

SUPPORTING INFORMATION FOR:

**A fluorescent light up probe as an inhibitor for
intracellular β -tryptase**

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1. General Synthetic Information:

All solvents were dried and distilled under argon before use. Pyrene-2-carboxylic acid and rhSkin β -Tryptase /Tos-Gly-Pro-Arg-AMC and Trypsin/Z-Phe-Arg-AMC were obtained from Aldrich. Trisisopropylsilane (99 %), Fmoc-protected amino acids and PyBOP (95%) were supplied from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). All melting points were measured with a Bruker Melting-Point B-450 apparatus with open end glass capillary tube. The melting points are not corrected. NMR-spectra were recorded at room temperature with a Bruker DRX 500 spectrometer. All IR spectra were measured on a *Jasco* FT/IR-430 spectrometer. All mass spectra were recorded with a Bruker BioTOF III spectrometer. Analytical "High Performance Liquid Chromatography" (HPLC) was done with the following parameters: Dionex HPLC system: P680 pump, ASI-100 automated sample injector, UVD-340U UV detector, UltiMate 3000 Column Compartment; Software: Dionex Chromeleon 6.80; Column: YMC-Pack ODS-A, reversed phase RP18, 150 mm length, 3.0 mm diameter, 5 μ m, 12 nm; type: AA12S05-1503QT.

2. General Procedures for Peptide Synthesis:

Fmoc Removal: Fmoc protecting groups were cleaved by treatment with 20% piperidine in DMF (2 \times 6 mL, 5 min each) under microwave irradiation (20 W, 50 \pm 5 $^{\circ}$ C, 5 min). The resin was washed with DMF (3 \times 8 mL), DCM (3 \times 8 mL) and DMF (3 \times 8 mL) to remove excess piperidine (each wash ca. 5 min). A positive Kaiser test confirmed the cleavage of the Fmoc group and the formation of a free amino function in each case.

Standard Fmoc solid phase peptide synthesis techniques (SPPS): Each peptide was synthesized on Fmoc Rink amide resin (loading 0.84 mmol/g). The reactions were conducted under microwave irradiation (20 W, 60 \pm 5 $^{\circ}$ C, 20 min), after which the resin was washed with DMF (3 \times 8 mL), DCM (3 \times 8 mL) and DMF (3 \times 8 mL) to remove excess reagents (each wash ca. 5 min). A negative Kaiser test confirmed the attachment of the protected amino acid.

Cleavage from the Resin: Cleavage of the product from the resin was achieved by treatment with a mixture of TFA/H₂O/triisopropylsilane (95:2.5:2.5) for 3 h. The yellow cleavage mixture was collected by filtration and the resin was washed twice with pure TFA (6 mL). The filtrates were combined and concentrated under vacuum to yield an oily residue. The peptide was precipitated by adding dry diethyl ether to the oil. The precipitate was isolated by centrifugation. The crude precipitate was dissolved in water (40 mL), acidified with hydrochloric acid (0.1 N, 5 mL) and lyophilized, yielding the corresponding HCl salts of the peptides as yellow solids. This step was repeated three times.

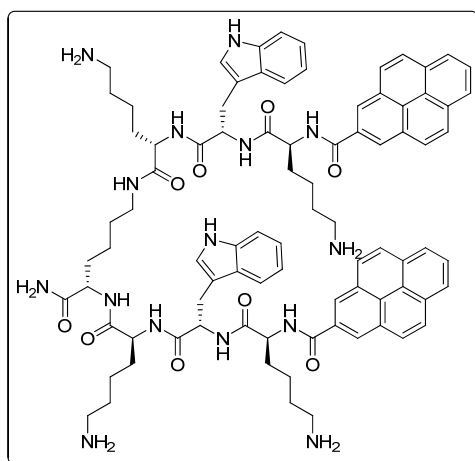
The purity of the peptides was checked by HPLC on a RP18-column using water/MeOH (with 0.05% TFA) as the eluent. If necessary, crude peptides were purified by RP18-MPLC using the same conditions.

The following Fmoc-based amino acids were used: Fmoc-Lys(Boc)-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(pbf)-OH.

3. Synthesis of Inhibitor 1:

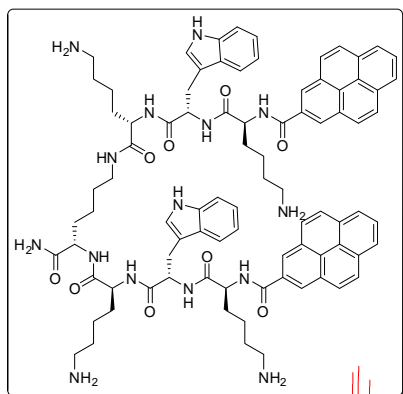
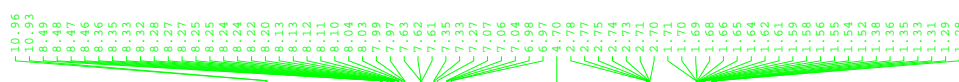
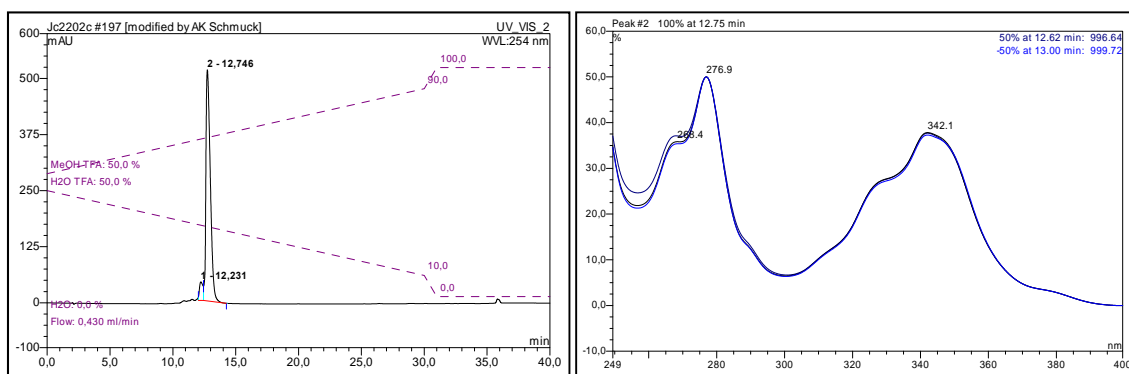
Rink amide resin (200 mg, 84 $\mu\text{mol/g}$, 168 μmol , 1 equiv) was weighed into plastic peptide synthesis vessel and allowed to swell in DCM/DMF (5.0/5.0 mL) for 1.5 h. Then, the Fmoc protection group was removed by agitation with piperidine (20%) in DMF under microwave irradiation. After an intensive wash cycle with DMF the following four amino acids and pyrene-2-carboxylic acid were attached under microwave irradiation following the general procedure: Fmoc-Lys(Fmoc)-OH (0.504 mmol, 3 equiv), PyBOP (0.504 mmol, 3 equiv) and DIPEA (1.01 mmol, 6 equiv); and pyrene-2-carboxylic acid (1.01 mmol, 6 equiv), PyBOP (1.01 mmol, 6 equiv) and DIPEA (2.02 mmol, 12 equiv) in DMF (8.0 mL), respectively. The product was transferred into a glass peptide synthesis vessel and was cleaved from the solid support according to the general procedure.

Peptide 1



Peptide 1 (60.0 mg, 36.7 μmol , Yield 21.9%, purity HPLC 95%). Mp: 210-212 °C. ¹H NMR (500 MHz, DMSO-d₆): δ [ppm] 1.23-1.77(m, 30H), 2.70-2.78(m, 8H), 2.94-3.12(m, 4H), 3.22-3.27(m, 2H), 4.17-4.26(m, 2H), 4.32-4.36(m, 1H), 4.57-4.61(m, 2H), 4.69-4.73(m, 2H), 6.96-6.99(m, 2H), 7.04-7.07(t, $J = 7.5$ Hz, 3H), 7.27(s, 2H), 7.33-7.35(d, $J = 8.0$ Hz, 2H), 7.45(br, 1H, NH), 7.61-7.65(dd, $J = 8.0$ Hz, 2H), 7.76-7.78(t, $J = 6.0$ Hz, 1H), 7.97-8.04(m, 12H), 8.10-8.13(m, 5H), 8.19-8.36(m, 16H), 8.46-8.49(dd, $J = 4.0$ Hz, 2H), 8.82-8.86(dd, $J = 8.0$ Hz, 2H), 10.93(br, 1H, NH), 10.96(br, 1H, NH). ¹³C NMR (125.8 MHz, DMSO-d₆) δ :

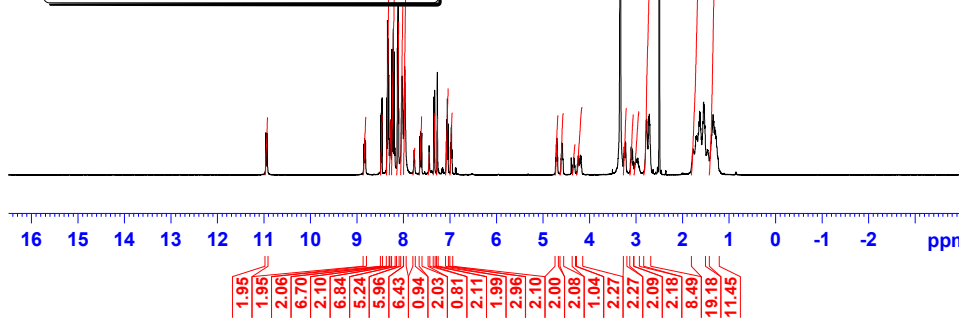
22.0, 22.2, 22.6, 22.7, 26.5, 26.6, 27.5, 28.8, 31.0, 31.3, 31.6, 31.7, 38.5, 38.6, 52.4, 52.5, 52.7, 53.4, 53.8, 109.8, 109.9, 111.3, 118.2, 118.4, 118.5, 120.8, 123.6, 123.7, 124.3, 124.8, 125.4, 125.5, 125.6, 125.8, 126.6, 127.2, 127.4, 127.8, 128.1, 128.3, 130.2, 130.7, 131.5, 131.6, 136.0, 136.1, 169.1, 171.0, 171.2, 171.5, 171.8, 173.7. FT-IR (pure):v [cm⁻¹] 3269, 3050, 2933, 1637, 1518, 1236, 849, 742, 645, 621. HRMS-ESI (m/z): calcd for C₈₆H₉₉N₁₅O₉ 1486.7823, found[M+H]⁺, 1486.7805.



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PROCNO        1
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Time          23.00
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FIDRES        0.157632 Hz
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RG            322.5
DW            48.400 usec
DE            6.00 usec
TE            298.0 K
D1            2.0000000 sec
TD0           1

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PL1           0.00 dB
SF01          500.2730894 MHz
SI            65536
SF            500.2700044 MHz
WDW           EM
SSB           0
LB            0.10 Hz
GB            0
PC            1.00
  
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4. Buffer solution

Depending on the enzyme different buffer systems had to be used. The following table lists the compositions of the buffers used (for 500 mL) and the adjusted pH value:

rhSkin β-Tryptase (pH = 7.4)		Trypsin (pH = 7.4)	
Reagent	Amount	Reagent	Amount
Tris	50 mM, 3.03 g	Tris	50 mM, 3.03 g
NaCl	100 mM, 2.92 g	NaCl	100 mM, 2.92 g
Heparin	50 μ g/mL, 250 μ g	EDTA	5 mM, 731 μ g
Triton-X	0.02%, 100 μ L	Dest. H ₂ O	500 mL
Dest. H ₂ O	500 mL		

5. Fluorescence experiments:

General procedures: All fluorescence and absorption spectra were recorded using a VARIAN Fluorescence Spectrophotometer at 20 °C. The samples were each excited at the wavelength appropriate for the fluorescent peptide **1**. The slit widths were set to 5 nm for excitation and emission. The data points were collected at 1 nm increments with a 0.1 s integration period. All spectra were corrected for intensity using the manufacturer-supplied correction factors and corrected for background fluorescence and absorption by subtracting a blank scan of the buffer system.

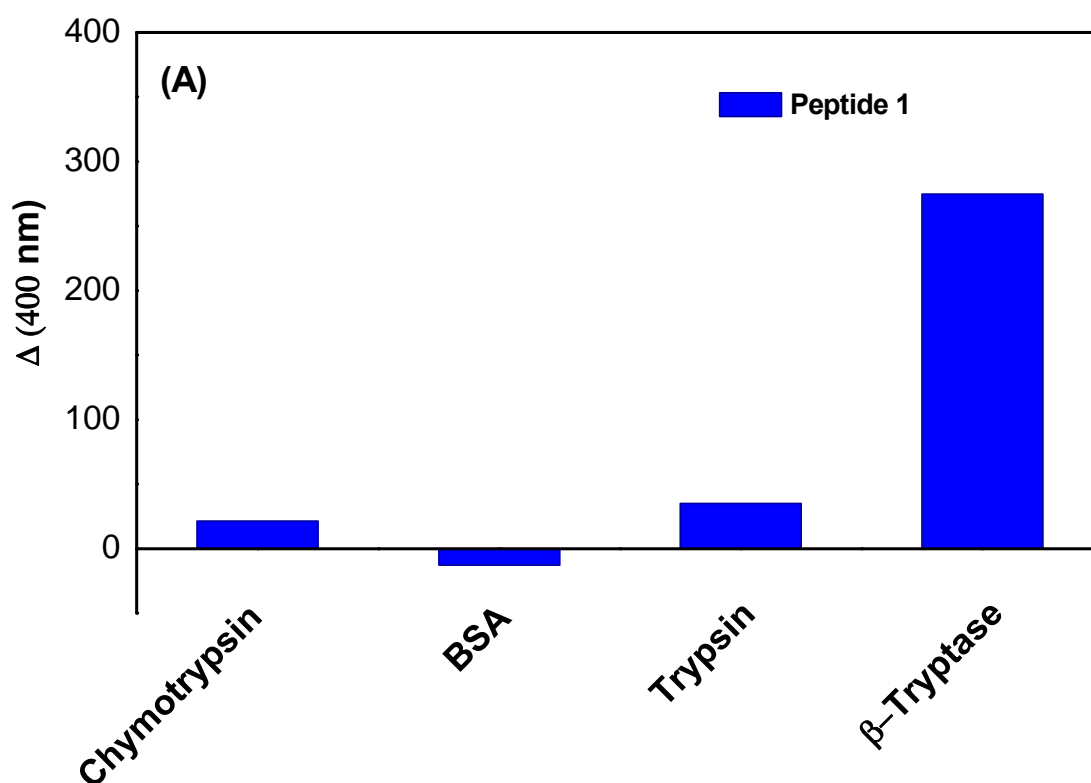


Figure S1. Changes in fluorescence intensity at 400 nm of an aqueous solution (TBS, pH = 7.4) of **1** (500nM) upon addition of as β -tryptase, trypsin, BSA and chymotrypsin, respectively (20 nM, $\lambda_{\text{ex}} = 345 \text{ nm}$).

6. Test for reversibility of enzyme inhibition:

The initial rate of the enzymatic reaction was measured at different enzyme concentrations (rhSkin β -tryptase: 1-10 nM), always with a fixed substrate concentration (Tos-Gly-Pro-Arg-AMC: 2.0 mM). The concentration of the inhibitor **1** was either 10 μ M or 0 (control).

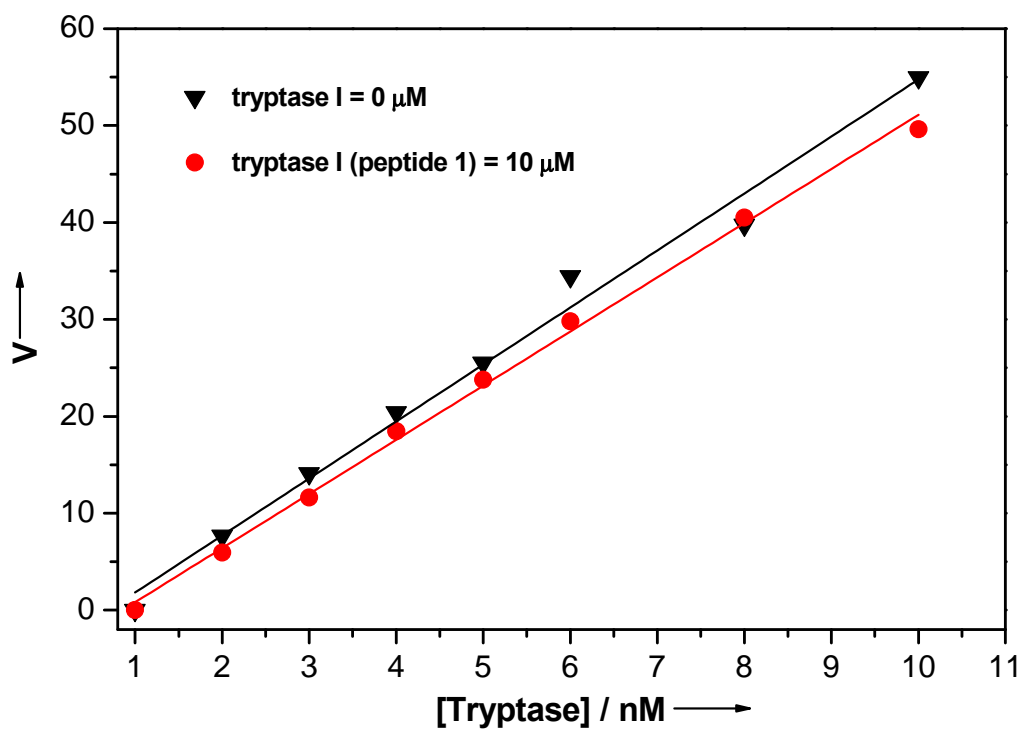


Figure S2. Test for reversible versus irreversible inhibition of the enzyme. Dependence of the initial reaction rate (V_0) on increasing enzyme concentration $[E]$ with or without excess of inhibitor.

7. Determination of K_m for rhSkin β -Tryptase / Tos-Gly-Pro-Arg-AMC:

The Michaelis constant K_m for this enzyme/substrate combination was determined experimentally. Therefore the rate of the enzyme reaction was measured at different substrate concentrations (0-800 μM), always with a fixed enzyme concentration of tryptase (2.0 nM) and without inhibitor. The obtained K_m -value was 300 μM .

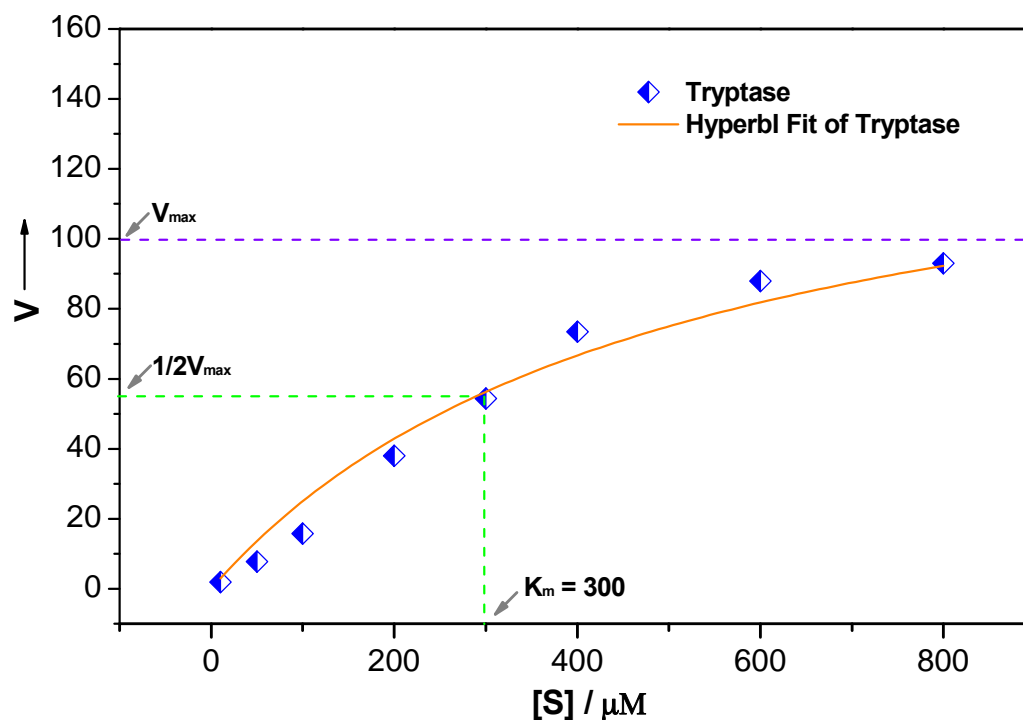


Figure S3. Graph of rate against total substrate concentration for a typical tryptase catalyzed reaction.

8.0 Determination of IC_{50}

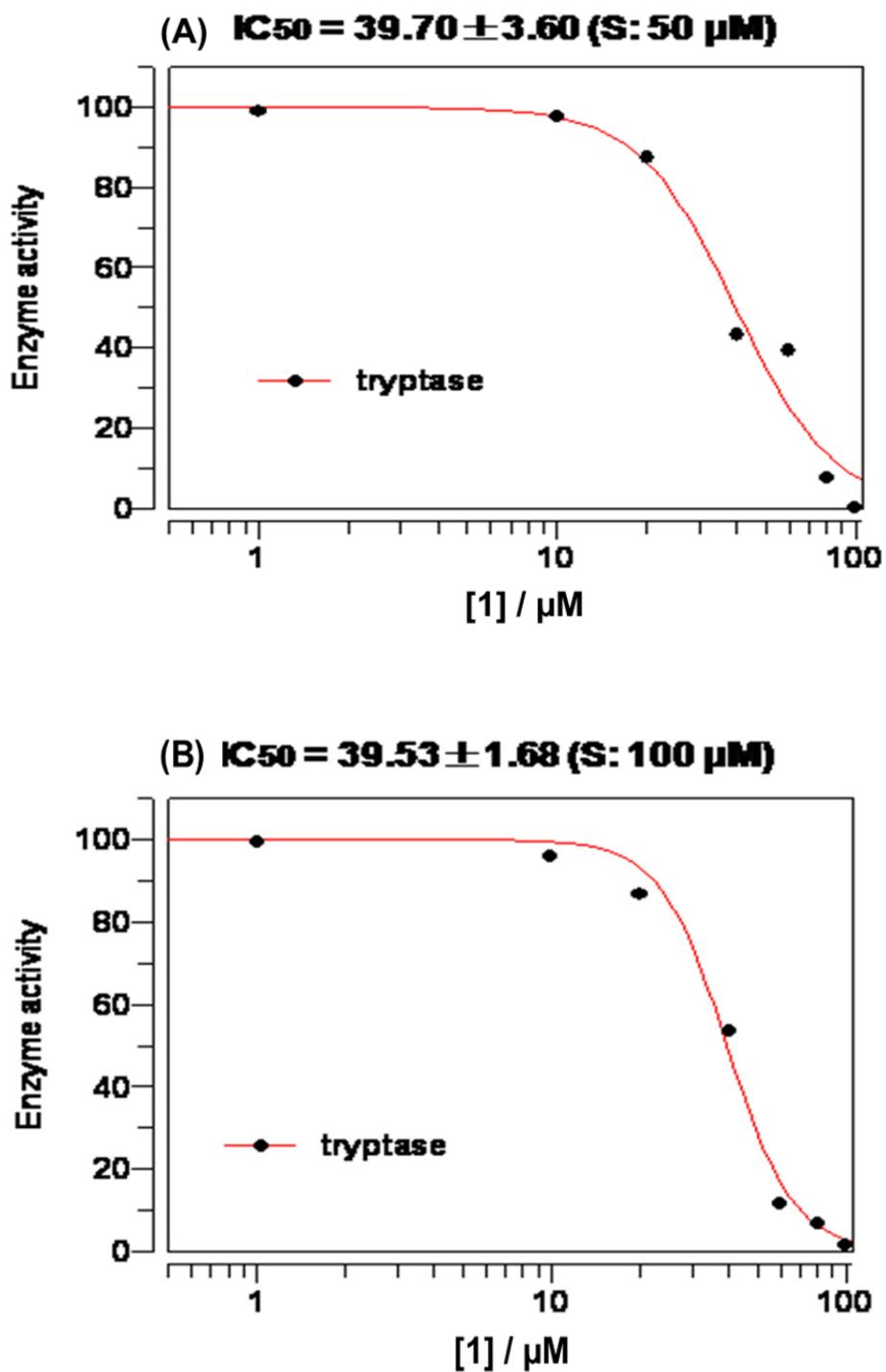


Figure S4. Determination of the IC_{50} values at two different substrate concentrations (50 and 100 μ M). A change of the IC_{50} value for different substrate concentrations is an indicator for competitive inhibition, no change – as in this case - for non-competitive inhibition.

9.0 Molecular Docking

Docking studies were performed using Autodock 4.2 and Autodocktools 4.2. The coordinate .pdbqt file for β -tryptase and trypsin was prepared from pdb 1A0L by adding polar hydrogens and Kollman charges using Autodock tools 4.2. A grid box of 86 \times 96 \times 86 Å centered on the active site of tryptase was determined. Energy minimized pdb-coordinates for each ligand were obtained with ChemBio3D Ultra 12.0. Gasteiger charges were added, non-polar hydrogens were merged and rotatable bonds were set using Autodocktools 4.2 to generate flexible coordinate.pdbqt files for each ligand. The flexible ligand coordinates were docked into the β -tryptase and trypsin coordinates using Autodock Vina employing a grid box consisting of 25000000 points. The resulting docking poses were visualized and overlaid with PyMol.

10.0 Cell Experiments

Cytotoxicity assay: The cytotoxicity on CHMAS cells was studied using a CCK-8 assay. Briefly, CHMAS cells suspension (50 μ L, 6 \times 10⁴ cells/mL) were seeded onto a 96-well plate with a cell density of 3 \times 10⁴ cells/well. Peptide **1** (50 μ L/well) in RPMI 1640 medium was added at concentrations of 0.5, 1, 1.5 and 2 μ M. and the cells were incubated for 24 h at 37 °C under 5 % CO₂. Subsequently, 10 μ L of CCK-8 solution were added and the absorbance was measured 4 h later using a Synergy H4 Hybrid Microplate reader (Biotek, USA) at 450 nm. The following formula was used to calculate the viability of the cells: Viability (%) = (mean absorbance value of treatment group - blank) / (mean absorbance value of control - blank) \times 100. Each sample was processed in triplicate and the IC₅₀ value was obtained from the respective cell viability curves.

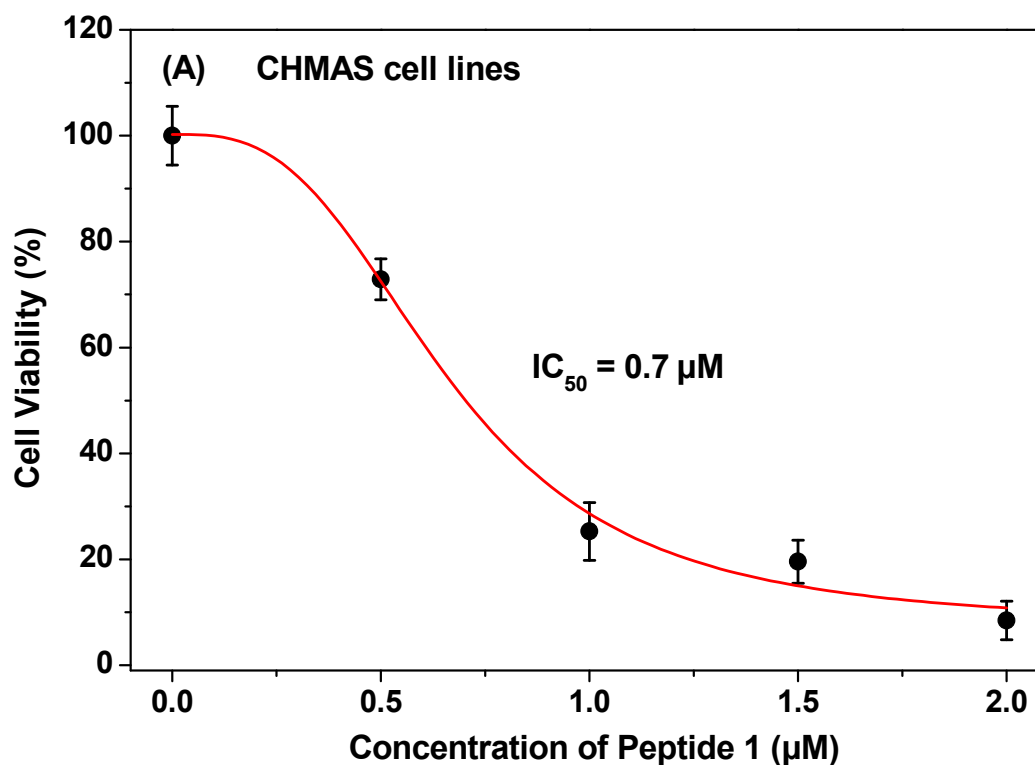


Figure S5. Cell survival curve (A) as measured by CCK-8 assay for peptide **1** against CHMAS cell lines. The cells were seeded at 3×10^4 cells/well on a 96-well plate and incubated with various concentrations of peptide **1** (0.5-2.0 μM) for 24 h at 37 °C. After this incubation time, a CCK-8 assay was performed. The data are presented as mean \pm SD (n = 5).

Confocal laser scanning microscopy (CLSM) images:

CHMAS cells were cultured in RPMI 1640 supplemented with 10 % heat-inactivated FBS. Cell culture was maintained at 37 °C in a humidified condition of 95% air and 5% CO₂ in culture medium. The cells were centrifuged to remove the medium and then incubated with the peptide in RPMI-1640 medium at a final concentration of 10 μM for 30 min at 37 ° C. Afterwards, the cells were washed with PBS and centrifuged three times to remove the peptide. Cell images were taken with a confocal laser scanning microscope (CLSM) Nikon A1 (Japan) with the excitation wavelengths of 404 nm and the emission collected at 410-425 nm.

MTT-assay

The cytotoxicity was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with KB cell lines. Cells growing in log phase were seeded into 96-well cell-culture plate at 3×10^4 cells/well. A solution of peptide 1 (100.0 μL /well) at concentrations of 1, 10, 20 and 40 μM in RPMI-1640 was added to the wells of the treatment group. The cells were incubated for 24 h at 37 °C under 5% CO_2 . A combined solution of 5 mg/mL MTT/PBS (10 μL /well) was added to each well of the 96-well plate assay, and the cells incubated for an additional 4 hours. Formazan extraction was performed with DMSO and its quantity was determined colorimetrically using a Synergy H4 Hybrid Microplate reader (Biotek, USA), which was used to measure the $\text{OD}_{490 \text{ nm}}$ (absorbance value). The following formula was used to calculate the viability of cell growth: $\text{Viability (\%)} = (\text{mean of Absorbance value of treatment group} - \text{blank} / \text{mean Absorbance value of control-blank}) \times 100$. Each sample was processed in quintuplicate and the IC_{50} value was obtained from the respective cell viability curves.

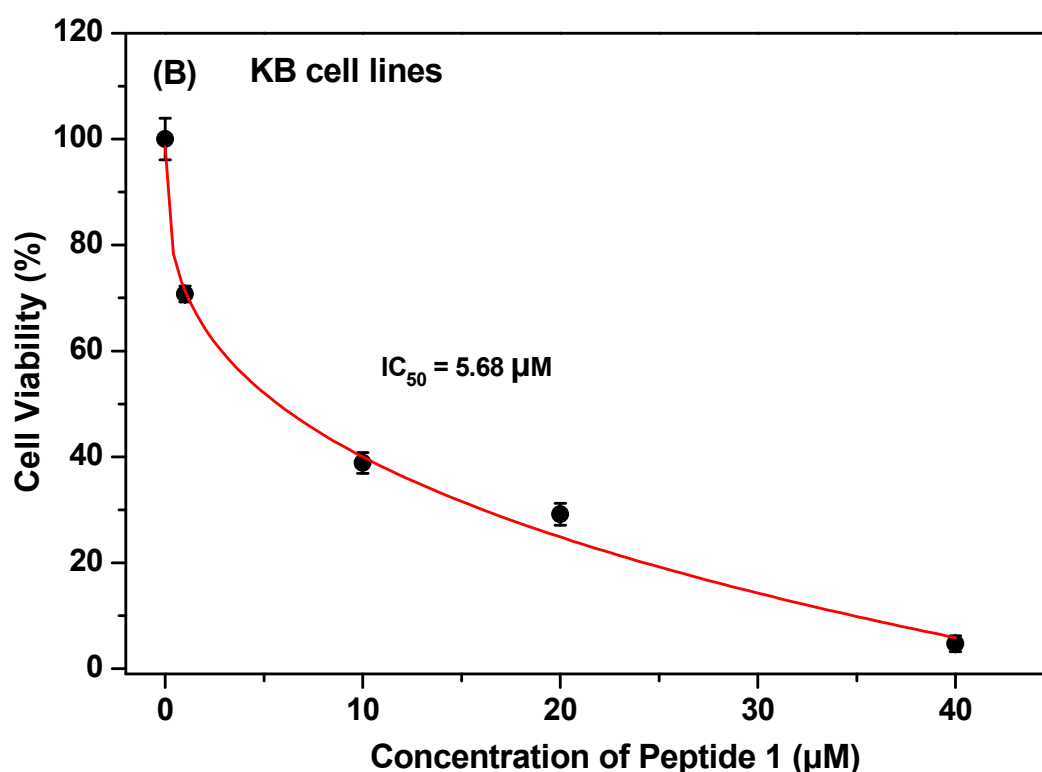


Figure S6. Cell survival curve (B) as measured by MTT assay for peptide 1 against KB cell lines. The cells were seeded at 3×10^4 cells/well on a 96-well plate and incubated with various concentrations of peptide1 for 24 h at 37 °C. After this incubation time, a MTT assay was performed. The data are presented as mean \pm SD (n = 5).