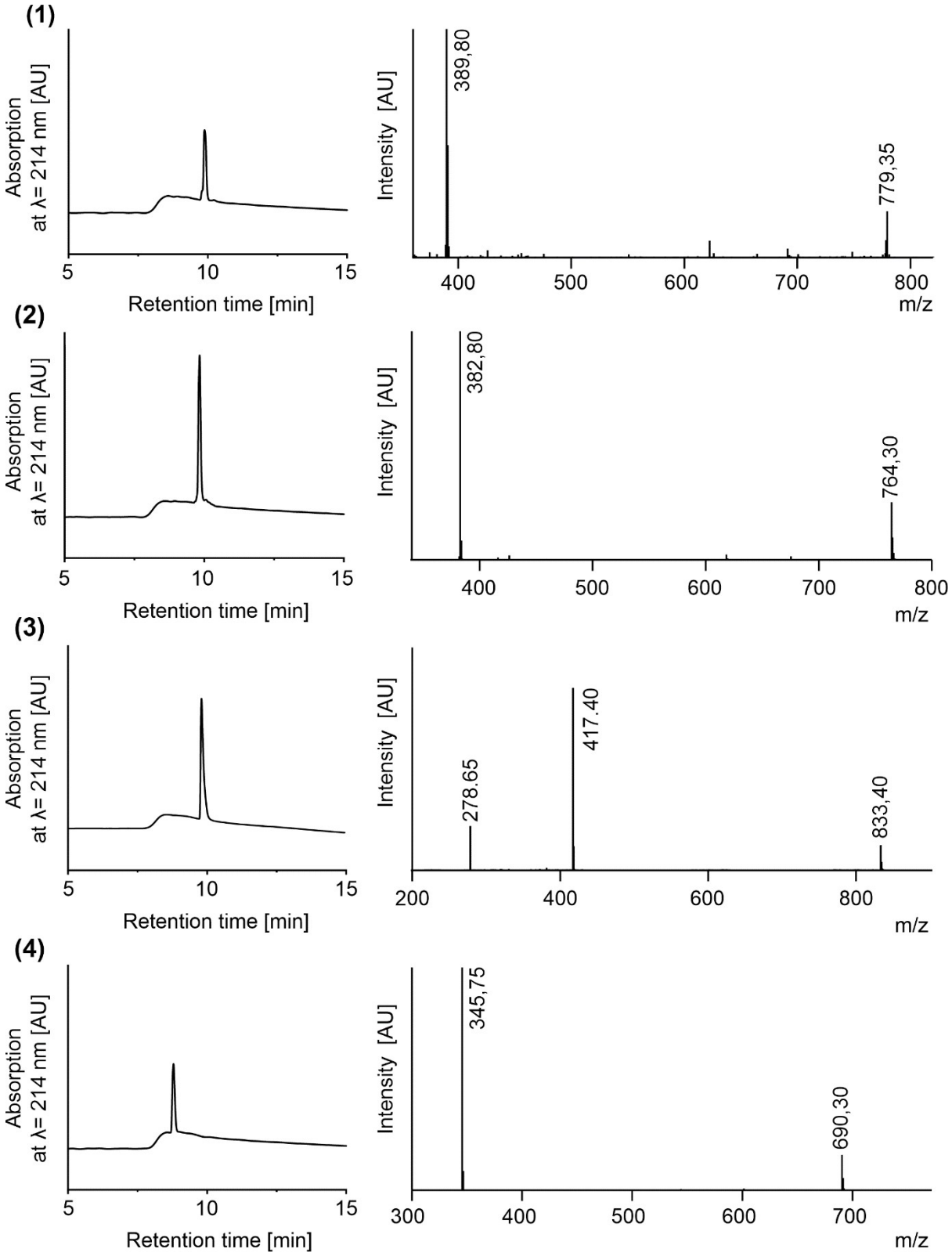


Figure S2: Characterization of peptides **a** and **b**. HPLC and LC–MS analysis of sequence **a** indicate a purity of > 95% and an observed m/z of 451.90 (z = 2) for a calculated m/z of 451.75 (z = 2). HPLC and LC–MS analysis of Sequence **b** indicate a purity of > 95% and an observed m/z of 764.35 for a calculated m/z of 764.37.


(1)
Absorption at λ= 214 nm [AU]
Retention time [min]
Intensity [AU]
389,80
779,35
m/z
(2)
Absorption at λ= 214 nm [AU]
Retention time [min]
Intensity [AU]
382,80
764,30
m/z
(3)
Absorption at λ= 214 nm [AU]
Retention time [min]
Intensity [AU]
278.65
417.40
833,40
m/z
(4)
Absorption at λ= 214 nm [AU]
Retention time [min]
Intensity [AU]
345,75
690,30
m/z

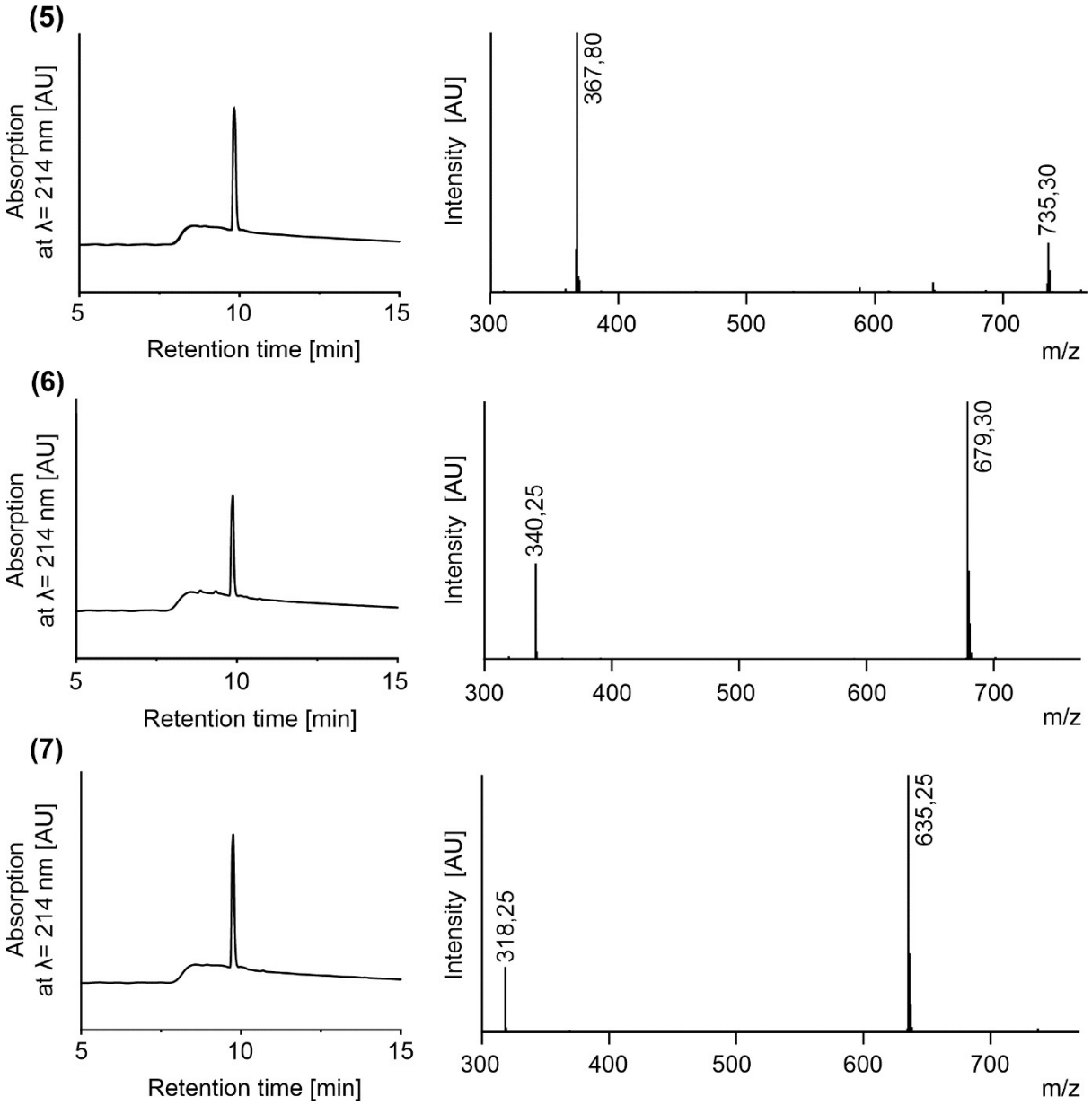


Figure S3: HPLC and LC–MS characterization of Peptide Sequences **1–7**. All peptides were synthesized with a purity of > 95% according to HPLC chromatogram (left). Observed mass m/z of (1) is 779.35 (calculated m/z = 778.37). Observed m/z of (2) is 764.30 (calculated m/z = 764.35). Observed m/z of (3) is 833.40 (calculated m/z = 833.42). Observed m/z of (4) is 690.30 (calculated m/z = 690.34). Observed m/z of (5) is 735.30 (calculated m/z = 734.34). Observed m/z of (6) is 679.30 (calculated m/z = 679.29). Observed m/z of (7) is 635.25 (calculated m/z = 635.26).

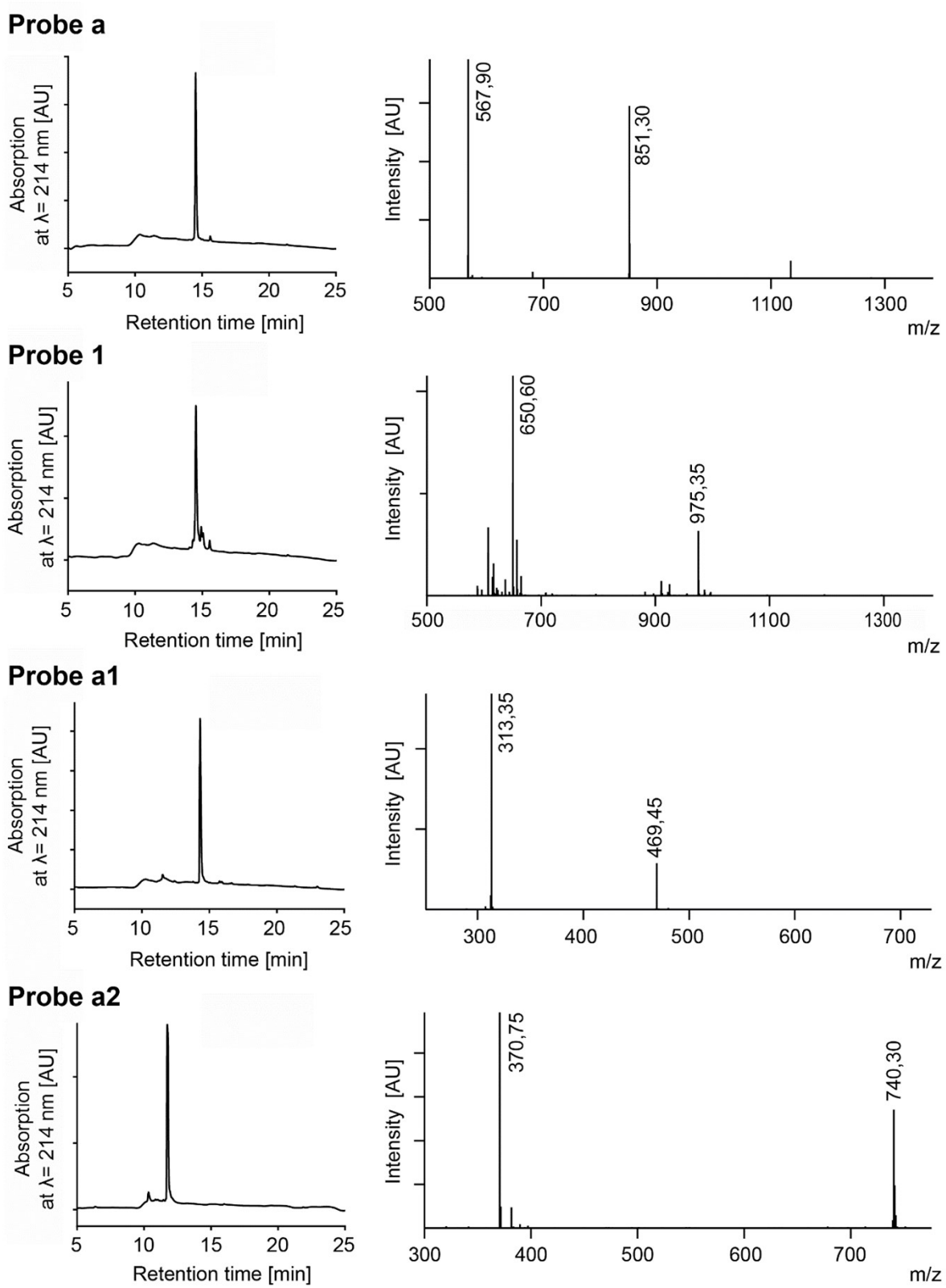


Figure S4: HPLC and LC–MS characterization of **Probe a** and **Probe 1** and synthesized cleavage fragments **Probe a1** and **Probe a2**. HPLC and LC–MS analysis of **Probe a** indicates a purity of > 95% and an observed m/z of 851.30 (z =2) for a calculated m/z of 851.11 (z = 2). **Probe 1** was synthesized with a purity of > 75% and an m/z of 975.35 (calculated m/z = 975.60; z = 2). **Probe a1** was synthesized with a purity of > 83% and an observed m/z of 469.45 (calculated m/z = 469.36; z = 2). **Probe a2** was synthesized with a purity of > 93% and an observed m/z of 740.30 (calculated m/z = 740.48; z = 2).

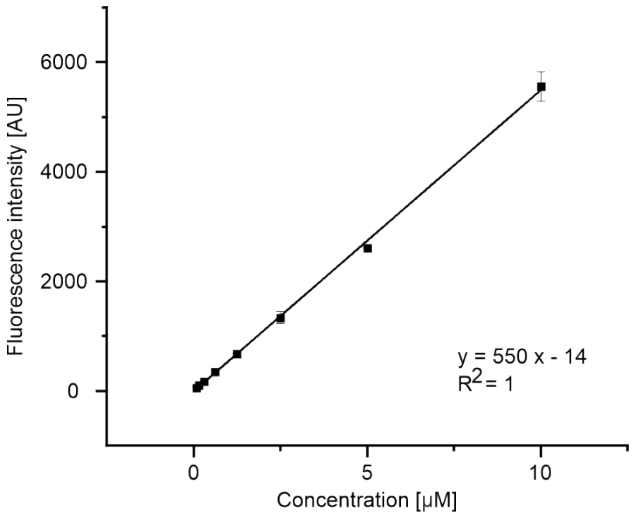
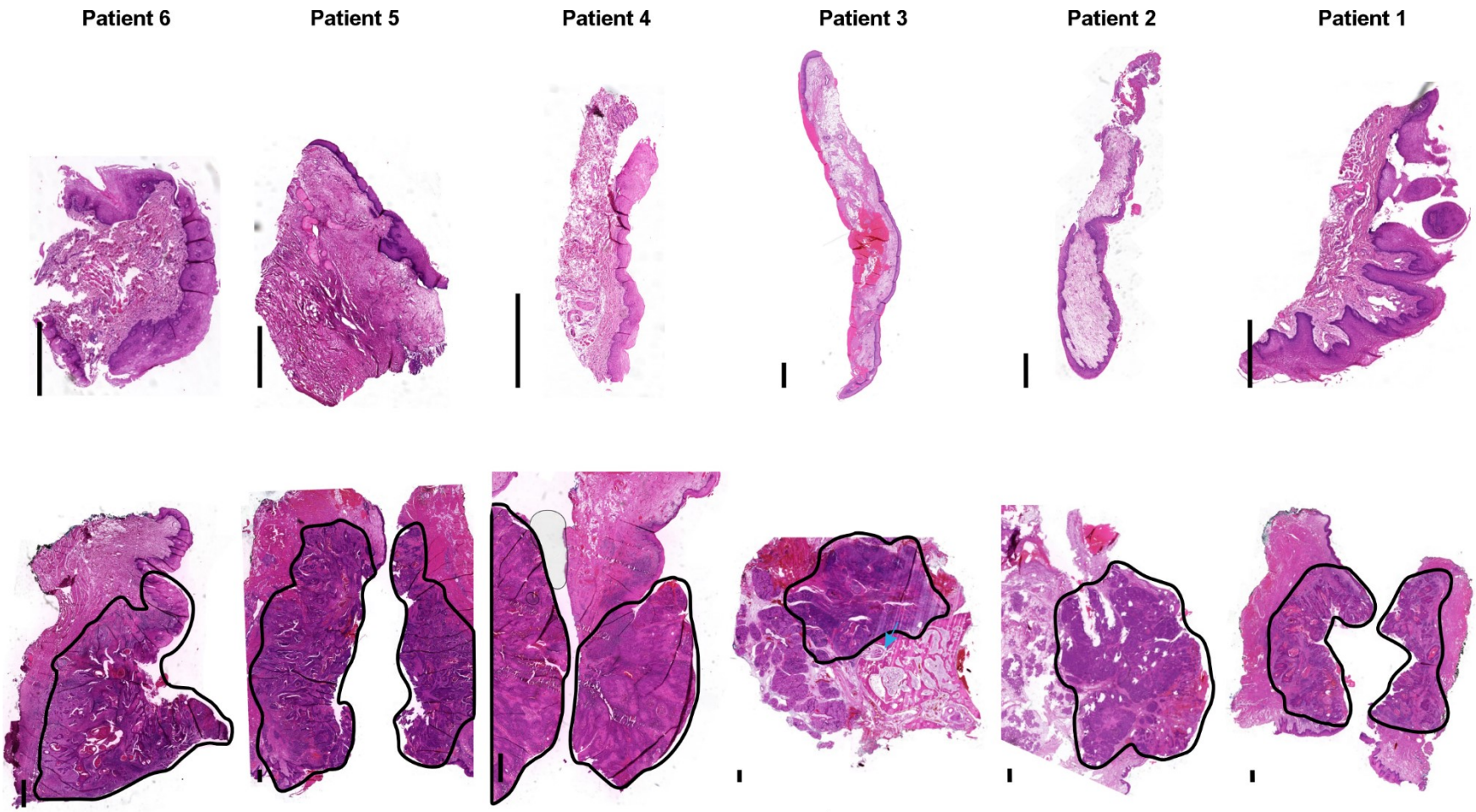


Figure S5: Concentration series of fragments **Probe a1** and **Probe a2** (1 µM represents 1 µM of each fragment). The excitation and emission wavelengths were set to 340 nm and 490 nm, respectively.


Patient 6
Patient 5
Patient 4
Patient 3
Patient 2
Patient 1

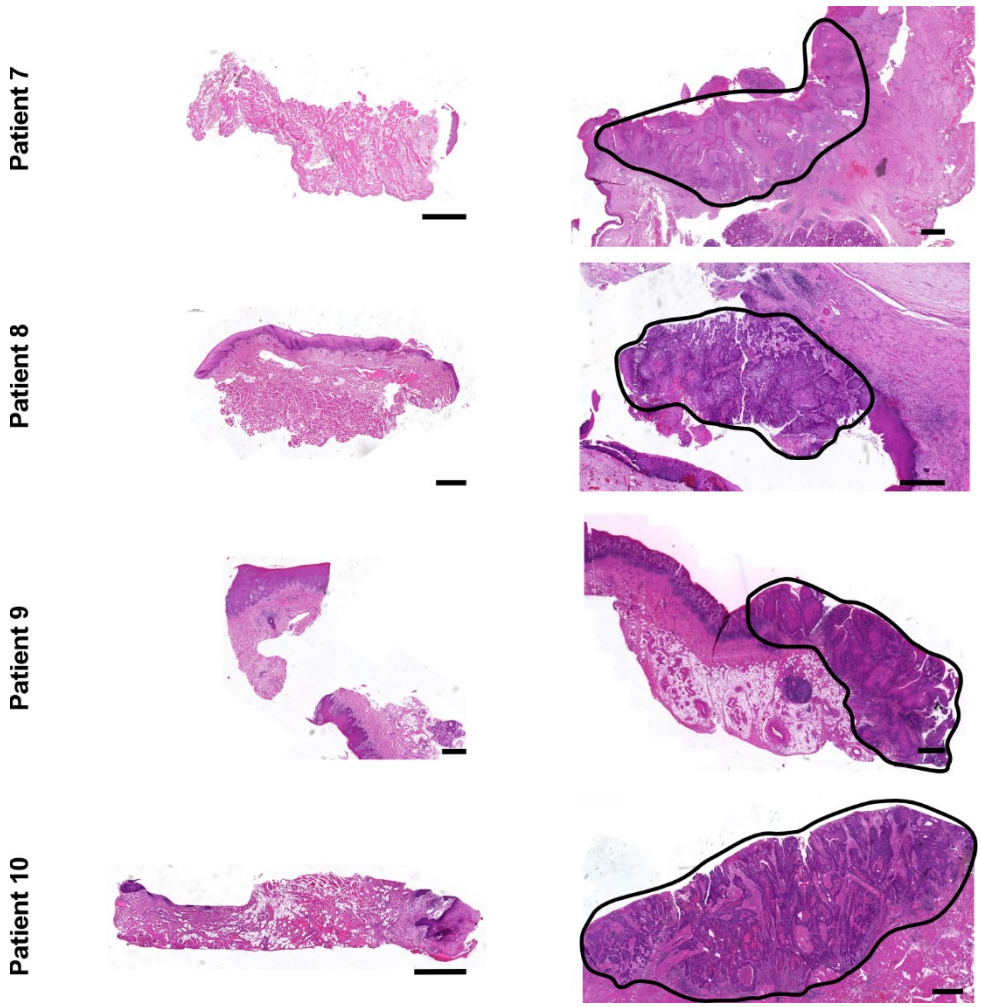


Figure S6: Microscopy images of collected healthy tissue (left) and HNSCC tissue (right). The black markings illustrate the morphologically abnormal tissue, which corresponds to squamous cell carcinomas and was separated from the surrounding tissue for further experiments. The tumor tissue is characterized by irregular squamous epithelial proliferations with variable and irregular keratinization (scale bar = 1 mm). Most cases show an abrupt junction between tumor tissue and healthy tissue. In Patient 3, bone can be seen adjacent to the carcinoma as a result of osseous tumor infiltration (blue arrow). Patient 10 is shown in Figure 2.

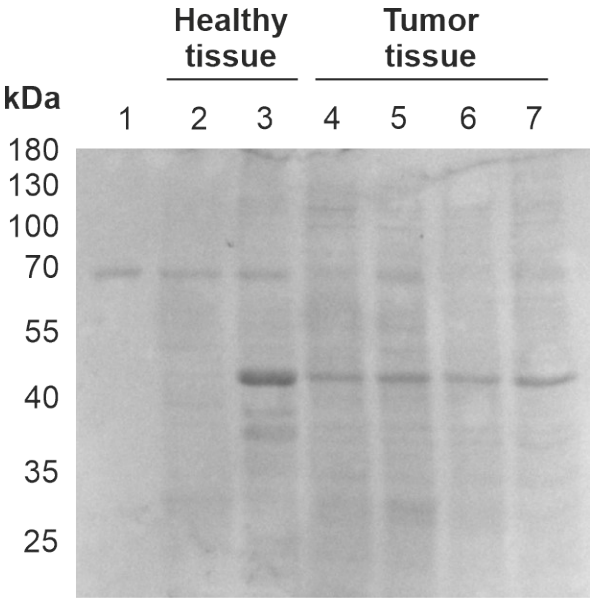


Figure S7: Ponceau S staining of western blot membrane.

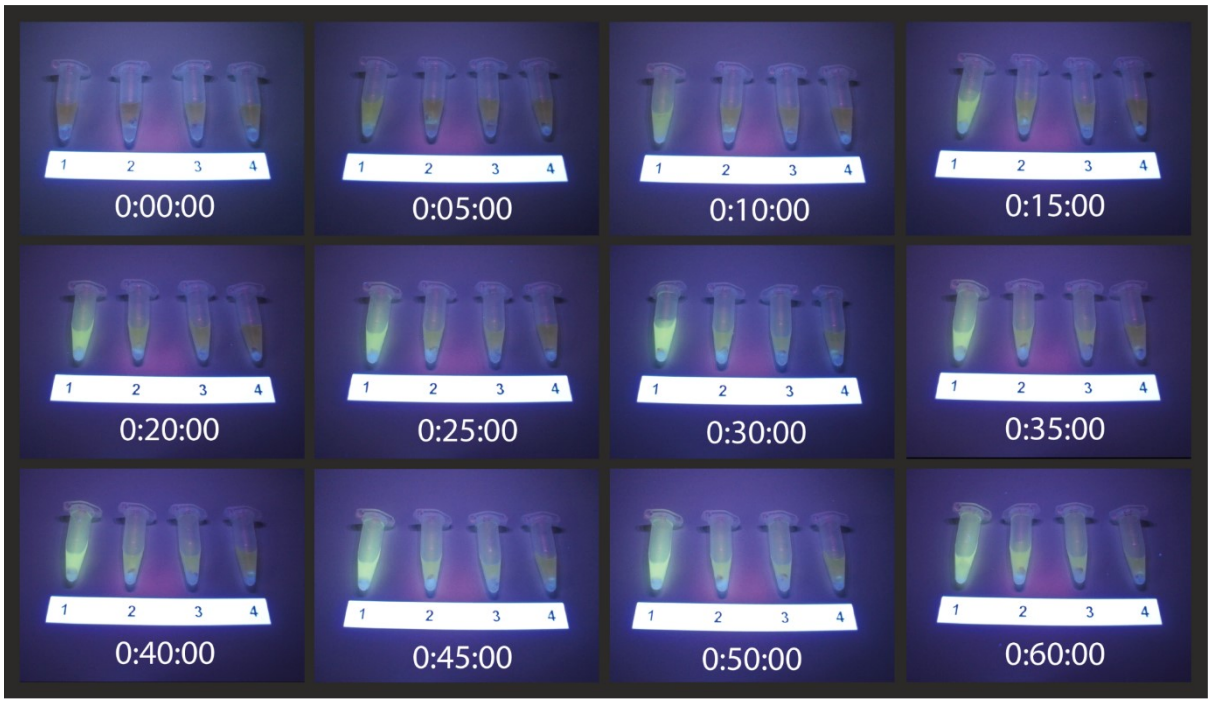


Figure S8: Timelapse incubation of tissues with probe. **Probe a** was incubated with tumor tissue (1) and healthy tissue (2). **Probe 1** was incubated with tumor tissue (3) and healthy tissue (4). The time points 0, 10, 20, and 50 min are shown in Figure 2.

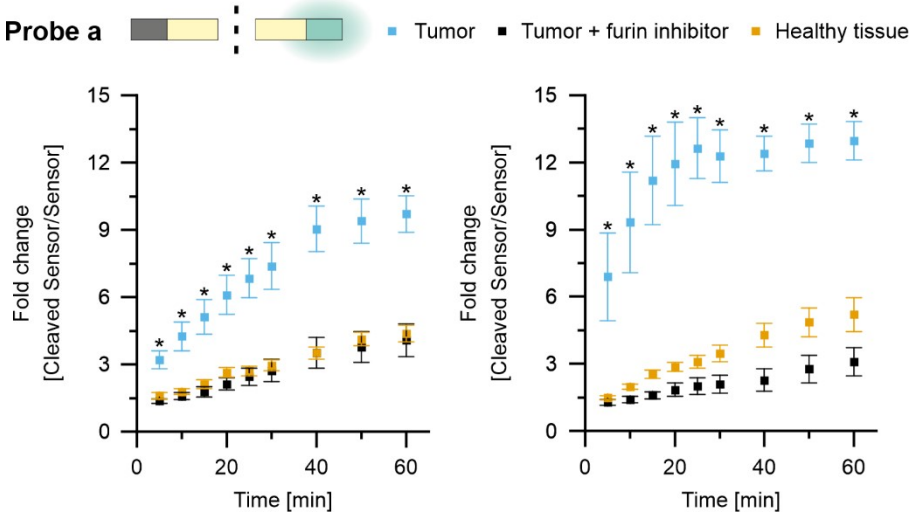


Figure S9: Time-dependent cleavage of **Probe a** by healthy tissue (orange), tumor tissue (blue), and tumor tissue in the presence of the furin inhibitor (black). Results are presented as means ± SD, n = 4 for tumor; n = 3 for healthy tissue. Pairwise comparisons of tumor tissue and healthy tissue by Student t-test; results were considered statistically significant at $p \leq 0.05$(*).

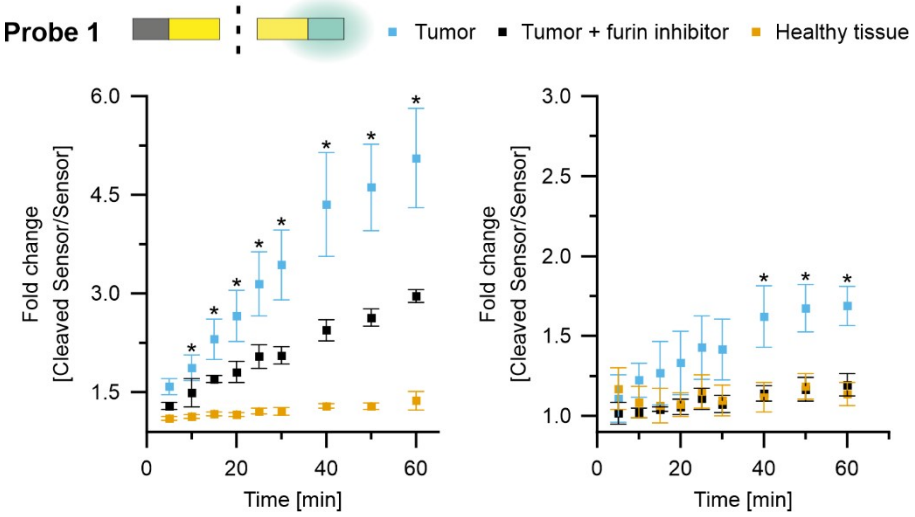


Figure S10: Time-dependent cleavage of **Probe 1** by healthy tissue (orange), tumor tissue (blue), and tumor tissue in the presence of the furin inhibitor (black). Results are presented as means ± SD, n = 3. Pairwise comparisons of tumor tissue and healthy tissue by Student t-test; results were considered statistically significant at $p \leq 0.05$(*).

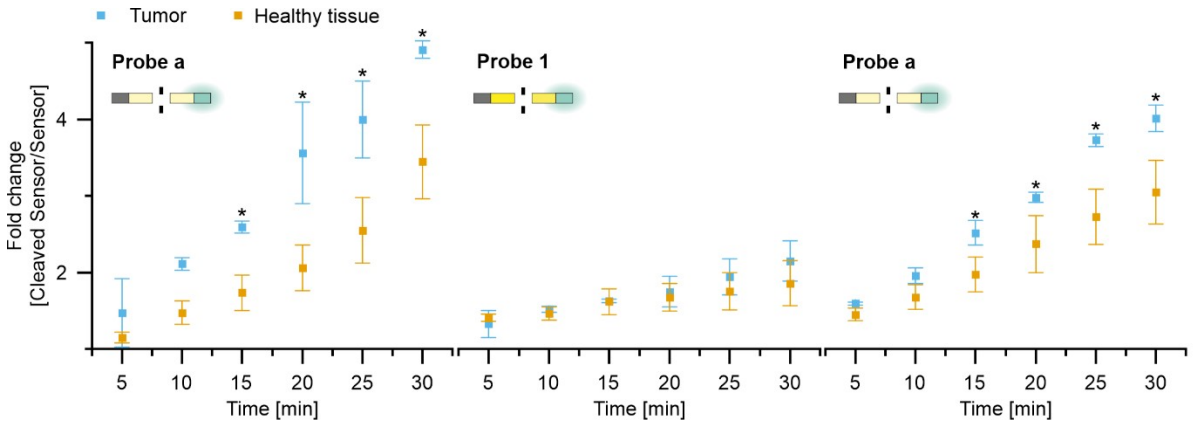


Figure S11: Comparison of both probes. Tissue samples were first incubated with **Probe a**, followed by incubation with **Probe 1** and a third incubation with **Probe a**. Pairwise comparisons by Student t-test; means ± standard deviation, n = 3; results were considered statistically significant at $p \leq 0.05$(*).

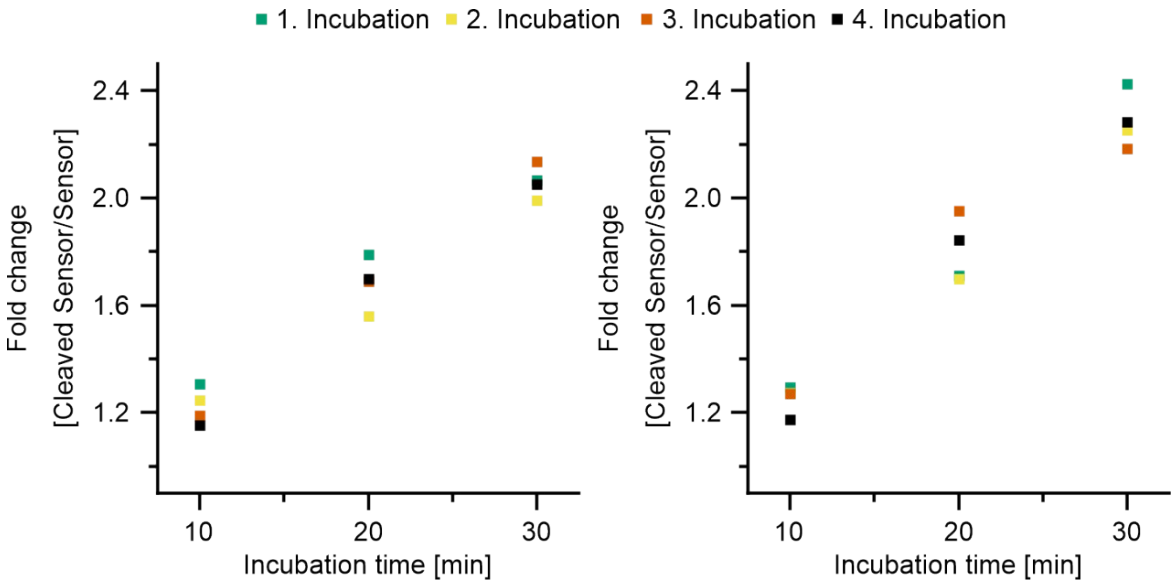


Figure S12: Stability of **Probe a** cleavage by tumor tissue over time. Two tumor specimens were incubated with **Probe a** four times each.

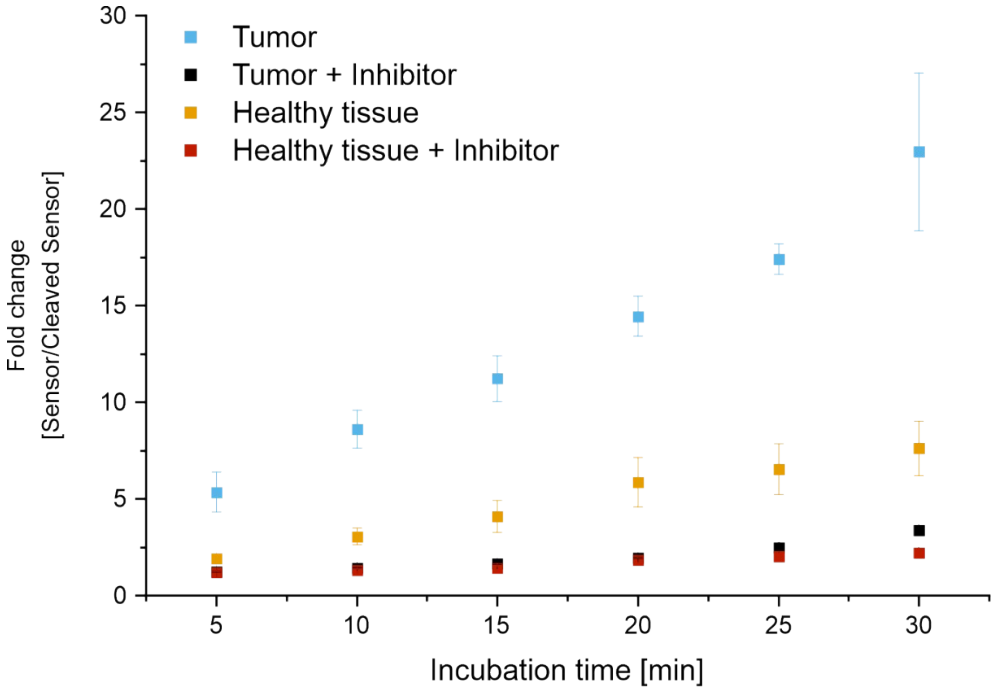


Figure S13: Cleavage of **Probe a** by tumor tissue and healthy tissue in the presence of a broad-range protease-inhibitor mixture. Results are presented as means ± SD, n = 3.

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