Supporting Information

Optimization of Cyclic Plasmin Inhibitors: From Benzamidines to Benzylamines

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1. General Information

Reagents for synthesis
All solvents, reagents and amino acid derivatives were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar or Iris Biotech GmbH. The amino acid derivatives Boc-Phe(p-NO₂)-OH, H-Phe(p-NO₂)-OMe, and H-dPhe(p-NO₂)-OH were purchased from Bachem.

Methods

Analytical HPLC
A Shimadzu LC-10A gradient HPLC system consisting of the subsystems CTO-10A column oven, LC-10ATvp pumps (2 x), DGU-14A degasser, SIL-10Axl auto injector, system controller SCL-10Avp, SPD-M 10Avp photodiode array detector, Shimadzu Class-VP software and a reversed phase column (NUCLEODUR C₁₈ ec, 5 µm, 5 Å, 4.6 mm x 250 mm, Macherey-Nagel, Düren, Germany) was used for analytical HPLC. The detection was performed at 220 nm. Water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B) served as eluents at a flow rate of 1 mL/min and a linear gradient (increase of 1% B/min). Different starting conditions were used for analytical HPLC depending on the properties of the compounds, which are indicated for each derivative. The indicated purity for all intermediates is based on HPLC detection at 220 nm.

Preparative HPLC
The final inhibitors were purified to > 95% by preparative HPLC (pumps: Varian PrepStar Model 218 gradient system, detector: ProStar Model 320, fraction collector: Varian Model 701). Most of the compounds were purified on a C₈ column (NUCLEODUR C₈ ec, 5 µm, 100 Å, 32 mm x 250 mm, Macherey-Nagel, Düren, Germany), in some cases also a C₁₈-column (ProntoSIL C18 SH, 5 µm, 120 Å, 32 mm x 250 mm, Bischoff Chromatography) was used. All purifications were performed using a linear gradient (increase of 1% B per 2 min) of acetonitrile/water containing 0.1% TFA at a flow rate of 20 mL/min. All inhibitors were finally obtained as TFA-salts after lyophilization.

Mass spectrometry
The molecular mass of the synthesized compounds was determined using a QTrap 2000 ESI spectrometer (Applied Biosystems), or using an Autospec spectrometer (Micromass).
Thin layer chromatography

Thin layer chromatography was performed on silica 60 plates (ALUGRAM SIL G/UV$_{254}$, Macherey-Nagel, Düren, Germany). For detection of the spots UV-absorbance was used, followed by treatment with ninhydrine reagent. In addition, after incubation of the TLC plates in a chlorine atmosphere, an o-toluidine reagent was used for detection.

NMR spectroscopy

NMR spectra for non-cyclic compounds were recorded on a JEOL ECX-400 or JEOL ECA-500. A Bruker DRX 400, Bruker Avance 500 or Bruker Avance 600 was used for NMR measurements of the cyclic derivatives. Chemical shifts (\(\delta\)) are reported in ppm and calibrated on the internal solvent signal (DMSO-$d_6$) or 3-(Trimethylsilyl)propionic-2,2,3,3-$d_4$ acid sodium salt. Multiplicities are indicated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplett). Coupling constants are reported in Hertz (Hz). Water suppression was achieved by using the Bruker WATERGATE pulse sequence.
2. Enzyme kinetic measurements

The conditions for the measurements with the fluorescence substrates have been described in the main manuscript.

The measurements with the chromogenic substrates were performed with the following volumina:
- 200 µL of 50 mM Tris·HCl buffer pH 8.0 (containing 0.154 M NaCl, 2% ethanol and inhibitor in appropriate concentrations).
- 25 µL of substrate
- start of reaction by adding 50 µL of enzyme solution

At appropriate time (approximately 5-10 min) the measurements were stopped by addition of 25 µL of 50% acetic acid and the $K_i$ was calculated according to the method of Dixon. The $K_i$ are the mean of at least two measurements. The following substrates and enzymes were used for kinetic measurements, as described previously (Table S1).
Table S1. Used enzymes and chromogenic substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma kallikrein (human), Enzyme Research South Bend</td>
<td>H-D-Pro-Phe-Arg-pNA (Haemochrom PK)</td>
</tr>
<tr>
<td>concentration in measurement of 0.132 nM</td>
<td>2 mM (182 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>1 mM (91 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM (45.5 μM in measurement)</td>
</tr>
<tr>
<td>thrombin (bovine), 1425 IE/mg</td>
<td>CH₃SO₂-d-Cha-Gly-Arg-pNA (Pefachrome tPA)</td>
</tr>
<tr>
<td>concentration in measurement of 0.677 nM</td>
<td>2 mM (182 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>1 mM (91 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM (45.5 μM in measurement)</td>
</tr>
<tr>
<td>factor Xa (human), 200.35 IE/mg, Enzyme Research South Bend</td>
<td>CH₃OCO-d-Cha-Gly-Arg-pNA (Pefachrome FXa)</td>
</tr>
<tr>
<td>concentration in measurement of 0.494 nM</td>
<td>2 mM (182 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>1 mM (91 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM (45.5 μM in measurement)</td>
</tr>
<tr>
<td>activated protein C (human), Kordia Laboratory Supplies, Leiden</td>
<td>H-D-Lys(Cbz)-Pro-Arg-pNA (Pefachrome PCa)</td>
</tr>
<tr>
<td>concentration in measurement of 0.373 nM</td>
<td>4 mM (364 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>2 mM (182 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>1 mM (91 μM in measurement)</td>
</tr>
<tr>
<td>trypsin (porcine) 8 U/mg, Merck, Darmstadt</td>
<td>CH₃SO₂-d-Cha-Gly-Arg-pNA (Pefachrome LAL)</td>
</tr>
<tr>
<td>concentration in measurement of 0.390 nM</td>
<td>2 mM (182 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>1 mM (91 μM in measurement)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM (45.5 μM in measurement)</td>
</tr>
</tbody>
</table>
3. Synthesis of inhibitors

3.1 $^1$H-NMR Spectra of final inhibitors fully described in the main manuscript

The syntheses of inhibitors 23, 24, 30, 31, 41 and 42 have been described in the main manuscript with all details, their $^1$H-NMR-spectra are shown below.

Inhibitor 23
Inhibitor 24
Inhibitor 30

Chemical structure of inhibitor 30 with a proton NMR spectrum.
Inhibitor 31
Inhibitor 41
3.2 Synthesis of additional cyclic inhibitors

Inhibitor 15

15a) Boc-\(d\)-Phe(4-NO\(_2\))-Phe(4-NO\(_2\))-OMe

Boc-\(d\)-Phe(4-NO\(_2\))-OH (1.00 g, 3.22 mmol, 1.0 equiv) and H-Phe(4-NO\(_2\))-OMe \(\times\) HCl (0.84 g, 3.22 mmol, 1.0 equiv) were suspended in 25 mL of DMF and stirred at 0 °C. The suspension was treated with PyBOP (1.66 g, 3.22 mmol, 1.0 equiv) and DIPEA (1.67 mL, 9.66 mmol, 3.0 equiv) (pH 7-8) and stirred for 2.5 h at 0 °C. The solvent was removed \textit{in vacuo} and the remaining yellow oil was dissolved in a mixture of 5% KHSO\(_4\)/EtOAc. The organic layer was washed 3 \times with 5% KHSO\(_4\), once with brine, 3 \times with saturated aq NaHCO\(_3\) and 3 \times with brine. The organic phase was dried over Na\(_2\)SO\(_4\), filtered and the solvent was evaporated.

Yield: 1.76 g of a crude yellow amorphous solid containing impurities.

HPLC: 52.5 min, start at 10% B (purity: 97.7%).

MS (ESI, positive): calcd: 516.19; \(m/z\): 539.20 [M + Na\]^+.

15b) Boc-\(d\)-Phe(4-NH\(_2\))-Phe(4-NH\(_2\))-OMe

Boc-\(d\)-Phe(4-NO\(_2\))-Phe(4-NO\(_2\))-OMe 15a (1.60 g, 2.93 mmol) was dissolved in 150 mL of 90% AcOH. The solution was treated with 160 mg of 10% Pd/C and hydrogenated overnight at rt. The suspension was filtered and the solvent was evaporated. The remaining residue was dissolved in a mixture of saturated aqueous NaHCO\(_3\)/EtOAc. The organic layer was washed 3 \times with saturated aq NaHCO\(_3\), twice with brine, dried over Na\(_2\)SO\(_4\), filtered, and the solvent removed by evaporation.
Yield: 1.36 g of a beige amorphous solid containing impurities.

HPLC: 15.6 min, start at 10% B (purity: 91.5%).


Compound 15c

2,2′-(piperazine-1,4-diyl)diacetic acid (443 mg, 2.19 mmol, 1.0 equiv) was dissolved in 50 mL of DMF and stirred at 0 °C. After the addition of PyBOP (2.28 g, 4.38 mmol, 2.0 equiv) and DIPEA (1.5 mL, 8.76 mmol, 4.0 equiv), the solution was stirred for 15 min at 0 °C. Then, a solution of Boc-D-Phe(4-NH₂)-Phe(4-NH₂)-OMe 15b (1.00 g, 2.19 mmol, 1.0 equiv) in 400 mL of DMF was added and stirred for 1 h at 0 °C and overnight at rt. The solvent was evaporated and the remaining residue was dissolved in a mixture of saturated aq NaHCO₃/EtOAc. The organic layer was washed 3 × with saturated aq NaHCO₃ and once with brine. The organic layer was dried over MgSO₄, filtered and the solvent was evaporated.

Yield: 2.18 g (crude yellow solid containing impurities).

HPLC: 27.7 min, start at 10% B (purity: 48.7%).


Inhibitor 15

The crude compound 15c (200 mg) was dissolved in 10 mL of 50% 1 M NaOH/EtOH (v/v) and stirred for 2 h at rt. The solution was neutralized with TFA, the solvent was evaporated and the remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol (Yield: 57.4 mg (34.2%) of light yellow lyophilized solid; HPLC: 22.2 min, start at 10% B (purity: 88.5%); MS (ESI, positive):
calcld: 608.30; \( m/z \): 609.29 [M + H]\(^+\), 631.31 [M + Na]\(^+\). The saponified intermediate (43.3 mg, 51.7 \( \mu \)mol, 1.0 equiv) and NMM (17.1 \( \mu \)L, 155 \( \mu \)mol, 3.0 equiv) were dissolved in 3.0 mL of DMF at -15 \(^\circ\)C and treated with isobutyl chloroformate (6.7 \( \mu \)L, 51.7 \( \mu \)mol, 1.0 equiv). The mixture was stirred 10 min at -15 \(^\circ\)C, afterwards the formed mixed anhydride was treated with 4-amidinobenzylamine \( \times 2 \) HCl\(^3\) (17.2 mg, 77.5 \( \mu \)mol, 1.5 equiv) and NMM (5.7 \( \mu \)L, 51.7 \( \mu \)mol, 1.0 equiv). The mixture was stirred for 1 h at -15 \(^\circ\)C and overnight at rt. The solvent was removed \textit{in vacuo}; the remaining residue (HPLC: 20.9 min, start at 10% B) was treated with 6.0 mL of 50% TFA/DCM (v/v) and stirred for 1.5 h at rt. The solvent was removed \textit{in vacuo} and to remove the residual TFA, the remaining residue was treated with water and the solvent was evaporated again. The residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 14.4 mg (25.4%) of a white lyophilized solid.

**HPLC:** 18.0 min, start at 1% B (purity: 95.3%).

**MS (ESI, positive):** calcld: 639.33; \( m/z \): 640.45 [M + H]\(^+\).

**TLC:** \( R_f = 0.05 \) (1-Butanol/EtOAc/1M NH\(_3\) (1/1/1, v/v/v, upper phase)).

**Inhibitor 16**

![Inhibitor 16](image)

The Cbz-protected inhibitor 20 (see below, 10.0 mg, 8.74 \( \mu \)mol) was treated with 200 \( \mu \)L of 32% HBr in AcOH. The mixture was sometimes shaken, after 1 h at rt the product was precipitated by addition of diethyl ether and obtained by centrifugation. The precipitate was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 8.2 mg (83.5%) of a white lyophilized solid.

**HPLC:** 18.4 min, start at 1% B (purity: 95.9%).

**MS (ESI, positive):** calcld: 667.36; \( m/z \): 668.2 [M + H]\(^+\), 690.1 [M + Na]\(^+\).

**TLC:** \( R_f = 0.04 \) (1-Butanol/EtOAc/1M NH\(_3\) (1/1/1, v/v/v, upper phase)).
Inhibitor 17

The formyl-inhibitor 17 was synthesized according to the strategy shown in Scheme S1.

**Scheme S1.** Synthesis of Inhibitor 17. (a) (i) 1 equiv H-Phe(p-NO2)-OMe × HCl, 1 equiv BOP, 3 equiv DIPEA, THF, 0 °C; (ii) zinc powder, 60% AcOH, rt, preparative HPLC; (b) (i) 1 equiv 10, 3 equiv of PyBOP, 5 equiv DIPEA, DMF, 30 min 0 °C, rt overnight; (ii) 1 M NaOH, dioxane, rt, preparative HPLC; (c) 1 equiv PyAOP, 1 equiv 6-Cl-HOBt, 1.4 equiv of 4-amidinobenzylamine × 2 HCl, 2 equiv DIPEA, DMF, 0°C 15 min, rt 1 h, preparative HPLC.

**Compound 17a (Formyl-DPhe(p-NO2)-OH)**

H-D-Phe(p-NO2)-OH (2.10 g, 10 mmol, 1.0 equiv) was dissolved in 20 mL of formic acid and cooled at 10 °C. 7 mL (74 mmol) of acetic anhydride were added to the solution in several portions within 30 min and the temperature of the solution was kept at 5 to 15°C. The mixture was stirred for 1 h at rt, treated with 30 mL of cooled water followed by evaporation. The yellowish solid was recrystallized from ethanol.

**Yield:** 1.76 g (74%) of yellowish crystals.

**HPLC:** 19.7 min, start at 10% B (purity 94.6%).

**MS (ESI, positive):** calcd, 238.06; m/z, found 239.11 [M + H]^+.
Compound 17b (Formyl-DPhe(p-NH2)-Phe(p-NH2)-OMe)

Compound 17a (1.40 g, 5.88 mmol, 1.0 equiv) and H-Phe(p-No2)-OMe × HCl (1.53 g, 5.88 mmol, 1.0 equiv) were suspended in 100 mL of THF and stirred on an ice bath. The suspension was treated with BOP (2.602 g, 5.88 mmol, 1.0 equiv) and DIPEA (3.069 mL, 17.64 mmol, 3.0 equiv) and was stirred for 15 min at 0 °C (in this time the suspension dissolved completely) and 3 h at rt, the pH was maintained at 7-8 by addition of a small amounts of DIPEA. The solvent was removed in vacuo and the remaining residue was suspended in a mixture of 5% KHSO4 and EtOAc. The product immediately precipitated in the shaking flask between the two phases as a yellowish solid, which was afterwards washed 3 × with 5% KHSO4, once with brine, 3 × with saturated aq NaHCO3 and 3 × with brine. The solid was isolated by filtration and dried in vacuo (Yield: 1.957 g (95.7%) of Formyl-DPhe(p-No2)-Phe(p-No2)-OMe, HPLC: 38.6 min, start at 10% B (purity, 96.0%). MS (ESI, positive): calcd, 444.13; m/z, 445.14 [M + H]+).

The intermediate (1.787 g, 4.02 mmol, 1.0 equiv) was suspended in 150 mL of 60% AcOH and after addition of zinc dust (2.6 g, 40.2 mmol, 10 equiv) the suspension was stirred for 1.5 h at rt (the suspension dissolves completely after 15-30 min. After centrifugation to remove remaining zinc, the solvent was evaporated. The remaining residue was suspended in 50 mL of 90% MeCN, the precipitated zinc acetate was removed by centrifugation and the supernatant was evaporated. The remaining residue (yellow oil) was purified by preparative HPLC and lyophilized from water.

Yield: 0.529 g (20%) of a white lyophilized solid.

HPLC: 14.6 min, start at 1% B (purity 93.4%).

1H NMR (500 MHz, DMSO-d6): δ = 8.61 (d, J = 8.0 Hz, 1 H), 8.20 (d, J = 9.2 Hz, 1 H), 7.88 (s, 1 H), 7.20 (d, J = 8.3 Hz, 2 H), 7.13 (d, J = 8.3 Hz, 2 H), 7.05 (d, J = 8.3 Hz, 2 H), 7.01 (d, J = 8.3 Hz, 2 H), 4.62 (td, J = 9.3, 4.3 Hz, 1 H), 4.46 (td, J = 8.6, 5.4 Hz, 1 H), 3.63 (s, 3 H), 3.02 (dd, J = 13.7, 5.2 Hz, 1 H), 2.85 (dd, J = 13.7, 9.5 Hz, 1 H), 2.77 (dd, J = 13.9, 4.2 Hz, 1 H), 2.53 ppm (m,1 H). The protons of both amino groups could not be identified.

13C NMR (125 MHz, DMSO-d6): δ = 171.68, 170.57, 160.62, 135.79, 134.54, 133.88, 132.82, 130.46, 130.24, 120.62, 119.90, 53.45, 51.95, 51.89, 37.33, 36.22 ppm.

MS (ESI, positive): calcd, 384.18; m/z, 385.18 [M + H]+.

Compound 17c

Intermediate 17b (1.657 g, 2.7 mmol, 1.0 equiv) and 3,3’-(piperazine-1,4-diyl)dipropanoic acid ×2 HCl 10 (0.867 g, 2.7 mmol, 1.0 equiv) were dissolved in 700 mL of DMF and stirred on an ice bath. The mixture was treated with PyBOP (4.215 g, 8.1 mmol, 3.0 equiv) and DIPEA (2 350 µL, 13.5
mmol, 5.0 equiv) were added and the mixture was stirred at pH 8 for 1 h at 0 °C and at rt overnight. The solvent was removed in vacuo and the remaining residue was purified by preparative HPLC and lyophilized. (Yield: 0.362 g of white lyophilized solid, 16.6%. HPLC: 14.1 min, start at 10% B (purity, 73.4%), MS (ESI, positive): calcd, 578.29; m/z, 579.25 [M + H]+, 601.20 [M + Na]+).

The cyclized intermediate (0.324 g) was dissolved in 8 mL of 50% 1 M NaOH/dioxane (v/v), the solution was stirred for 1 h at rt, neutralized with TFA, and the solvent was removed in vacuo. The residue was purified by preparative HPLC and lyophilized.

Yield: 485 mg (25.0%) of a white lyophilized solid.
HPLC: 19.1 min, start at 1% B (purity, 90.5%).
MS (ESI, positive): calcd, 564.27; m/z, 565.27 [M + H]+, 587.23 [M + Na]+.

Inhibitor 17

Compound 17c (40 mg, 0.050 mmol, 1.0 equiv) and 4-amidinobenzylamine × 2 HCl (15.8 mg, 0.071 mmol, 1.4 equiv) were suspended in 2.0 mL of DMF and stirred on an ice bath. The mixture was treated with PyAOP (26.1 mg, 0.050 mmol, 1.0 equiv), 6-Cl-HOBt (8.5 mg, 0.050 mmol, 1.0 equiv) and DIPEA (17.4 µL, 0.100 mmol, 2.0 equiv), was stirred on an ice bath for another 15 min and at rt for 4 h. The product was purified by preparative HPLC and lyophilized.

Yield: 47.4 mg (73.0%) of a white lyophilized solid.
HPLC: 19.8 min, start at 1% B (purity 98.0%).
MS (ESI, positive): calcd, 695.35; m/z, 696.38 [M + H]+, 348.74 [M + 2H]2+/2.
Inhibitor 18

Compound 18a

The synthesis of compound 18a was performed as described for compound 12 in the main manuscript, whereas methyl chloroformate instead of phenylpropionic acid was used for coupling to compound 6b (obtained intermediate: HPLC: 45.29 min, start at 10% B; MS (ESI, positive): calcd: 574.18; m/z: 575.13 [M + H]+), followed by reduction of both nitro groups (obtained intermediate: HPLC: 14.93 min, start at 10% B; MS (ESI, positive): calcd: 514.23; m/z: 515.20 [M + H]+) and cyclization with 3,3’-(piperazine-1,4-diyl)dipropanoic acid providing a white solid (compound 18a: HPLC: 23.2 min, start at 10% B; MS (ESI, positive): calcd: 708.34; m/z: 709.25 [M + H]+).

Inhibitor 18

40.0 mg intermediate 18a (0.056 mmol, 1.0 eq.) were converted into the amidine as described for the synthesis of inhibitor 24 in the main manuscript. The product was purified by preparative HPLC providing 12.0 mg (0.0112 mmol) of a white lyophilized solid. MS (ESI, positive): calc.: 725.36, found: 363.70 (M/2+2H+), 726.27 (M+H+). HPLC: 13.34 min (start at 10% B).
**Inhibitor 19**

**Compound 19a**

![Chemical Structure](image1)

Compound 15c (1.00 g, 1.01 mmol) was treated with 60 mL of 50% TFA/DCM (v/v) and stirred for 1.25 h at rt. The solvent was removed *in vacuo*. To remove the residual acid, the remaining residue was treated with water and the solvent was evaporated again. The remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 138.2 mg (15.9%) of a white lyophilized solid.

**HPLC:** 11.4 min, start at 10% B (purity: 89.1%).

**MS (ESI, positive):** calcd: 522.26; *m/z*: 523.5 [M + H]+.

**Compound 19b**

![Chemical Structure](image2)

Compound 19a (25.7 mg, 29.7 µmol, 1.0 equiv) was dissolved in 2.0 mL of MeCN with NMM (13.1 µL, 119 µmol, 4.0 equiv) and stirred at 0 °C. After the addition of Cbz-OSu (7.4 mg, 29.7 µmol, 1.0 equiv), the solution was stirred overnight, 0 °C → rt. The solvent was evaporated; the remaining residue (**HPLC:** 30.1 min, start at 10% B) was dissolved in 3.0 mL of 50% 1 M NaOH/EtOH (v/v) and stirred for 3 h at rt. After neutralization with TFA, the solvent was removed *in vacuo* and the product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 7.6 mg (29.4%) of a white lyophilized solid.

**HPLC:** 25.3 min, start at 10% B (purity: 93.6%).

**MS (ESI, positive):** calcd: 642.28; *m/z*: 643.35 [M + H]+.
Inhibitor 19

The 4-amidinobenzylamine × 2 HCl was coupled by the mixed anhydride procedure as described for inhibitor 15 using compound 19b (7.6 mg, 8.72 µmol, 1.0 equiv), NMM (2.9 µL, 26.2 µmol, 3.0 equiv) and isobutyl chloroformate (1.1 µL, 8.72 µmol, 1.0 equiv) and 4-amidinobenzylamine × 2 HCl (2.88 mg, 13.0 µmol, 1.5 equiv) and NMM (6.4 µL, 58.5 µmol, 1.0 equiv) in 1.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

Yield: 5.9 mg (60.9%) of a white lyophilized solid.

HPLC: 21.7 min, start at 10% B (purity: 95.3%).

MS (ESI, positive): calcd: 773.36; m/z: 387.7 [M + 2H]²⁺, 774.3 [M + H]⁺.

TLC: Rᵣ = 0.32 (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

Inhibitor 20

Compound 20a

Boc-d-Phe(4-NH₂)-Phe(4-NH₂)-OMe 15b (1.26 g, 2.76 mmol, 1.0 equiv) and 3,3’-(piperazine-1,4-diyl)dipropanoic acid (630 mg, 2.76 mmol, 1.0 equiv) were suspended in 630 mL of DMF and stirred at 0 °C. After 5 min, PyBOP (2.87 g, 5.52 mmol, 2.0 equiv) and DIPEA (1.92 mL, 11.0 mmol, 4.0 equiv) were added (pH 7-8) and stirred for 6 h, 0 °C → rt. Due to incomplete reaction (HPLC control), additional PyBOP (1.15 g, 2.21 mmol, 0.8 equiv) and DIPEA (768 µL, 4.41 mmol, 1.6 equiv) were added and stirred at 0 °C. After stirring overnight at rt, the solvent was evaporated and the remaining brown oil
Compound 20b

Compound 20a (150 mg, 0.168 mmol, 1.0 equiv) was dissolved in 7.0 mL of MeCN with NMM (73.9 µL, 0.672 mmol, 4.0 equiv) and stirred at 0 °C. After the addition of Cbz-OSu (46.0 mg, 0.185 mmol, 1.1 equiv), the solution was stirred for 1 h at 0 °C and overnight at rt. The solvent was evaporated and the remaining residue was dissolved in a mixture of saturated aq NaHCO₃/EtOAc. The organic layer was washed 3 × with saturated aq NaHCO₃ and 3 × with brine, dried over MgSO₄ and the solvent was evaporated. The remaining yellow residue (HPLC: 29.8 min, start at 10% B) was dissolved in 10 mL of 50% 1 M NaOH/EtOH (v/v) and stirred for 3 h at rt. The solution was neutralized with TFA and the solvent was removed in vacuo. The remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

Yield: 51.0 mg (33.8%) of a white lyophilized solid.

HPLC: 25.5 min, start at 10% B (purity: 99.2%).

Inhibitor 20

![Chemical structure of Inhibitor 20](image)

The compound was prepared according to the mixed anhydride procedure described for inhibitor 15 from compound 20b (40.0 mg, 44.5 µmol, 1.0 equiv), NMM (14.7 µL, 134 µmol, 3.0 equiv), isobutyl chloroformate (5.8 µL, 44.5 µmol, 1.0 equiv), 4-amidinobenzylamine × 2 HCl (14.8 mg, 66.8 µmol, 1.5 equiv) and NMM (4.9 µL, 44.5 µmol, 1.0 equiv) in 2.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 21.1 mg (41.4%) of a white lyophilized solid.

**HPLC:** 22.6 min, start at 10% B (purity: 95.3%).

**MS (ESI, positive):** calcd: 801.40; m/z: 401.81 [M + 2H]^2+, 802.50 [M + H]^+.

**TLC:** R_f = 0.13 (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

Inhibitor 21

**Compound 21a**

![Chemical structure of Compound 21a](image)

Phenylacetic acid (15.7 mg, 0.115 mmol, 1.0 equiv) was dissolved in 3.0 mL of DMF at -15 °C and treated with NMM (12.7 µL, 0.115 mmol, 1.0 equiv) and isobutyl chloroformate (15.0 µL, 0.115 mmol, 1.0 equiv). After 15 min, compound 19a (100 mg, 0.116 mmol, 1.0 equiv) and NMM (50.6 µL, 0.460 mmol, 4.0 equiv) were added, stirred for 1 h at -15 °C and overnight at rt. The solvent was removed in vacuo; the remaining residue (**HPLC**: 25.8 min, start at 10% B) was dissolved in 4.0 mL of 50% 1 M
NaOH/EtOH and stirred for 3 h at rt. After neutralization with TFA, the solvent was evaporated and the remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 61.2 mg (62.3%) of a white lyophilized solid.

**HPLC:** 21.0 min, start at 10% B (purity: 94.5%).

**MS (ESI, positive):** calcd: 626.29; \( m/z \): 314.03 [M + 2H]^{2+}, 627.31 [M + H]^{+}, 649.22 [M + Na]^{+}.

**Inhibitor 21**

![Inhibitor 21](image)

The compound was prepared according to the mixed anhydride procedure described for inhibitor 15 from compound 21a (50.0 mg, 58.5 µmol, 1.0 equiv), NMM (19.3 µL, 176 µmol, 3.0 equiv) and isobutyl chloroformate (7.6 µL, 58.5 µmol, 1.0 equiv), 4-amidinobenzylamine \( \times 2 \) HCl (19.4 mg, 87.3 µmol, 1.5 equiv), and NMM (6.4 µL, 58.5 µmol, 1.0 equiv) in 4.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 37.5 mg (58.3%) of a white lyophilized solid.

**HPLC:** 18.8 min, start at 10% B (purity: 97.6%).

**MS (ESI, positive):** calcd: 757.37; \( m/z \): 379.69 [M + 2H]^{2+}, 758.62 [M + H]^{+}.

**TLC:** R\(_t\) = 0.24 (n-butanol/acetic acid/water 4/1/1 (v/v/v)).
**Inhibitor 22**

**Compound 22a**

The compound was prepared according to the mixed anhydride procedure described for compound 15 from phenylacetic acid (15.3 mg, 0.112 mmol, 1.0 equiv), NMM (12.3 µL, 0.112 mmol, 1.0 equiv), isobutyl chloroformate (14.6 µL, 0.112 mmol, 1.0 equiv) and compound 20a (100 mg, 0.112 mmol, 1.0 equiv) and NMM (36.9 µL, 0.336 mmol, 3.0 equiv) in 3.0 mL of DMF (HPLC: 25.7 min, start at 10% B; MS (ESI, positive): calcd: 668.33; m/z: 669.37 [M + H]+). After saponification and neutralization, the product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 50.0 mg (50.6%) of a white lyophilized solid.

**HPLC:** 21.2 min, start at 10% B (purity: 98.2%).


**Inhibitor 22**

The compound was prepared according to the mixed anhydride procedure described for inhibitor 15 from compound 22a (40.1 mg, 45.3 µmol, 1.0 equiv), NMM (15.0 µL, 136 µmol, 3.0 equiv), isobutyl chloroformate (5.9 µL, 45.3 µmol, 1.0 equiv), 4-amidinobenzylamine × 2 HCl (15.1 mg, 68.0 µmol, 1.5 equiv), and NMM (5.0 µL, 45.3 µmol, 1.0 equiv) in 2.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 16.5 mg (32.3%) of a white lyophilized solid.

**HPLC:** 19.3 min, start at 10% B (purity: 97.7%).

**MS (ESI, positive):** calcd: 785.40; m/z: 393.81 [M + 2H]2+, 786.30 [M + H]+.
TLC: R$_f$ = 0.13 (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

**Inhibitor 23**

Its synthesis is fully described in the main manuscript.

**Inhibitor 24**

Its synthesis is fully described in the main manuscript.

**Inhibitor 25**

*Compound 25a*

The synthesis of compound 25a was performed as described for compound 11 in the main manuscript, whereas phenylbutyric acid instead of phenylpropionic acid was used for coupling to intermediate 6b, followed by reduction of both nitro groups and cyclization with 2,2’-(piperazine-1,4-diyl)diacetic acid providing a white solid.

**$^1$H-NMR:** 500 MHz, DMSO-$d_6$; $\delta$ = 1.59-1.75 (m, 2H, 4-Phbut$_2$), 1.92-2.05 (m, 2H, 4-Phbut$_1$), 2.45-2.52 (m, 2H, 4-Phbut$_3$), 2.65-2.80 (m, 9H, Piz, D-Phe$_\beta$), 2.88-2.97 (m, 1H, L-Phe$_\beta$), 2.99-3.07 (m, 1H, L-Phe$_\beta$), 3.08-3.26 (m, 5H, Ac$_1,2$, D-Phe$_\beta$), 4.32-4.40 (m, 2H, 4-Cnbam-CH$_2$). 4.40-4.48 (m, 1H, L-Phe$_\alpha$), 4.53-4.61 (m, 1H, D-Phe$_\alpha$), 6.91-6.98 (m, 2H, D-Phe$_\text{arom.1}$), 7.10-7.17 (m, 3H, 4-Phbut$_\text{arom.1,3}$), 7.17-7.21 (m, 2H, L-Phe$_\text{arom.1}$), 7.23-7.31 (m, 4H, D-Phe$_\text{arom.2}$, 4-Phbut$_\text{arom.2}$), 7.37-7.46 (m, 4H, L-Phe$_\text{arom.2}$, 4-Cnbam$_\text{arom.2}$), 7.73-7.80 (m, 2H, 4-Cnbam$_\text{arom.1}$), 8.14 (d, $^3$J = 6.1 Hz, 1H, D-Phe-NH), 8.49-8.57 (m, 1H, 4-Cnbam-NH), 8.81 (d, $^3$J = 7.4 Hz, 1H, L-Phe-NH), 9.92 (s, 1H, D-Phe$_\text{arom.NH}$), 9.96 (s, 1H, D-Phe$_\text{arom.NH}$).
13C-NMR: 125 MHz, DMSO-d6; δ = 26.9 (4-Phbut2), 34.4 (4-Phbut1), 34.6 (4-Phbut3), 35.9 (d-Pheβ), 36.1 (L-Pheβ), 41.9 (4-Cnbam-CH2), 51.6 (Piz), 53.1 (d-Pheα), 55.0 (L-Pheα), 60.2 (Ac1), 60.6 (Ac2), 109.5 (CAr-CN), 118.1 (d-Phearom.2), 118.5 (L-Phearom.2), 118.9 (CN), 125.7 (4-Phbutarom.3), 127.6 (4-Cnbamarom.2), 128.2 (4-Phbutarom.1), 128.9 (d-Phearom.1 oder L-Phearom.1), 132.1 (CAr-d-Pheβ), 132.2 (4-Cnbamarom.1), 133.5 (CAr-L-Pheβ), 136.4 (CAr-d-Phearom.NH oder CAr-L-Phearom.NH), 136.5 (CAr-d-Phearom.NH oder CAr-L-Phearom.NH), 141.7 (CAr-4-Phbut3), 145.2 (CAr-4-Cnbam-CH2), 166.9 (Ac-C1 oder Ac-C2), 167.5 (Ac-C1 oder Ac-C2), 171.4 (d-Phe1), 171.8 (L-Phe1), 172.4 (4-Phbut-C1).

MS (ESI, positive): calcld: 768.37; m/z: 769.70 (M + H+).

TLC: Rf = 0.23 (DCM/MeOH 9/1 containing 0.5% NH4OH (25%)).

HPLC: 35.6 min (start at 10% B).

Inhibitor 25

35.0 mg of intermediate 25a (0.046 mmol, 1.0 eq.) were converted into the amidine as described for the synthesis of inhibitor 23 in the main manuscript. The product was purified by preparative HPLC providing 11.0 mg (0.011 mmol, 23.5%) of a white lyophilized solid.

1H-NMR: 500 MHz, DMSO-d6; δ = 1.66-1.76 (m, 2H, 4-Phbut2), 1.94-2.03 (m, 2H, 4-Phbut1), 2.06-2.15 (m, 1H, 4-Phbut3), 2.51-2.55 (m, 1H, 4-Phbut3), 2.61-2.82 (m, 9H, Piz, d-Pheβ), 2.85-2.94 (m, 1H, L-Pheβ), 2.98-3.06 (m, 1H, L-Pheβ), 3.09-3.25 (m, 5H, d-Pheβ, Ac1, Ac2), 4.26-4.35 (m, 2H, 4-Amba-CH2), 4.41-4.47 (m, 1H, L-Pheα), 4.51-4.58 (m, 1H, d-Pheα), 6.88-6.97 (m, 2H, d-Phearom.1), 7.10-7.20 (m, 5H, 4-Phbutarom.1, 4-Phbutarom.3, L-Phearom.1), 7.20-7.31 (m, 6H, 4-Phbutarom.2, d-Phearom.2, 4-Ambarom.2), 7.36-7.45 (m, 2H, L-Phearom.2), 7.76-7.86 (m, 2H, 4-Ambarom.1). Due to broad signals no amide protons could be identified.

13C-NMR: 125 MHz, DMSO-d6; δ = 26.9 (4-Phbut2), 34.4 (4-Phbut3), 34.6 (4-Phbut1), 36.4 (d-Pheβ), 37.0 (L-Pheβ), 41.8 (4-Amba-CH2), 51.6 (Piz), 53.2 (d-Pheα), 54.9 (L-Pheα), 60.3 (Ac1 oder Ac2), 60.7
(Ac₁ oder Ac₂), 118.2 (d-Phe₁arom.2), 118.5 (l-Phe₁arom.2), 125.7 (4-Phbut₁arom.3), 126.3 (4-Amba₂arom.2), 126.7 (4-Amba₂arom.1), 128.2 (4-Phbut₁ oder 4-Phbut₂), 128.3 (4-Phbut₁arom.1 oder 4-Phbut₂arom.2), 128.8 (d-Phe₁arom.1 oder l-Phe₂arom.1), 128.9 (d-Phe₁arom.1 oder l-Phe₂arom.1), 141.8 (C₁Ar.-4-Phbut₃). Due to broad signals and unsufficiently resolved spectrum no quaternary carbon atoms could be identified for the final inhibitor 25.

**MS (ESI, positive):** calcd: 785.40; m/z: 393.77 (M/2 + H⁺), 786.51 (M + H⁺).

**HPLC:** 23.2 min (start at 10% B).

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**Inhibitor 26**

**Compound 26a**

The synthesis of compound 26a was performed as described for compound 12 in the main manuscript, whereas phenylbutyric acid instead of phenylpropionic acid was used for coupling to intermediate 6b, followed by reduction of both nitro groups and cyclization with 3,3′-(piperazine-1,4-diyl)dipropanoic acid providing a white solid.

**¹H-NMR:** 500 MHz, DMSO-d₆; δ = 1.69-1.82 (m, 2H, 4-Phbut₂), 2.08-2.24 (m, 3H, 4-Phbut₁, Prop₂), 2.32-2.39 (m, 2H, Prop₁), 2.50-2.67 (m, 11H, 4-Phbut₃, Piz, Prop₂), 2.69-2.82 (m, 5H, d-Phe₁β, l-Phe₂β, Prop₃, Prop₄), 2.91 (dd, ³J = 13.4 Hz, ²J = 6.6 Hz, 1H, d-Phe₁β), 3.02-3.09 (m, 1H, l-Phe₂β), 3.11-3.20 (m, 1H, Prop₄), 4.34-4.44 (m, 3H, 4-Cnbam-CH₂, l-Phe₂α), 4.58-4.65 (m, 1H, d-Phe₁α), 6.20-6.24 (m, 2H, d-Phe₂arom.1), 7.02-7.06 (m, 2H d-Phe₂arom.2), 7.13-7.19 (m, 3H, 4-Phbut₂arom.1,3), 7.20-7.24 (m, 2H, l-Phe₂arom.1), 7.24-7.30 (m, 3H, 4-Phbut₂arom.2, d-Phe-NH), 7.39-7.44 (m, 4H, l-Phe₂arom.2, 4-Cnbam₂arom.2), 7.74-7.78 (m, 2H, 4-Cnbam₂arom.1), 8.60 (d, ³J = 8.6 Hz, 1H, l-Phe-NH), 8.70 (t, ³J = 6.0 Hz, 1H, 4-Cnbam-NH), 10.10 (s, 1H, l-Phe₂arom.NH), 10.47 (s, 1H, d-Phe₂arom.NH).

**¹³C-NMR:** 125 MHz, DMSO-d₆; δ = 27.0 (4-Phbut₂), 31.5 (Prop₂), 32.2 (Prop₁), 34.5 (4-Phbut₁), 34.6 (4-Phbut₃), 36.8 (l-Phe₁β), 36.9 (d-Phe₁β), 41.9 (4-Cnbam-CH₂), 50.7 (Piz), 51.4 (Piz), 52.2 (Prop₄), 52.8 (Prop₅), 53.1 (d-Phe₂α), 55.3 (l-Phe₂α), 109.5 (C₁Ar.-CN), 118.8 (CN), 119.2 (d-Phe₂arom.2), 119.8 (l-
Phe_{arom.2}, 125.7 (4-Phbut_{arom.3}, 127.8 (4-Cnbam_{arom.2}, 128.2 (4-Phbut_{arom.1,2}, 129.6 (L-Phe_{arom.1}), 129.8 (D-Phe_{arom.1}), 130.9 (C_{Ar}-D-Phe{\beta}), 132.2 (4-Cnbam_{arom.1}, 133.3 (C_{Ar}-L-Phe{\beta}), 137.1 (C_{Ar}-D-Phe_{arom.NH}), 137.6 (C_{Ar}-D-Phe_{arom.NH}), 141.8 (C_{Ar}-4-Phbut3), 145.2 (C_{Ar}-4-Cnbam-CH2), 170.1 (d-Phe1), 170.2 (Prop-C1), 170.4 (Prop-C2), 171.5 (L-Phe1), 171.6 (4-Phbut-C1).

**MS (ESI, positive):** calcld: 796.41; m/z: 797.35 (M + H+).

**TLC:** Rf = 0.16 (DCM/MeOH 9/1 containing 0.5% NH4OH (25%)).

**HPLC:** 34.3 min (start at 10% B).

**Inhibitor 26**

40.0 mg of intermediate **26a** (0.05 mmol, 1.0 eq.) were converted into the amidine as described for the synthesis of inhibitor **24** in the main manuscript. The product was purified by preparative HPLC providing 16.0 mg (0.014 mmol, 27.6%) of a white lyophilized solid.

**1H-NMR:** 600 MHz, D2O/H2O 1:5 (H3PO4/NaH2PO4; pH = 3); δ = 1.65-1.80 (m, 2H, 4-Phbut2), 2.14-2.24 (m, 2H, 4-Phbut1), 2.47 (t, 2H, J = 8.1 Hz, 4-Phbut3), 2.70-2.80 (m, 4H, Prop1, Prop2), 2.89-2.99 (m, 3H, L-Phe{\beta}, d-Phe{\beta}), 2.99-3.26 (m, 9H, L-Phe{\beta}, Piz), 3.26-3.37 (m, 2H, Prop3), 3.37-3.52 (m, 2H, Prop4) 4.35-4.45 (m, 2H, 4-Amba-CH2), 4.53-4.60 (m, 1H, d-Phe_a), 4.65-4.70 (m, 1H, L-Phe_a), 6.60-6.70 (m, 2H, d-Phe_{arom.1}), 7.03-7.09 (m, 2H, L-Phe_{arom.1}), 7.10-7.15 (m, 2H, 4-Phbut_{arom.1}), 7.16-7.21 (m, 2H, d-Phe_{arom.2}), 7.22-7.25 (m, 1H, 4-Phbut_{arom.3}), 7.26-7.30 (m, 2H, 4-Phbut_{arom.2}), 7.30-7.34 (m, 2H, L-Phe_{arom.2}), 7.35-7.40 (m, 2H, 4-Amba_{arom.2}), 7.54-7.60 (m, 2H, 4-Amba_{arom.1}), 7.76 (d, 1H, J = 6.1 Hz, d-Phe-NH), 8.35 (bs, 2H, 4-Amba-NH oder 4-Amba-NH2), 8.56-8.74 (m, 4H, L-Phe-NH, 4-Amba-CH2-NH, 4-Amba-NH oder 4-Amba-NH2), 10.11 (s, 1H, L-Phe_{arom.NH}), 10.14 (s, 1H, D-Phe_{arom.NH}).

**13C-NMR:** 150 MHz, D2O/H2O 1.5:5 (H3PO4/NaH2PO4; pH = 3); δ = 26.8 (4-Phbut2), 31.1 (Prop1 oder Prop2), 31.6 (Prop1 oder Prop2), 34.6 (4-Phbut3), 34.7 (4-Phbut1), 36.2 (L-Phe_a), 36.6 (D-Phe_a), 42.8 (4-Amba-CH2), 49.3 (Piz), 49.5 (Piz), 51.2 (Prop4), 51.3 (Prop3), 54.6 (d-Phe_a), 55.2 (L-Phe_a), 122.1 (d-Phe_{arom.2}), 122.3 (L-Phe_{arom.2}), 126.1 (4-Phbut_{arom.3}), 126.7 (C_{Ar}-C(NH)-NH2), 127.5 (4-Amba_{arom.2}), 127.9 (4-Amba_{arom.1}), 128.5 (4-Phbut_{arom.1 oder 4-Phbut_{arom.2}), 128.6 (4-Phbut_{arom.1 oder 4-Phbut_{arom.2}), 129.9 (L-Phe_{arom.1}), 130.1 (D-Phe_{arom.1}), 132.9 (C_{Ar}-D-Phe{\beta}), 134.4 (C_{Ar}-L-Phe{\beta}), 135.5 (C_{Ar}-D-Phe_{arom.NH} oder C_{Ar}-D-Phe_{arom.NH}).
L-Phe$_{\text{arom,NH}}$, 135.6 (C$_{\text{A}}$-D-Phe$_{\text{arom,NH}}$ oder C$_{\text{A}}$-L-Phe$_{\text{arom,NH}}$), 141.8 (C$_{\text{A}}$-4-Phbut$_{3}$), 144.3 (C$_{\text{A}}$-4-AmbaCH$_{2}$), 166.3 (C(NH)-NH$_{2}$), 171.9 (Prop-C2), 172.2 (Prop-C1), 172.8 (L-Phe1 oder D-Phe1), 173.2 (L-Phe1 oder D-Phe1), 176.2 (4-Phbut-C1).

**MS (ESI, positive):** calcd: 813.43; m/z: 407.82 (M/2+H$^+$), 814.47 (M+H$^+$).
**HPLC:** 24.6 min (start at 10% B).

**Inhibitor 27**

*Compound 27a (Bus-D-Phe(4-NH$_2$)-Phe(4-NH$_2$)-OMe)*

Bus-D-Phe(4-NO$_2$)-OH (1.01 g, 3.06 mmol, 1.0 equiv) and H-Phe(4-NO$_2$)-OMe·HCl (0.80 g, 3.07 mmol, 1.0 equiv) were dissolved in 30 mL of DMF and stirred at 0 °C. The solution was treated with PyBOP (1.58 g, 3.04 mmol, 1.0 equiv) and DIPEA (1.58 mL, 9.08 mmol, 3.0 equiv) (pH 7-8), the mixture was stirred for 4 h. The solvent was removed in vacuo and the remaining residue was dissolved in a mixture of 5% KHSO$_4$/EtOAc. The organic phase was washed 3 × with 5% KHSO$_4$, once with brine, 3 × with saturated aq NaHCO$_3$ and 3 × with brine, dried over Na$_2$SO$_4$, filtered and the solvent removed in vacuo. The reddish brown oil (HPLC: 50.4 min, start at 10% B (purity: 90.8%); **MS (ESI, positive):** calcd: 536.16; m/z: 537.37 [M + H]$^+$) was dissolved in 150 mL of 90% AcOH and treated with zinc dust (1.60 g, 24.5 mmol, 8.0 equiv). The suspension was stirred for 3 h at rt. After filtration, the solvent was evaporated and the remaining residue was suspended in 25 mL of 90% MeCN. The precipitated zinc acetate was removed by centrifugation and the supernatant was evaporated. The remaining brown oil was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 878 mg (60.2%) of a yellow lyophilized solid.

**HPLC:** 15.1 min, start at 10% B (purity: 94.1%).
**MS (ESI, positive):** calcd: 476.21; m/z: 477.21 [M + H]$^+$, 499.18 [M + Na]$^+$. 
**Compound 27b**

The cyclic compound was prepared according to the synthesis procedure described for intermediate 12 in the main manuscript from Bus-D-Phe(4-NH$_2$)-Phe(4-NH$_2$)-OMe 27a (100 mg, 0.210 mmol, 1.0 equiv) and 3,3’-(piperazine-1,4-diyl)dipropanoic acid (49.0 mg, 0.213 mmol, 1.0 equiv) suspended in 60 mL of DMF, followed by treatment with PyBOP (219 mg, 0.421 mmol, 2.0 equiv) and DIPEA (146 µL, 0.840 mmol, 4.0 equiv). The solvent was evaporated and the remaining residue (HPLC: 25.7 min, start at 10% B; MS (ESI, positive): calcd: 670.31; m/z: 671.35 [M + H]$^+$) was saponified with 8.0 mL of 50% 1 M NaOH/EtOH (v/v) for 2 h at rt. After neutralization with TFA, the solvent was evaporated; the remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 49.7 mg (26.7%) of a white lyophilized solid.

**HPLC:** 20.5 min, start at 10% B (purity: 87.8%).

**MS (ESI, positive):** calcd: 656.30; m/z: 657.35 [M + H]$^+$.

**Inhibitor 27**

The compound was prepared according to the synthesis procedure described for inhibitor 15 from compound 27b (40.0 mg, 45.2 µmol, 1.0 equiv), NMM (15.0 µL, 136 µmol, 3.0 equiv), isobutyl chloroformate (5.9 µL, 45.2 µmol, 1.0 equiv), 4-amidinobenzylamine $\times$ 2 HCl (15.0 mg, 67.5 µmol, 1.5 equiv), and NMM (5.0 µL, 45.2 µmol, 1.0 equiv) in 2.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 11.6 mg (22.7%) of a white lyophilized solid.

**HPLC:** 18.7 min, start at 10% B (purity: 95.8%).

**MS (ESI, positive):** calcd: 787.38; m/z: 394.84 [M + 2H]$^{2+}$, 788.20 [M + H]$^+$.
TLC: $R_f = 0.09$ (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

Inhibitor 28

*Compound 28a (Phs-d-Phe(4-NO$_2$)-OH)*

The compound was prepared from H-d-Phe(4-NO$_2$)-OH (2.00 g, 9.52 mmol, 1.0 equiv), DIPEA (3.64 mL, 20.9 mmol, 2.2 equiv) and benzenesulfonyl chloride (1.34 mL, 10.5 mmol, 1.1 equiv) in dioxane/water.

**Yield:** 2.81 g (84.2%) crude brown oil.

**HPLC:** 36.9 min, start at 10% B (purity: 67.0%).

**MS (ESI, positive):** calcd: 350.35; $m/z$: 351.03 [M + H]$^+$, 368.04 [M + NH$_4$]$^+$.

*Compound 28b (Phs-d-Phe(4-NH$_2$)-Phe(4-NH$_2$)-Ome)*

Phs-d-Phe(4-NO$_2$)-OH 28a (672 mg, 1.92 mmol, 1.0 equiv) and H-Phe(4-NO$_2$)-OMe·HCl (500 mg, 1.92 mmol, 1.0 equiv) were dissolved in 25 mL DMF and stirred at 0 °C. The solution was treated with PyBOP (998 mg, 1.92 mmol, 1.0 equiv) and DIPEA (1.00 mL, 5.75 mmol, 3.0 equiv) (pH 7-8), the mixture was stirred overnight, 0 °C → rt. The solvent was removed in vacuo and the remaining residue was dissolved in a mixture of 5% KHSO$_4$/EtOAc. The organic layer was washed 3 × with 5% KHSO$_4$, once with brine, 3 × with saturated aq NaHCO$_3$ and 3 × with brine, dried over MgSO$_4$, filtered and the solvent was removed in vacuo (**Yield:** 1.31 g crude brown oil containing impurities; **HPLC:** 50.3 min, start at 10% B (purity: 50.3%); **MS (ESI, positive):** calcd: 556.13; $m/z$: 579.1 [M + Na]$^+$). The remaining residue was dissolved in 700 mL of 90% AcOH and treated with 150 mg of 10% Pd/C. The suspension was hydrogenated for 20 h at rt. After filtration, the solvent was evaporated. The remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.
Yield: 559 mg (58.6%) of a beige lyophilized solid.

HPLC: 14.9 min, start at 10% B (purity: 82.8%).


**Compound 28c**

The cyclic compound was prepared from Phs-D-Phe(4-NH$_2$)-Phe(4-NH$_2$)-OMe **28b** (100 mg, 0.201 mmol, 1.0 equiv) and 2,2’-(piperazine-1,4-diyl)diacetic acid (40.7 mg, 0.201 mmol, 1.0 equiv) treated with PyBOP (210 mg, 0.403 mmol, 2.0 equiv) and DIPEA (140 µL, 0.806 mmol, 4.0 equiv) in 70 mL of DMF (HPLC: 25.3 min, start at 10% B; MS (ESI, positive): calcd: 662.25; m/z: 663.58 [M + H]^+, 685.39 [M + Na]^+). After saponification for 1.5 h with 10 mL of 50% 1 M NaOH/EtOH (v/v) and neutralization with TFA, the solvent was evaporated and the remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

Yield: 56.7 mg (32.2%) of a white lyophilized solid.

HPLC: 20.3 min, start at 10% B (purity: 98.7%).


**Inhibitor 28**

Inhibitor **28** was prepared according to the synthesis procedure described for inhibitor **15** from intermediate **28c** (50.0 mg, 57.0 µmol, 1.0 equiv), NMM (18.8 µL, 171 µmol, 3.0 equiv) and isobutyl chloroformate (7.4 µL, 57.0 µmol, 1.0 equiv) and 4-amidinobenzylamine × 2 HCl (19.0 mg, 85.5 µmol,
1.5 equiv) and NMM (6.3 µL, 57.0 µmol, 1.0 equiv) in 3.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield**: 32.0 mg (50.0%) of a white lyophilized solid.

**HPLC**: 18.7 min, start at 10% B (purity: 98.1%).

**MS (ESI, positive)**: calcd: 779.32; m/z: 390.76 [M + 2H]^2+, 780.33 [M + H]+.

**TLC**: R_t = 0.23 (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

**Inhibitor 29**

**Compound 29a**

![Chemical Structure](image)

The compound was prepared according to the synthesis procedure described for intermediate 15c from Phs-D-Phe(4-NH2)-Phe(4-NH2)-OMe 28b (100 mg, 0.201 mmol, 1.0 equiv) and 3,3’-(piperazine-1,4-diyl)dipropanoic acid (46.3 mg, 0.201 mmol, 1.0 equiv) treated with PyBOP (210 mg, 0.403 mmol, 2.0 equiv) and DIPEA (140 µL, 0.806 mmol, 4.0 equiv) in 70 mL of DMF (HPLC: 25.4 min, start at 10% B; MS (ESI, positive): calcd: 690.28; m/z: 691.49 [M + H]+, 713.39 [M + Na]+). After saponification for 1.25 h with 10 mL of 50% 1 M NaOH/EtOH (v/v) and neutralization with TFA, the solvent was evaporated and the remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield**: 42.6 mg (23.4%) of a white lyophilized solid.

**HPLC**: 20.4 min, start at 10% B (purity: 91.0%).

**MS (ESI, positive)**: calcd: 676.27; m/z: 677.40 [M + H]+, 699.32 [M + Na]+.
Inhibitor 29

Inhibitor 29 was prepared according to the synthesis procedure described for inhibitor 15 from compound 29a (35.0 mg, 38.7 µmol, 1.0 equiv), NMM (12.8 µL, 117 µmol, 3.0 equiv), isobutyl chloroformate (5.0 µL, 38.7 µmol, 1.0 equiv), 4-amidinobenzylamine × 2 HCl (12.9 mg, 58.1 µmol, 1.5 equiv), and NMM (4.3 µL, 38.7 µmol, 1.0 equiv) in 3.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40% tert-butanol.

Yield: 20.1 mg (45.2%) of a white lyophilized solid.

HPLC: 19.2 min, start at 10% B (purity: 96.9%).

MS (ESI, positive): calcd: 807.35; m/z: 404.83 [M + 2H]^2+, 808.42 [M + H]^+.

TLC: R_f = 0.05 (1-Butanol/EtOAc/1M NH_3 (1/1/2, v/v/v, upper phase)).

Inhibitor 30

Its synthesis is fully described in the main manuscript.

Inhibitor 31

Its synthesis is fully described in the main manuscript.
Inhibitor 32

Compound 32a

\[
\text{\text{H}_3\text{C}}\quad \text{\text{O}}\quad \text{\text{NH}}\quad \text{\text{O}}\quad \text{\text{\text{(R)}}}\quad \text{\text{OH}} \\
\text{\text{\text{N}}}\quad \text{\text{\text{N}}}\quad \text{\text{\text{\text{(S)}}}\quad \text{\text{NH}}}\quad \text{\text{\text{\text{HN}}}\quad \text{\text{\text{\text{O}}}}} \\
\text{\text{\text{O}}}\quad \text{\text{\text{\text{NH}}}\quad \text{\text{\text{\text{HN}}}\quad \text{\text{\text{\text{N}}}}}} \\
\text{\text{\text{-2 TFA}}} \\
\]

Compound 19a (100 mg, 116 \( \mu \)mol, 1.0 equiv) was dissolved in 2.0 mL of DMF, treated with DIPEA (80.7 \( \mu \)L, 464 \( \mu \)mol, 4.0 equiv) and \( \text{N,N-}\text{dimethylsulfamoyl chloride} \) (12.3 \( \mu \)L, 116 \( \mu \)mol, 1.0 equiv) and stirred overnight, 0 \(^\circ\)C \( \rightarrow \) rt. The solvent was evaporated; the remaining residue (HPLC: 19.5 min, start at 10\% B; MS (ESI, positive): calcd: 629.26; \( m/z \): 630.21 \([M + H]^+\), 647.20 \([M + NH_4]^+\), 652.48 \([M + Na]^+\)) was dissolved in 8.0 mL of 50\% 1 M NaOH/EtOH (v/v) and stirred for 2 h at rt. After neutralization with TFA, the solvent was removed \textit{in vacuo} and the product was purified by preparative HPLC and lyophilized from 40\% tert-butanol.

\textbf{Yield}: 18.5 mg (18.9\%) of a white lyophilized solid.

HPLC: 14.9 min, start at 10\% B (purity: 94.2\%).

MS (ESI, positive): calcd: 615.25; \( m/z \): 330.66 \([M + 2Na]^{2+}\), 616.30 \([M + H]^+\), 638.20 \([M + Na]^+\).

Inhibitor 32

\[
\text{\text{H}_3\text{C}}\quad \text{\text{O}}\quad \text{\text{NH}}\quad \text{\text{O}}\quad \text{\text{\text{(R)}}}\quad \text{\text{\text{(S)}}}\quad \text{\text{NH}}}\quad \text{\text{\text{\text{N}}}\quad \text{\text{\text{\text{N}}}\quad \text{\text{\text{\text{\text{N}}}}}} \\
\text{\text{\text{O}}}\quad \text{\text{\text{\text{NH}}}\quad \text{\text{\text{\text{HN}}}\quad \text{\text{\text{\text{\text{N}}}}}} \\
\text{\text{\text{-3 TFA}}} \\
\]

Inhibitor 32 was prepared according to the synthesis procedure described for inhibitor 15 from compound 32a (18.0 mg, 21.3 \( \mu \)mol, 1.0 equiv), NMM (7.0 \( \mu \)L, 63.9 \( \mu \)mol, 3.0 equiv) and isobutyl chloroformate (2.8 \( \mu \)L, 21.3 \( \mu \)mol, 1.0 equiv) and 4-amidinobenzylamine \( \times 2 \) HCl (7.1 mg, 32.0 \( \mu \)mol, 1.5 equiv) and NMM (2.3 \( \mu \)L, 21.3 \( \mu \)mol, 1.0 equiv) in 2.0 mL of DMF. The product was purified by preparative HPLC and lyophilized from 40\% tert-butanol.

\textbf{Yield}: 16.5 mg (71.1\%) of a white lyophilized solid.
**HPLC:** 14.2 min, start at 10% B (purity: 98.5%).

**MS (ESI, positive):** calcd: 746.33; m/z: 374.25 [M + 2H]^{2+}, 747.67 [M + H].

**TLC:** $R_f = 0.18$ (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

### Inhibitor 38

**Compound 38a (Chas-D-Phe(4-NO₂)-OH)**

\[
\text{H-d-Phe(4-NO₂)-OH (2.00 g, 9.52 mmol, 1.0 equiv) was suspended in 25 mL of dry DCM and treated with TMS-Cl (2.68 mL, 20.9 mmol, 2.2 equiv) and DIPEA (3.64 mL, 20.9 mmol, 2.2 equiv). The suspension was refluxed for 1 h providing a clear solution. The mixture was cooled to 0 °C and cyclohexylsulfamoyl chloride}^4 \text{ (2.54 g, 12.4 mmol, 1.3 equiv) was added in several portions within 35 min and the pH was maintained at 7-8 by addition of DIPEA (2.23 mL, 12.4 mmol, 1.3 equiv). The solution was stirred for 4 h at 0 °C and overnight at rt. The solvent was removed \textit{in vacuo} and the brown residue was dissolved in a mixture of 5% KHSO₄/EtOAc. The aqueous phase was extracted twice with EtOAc. The combined organic phases were washed twice with 5% KHSO₄ and 3 × with brine. The organic layer was dried over MgSO₄, filtered and the solvent was removed \textit{in vacuo}.}

**Yield:** 3.10 g (87.6%) of a beige amorphous solid.

**HPLC:** 38.0 min, start at 10% B (purity > 99%).

**MS (ESI, positive):** calcd: 371.12; m/z: 372.17 [M + H].

**Compound 38b (Chas-dPhe(p-NO₂)-Phe(p-NO₂)-OMe)**

\[
\text{Chas-dPhe(p-NO₂)-OH} \text{ 38a (2.00 g, 5.39 mmol, 1.0 equiv) and H-Phe(p-NO₂)-OMe·HCl (1.42 g, 5.39 mmol, 1.0 equiv) were suspended in 30 mL of DMF and treated with PyBOP (2.82 g, 5.39 mmol, 1.0 equiv) and DIPEA (2.81 mL, 16.2 mmol, 3.0 equiv) at 0 °C. The solution was stirred for 2 h at 0 °C,}
\]
the solvent was removed in vacuo and the brown residue was dissolved in a mixture of in 5% KHSO₄/EtOAc. The organic layer was washed 3 × with 5% KHSO₄, once with brine, 3 × with saturated aq NaHCO₃ and 3 × with brine, dried over MgSO₄, filtered and the solvent was removed in vacuo.

**Yield:** 3.66 g of brown oil containing impurities.

**HPLC:** 50.0 min, start at 10% B (purity: 81.9%).

**MS (ESI, positive):** caled: 577.18; m/z: 600.29 [M + Na]⁺.

*Compound 38c (Chas-DPhe(p-NH₂)-Phe(p-NH₂)-OMe)*

![Chemical Structure]

Chas-dPhe(p-NO₂)-Phe(p-NO₂)-OMe 38b (1.0 g, 1.73 mmol) was dissolved in 200 mL of 90% AcOH, treated with 101 mg of Pd/C and hydrogenated overnight at rt. The catalyst was removed by filtration and the solvent was evaporated. The red oil was dissolved in 8.0 mL of 25% B, filtered with 0.2 μm membrane filter and the filtrate was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 328 mg (36.7%) of a light yellow lyophilized solid.

**HPLC:** 18.4 min, start at 10% B (purity: 92.3%).

**MS (ESI, positive):** caled: 517.24; m/z: 518.13 [M + H]⁺.

*Compound 38d*

![Chemical Structure]

The compound was prepared from Chas-dPhe(p-NH₂)-Phe(p-NH₂)-OMe 38c (100 mg, 0.193 mmol, 1.0 equiv), 3,3’-(piperazine-1,4-diyl)dipropanoic acid (44.5 mg, 0.193 mmol, 1.0 equiv) and PyBOP (202 mg, 0.386 mmol, 2.0 equiv) and DIPEA (134 μL, 0.773 mmol, 4.0 equiv) in 50 mL of DMF (HPLC: 28.7 min, start at 10% B; **MS (ESI, positive):** caled: 711.34; m/z: 712.36 [M + H]⁺). After...
saponification and neutralization, the solvent was evaporated and the remaining residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 40.0 mg (22.5%) of a white lyophilized solid.

**HPLC:** 23.9 min, start at 10% B (purity: 87.8%).

**MS (ESI, positive):** calcd: 697.33; \( m/z \): 698.27 \([M + H]^+\).

**Inhibitor 38**

![Inhibitor 38](image)

Compound 38d (20.0 mg, 21.6 \( \mu \)mol, 1.0 equiv) and N-Boc-1,4-butanediamine (6.1 mg, 32.4 \( \mu \)mol, 1.5 equiv) were dissolved 10 mL of DMF and stirred at 0 °C. The solution was treated with PyBOP (22.5 mg, 43.2 \( \mu \)mol, 2.0 equiv) and DIPEA (14.8 \( \mu \)L, 86.4 \( \mu \)mol, 4.0 equiv) and stirred for 1 h at 0 °C and overnight at rt. The solvent was evaporated and the remaining residue (**HPLC**: 34.2 min, start at 10% B) was treated with 10 mL of 50% TFA/DCM (v/v) and stirred for 1 h at rt. The solvent was removed *in vacuo*, and to remove the residual acid, the remaining residue was treated with water and the solvent was evaporated again. The residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 16.7 mg (69.7%) of a white lyophilized solid.

**HPLC:** 19.6 min, start at 10% B (purity: 97.0%).

**MS (ESI, positive):** calcd: 767.42; \( m/z \): 384.75 \([M + 2H]^{2+}\), 768.06 \([M + H]^+\).

**TLC:** \( R_f = 0.12 \) (n-butanol/acetic acid/water 4/1/1 (v/v/v)).
Inhibitor 39

\[
\text{Inhibitor 39}
\]

Inhibitor 38 (11.0 mg, 9.91 µmol, 1.0 equiv) was dissolved in 200 µL of DMF, treated with 1H-Pyrazol-1-carboxamidin × HCl (5.8 mg, 39.6 µmol, 4.0 equiv) followed by DIPEA (6.9 µL, 39.6 µmol, 4.0 equiv), and was stirred for 7 h at rt. The solvent was evaporated and the residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

Yield: 10.4 mg (91.1%) of a white lyophilized solid.

HPLC: 21.1 min, start at 10% B (purity > 99%).

MS (ESI, positive): calcd: 809.44; m/z: 405.80 [M + 2H]^{2+}, 810.47 [M + H]^{+}.

TLC: R_{f} = 0.16 (n-butanol/acetic acid/water 4/1/1 (v/v/v)).

Inhibitor 40

Inhibitor 40 was prepared from compound 40a, which was originally described as intermediate 23 in our previous publication.5

\[
\text{Inhibitor 40}
\]

Compound 40a (100 mg, 0.109 mmol, 1.0 equiv) was dissolved in 4.0 mL of DMF at -15 °C, treated with NMM (36.0 µL, 0.327 mmol, 3.0 equiv) and isobutyl chloroformate (14.2 µL, 0.109 mmol, 1.0 equiv). After 15 min, Boc-p-diaminoxylene (40.0 mg, 0.169 mmol, 1.6 equiv) and NMM (12.0 µL,
0.109 mmol, 1.0 equiv) were added, the solution was stirred for 1 h at 0 °C and overnight at rt. The solvent was removed in vacuo; the remaining residue (HPLC: 36.6 min, start at 10% B; MS (ESI, positive): calcd: 908.43; m/z: 909.49) was treated with 1.0 mL of 90% TFA and stirred for 2 h at rt. After evaporation, the residue was purified by preparative HPLC and lyophilized from 40% tert-butanol.

**Yield:** 39 mg (31.1%) of a white lyophilized solid.

**HPLC:** 20.3 min, start at 10% B (purity: 96.5%).

**MS (ESI, positive):** calcd: 808.37; m/z: 405.34 [M + 2H]^2+, 809.10 [M + H]^+.

**TLC:** Rᵓ = 0.18 (1-Butanol/EtOAc/1M NH₃ (1/1/2, v/v/v, upper phase)).

**Inhibitor 41**

Its synthesis is fully described in the main manuscript.

**Inhibitor 42**

Its synthesis is fully described in the main manuscript.

**Inhibitor 43**

*Compound 43a*

3,3’-(piperazine-1,4-diyl)dipropanoic acid × 2 HCl (110.8 mg, 0.37 mmol, 1.0 equiv) was dissolved in 3 mL DMF, DIPEA (255 µL, 1.46 mmol, 4.0 equiv) was added, the solution was cooled to 0 °C. After addition of HATU (270.8 mg, 0.74 mmol, 2.0 equiv), the reaction mixture was stirred for 15 min, then diluted with 250 mL DMF. A solution of compound 36 (240 mg, 0.37 mmol, 1.0 equiv, see main manuscript) in 60 mL DMF was added dropwise within 1 h, the mixture was stirred for additional 1 h at 0 °C, then at rt overnight. The solvent was removed in vacuo, the residue was dissolved in EtOAC. The
organic layer was washed 3 × with saturated aq NaHCO₃ and 3 × with small amounts of brine, then dried over MgSO₄ and filtered. The solvent was removed in vacuo.

**Yield:** 435.7 mg of crude Boc-protected intermediate containing impurities as yellow solid.

**HPLC:** 33.1 min, start at 10% B.

**MS (ESI, positive):** calcd, 850.40; m/z, 851.65 [M + H]+.

*Inhibitor 43*

294.4 mg of crude intermediate **43a** (0.25 mmol) were converted to the final inhibitor by dissolving the residue in a mixture of dioxane/1 M NaOH (9:1, v/v). The reaction mixture was stirred for 3 h at rt, then carefully neutralized with 1 M HCl (pH 7). The solvent was removed in vacuo, the residue was dissolved with small amounts of MeOH, then precipitated in cold diethyl ether. The product was purified by preparative HPLC, the product containing fractions were directly freeze-dried.

**Yield:** 30.9 mg (12%, 2 steps) of a white lyophilized solid.

**HPLC:** 19.9 min, start at 10% B.

**MS (ESI, positive):** calcd, 754.41; m/z, 755.59 [M + H]+.
4. Synthesis of substrates

The synthesis of the AMC-substrates was performed by standard methods according to a strategy described previously. In Scheme S2 the synthesis strategy for the most suitable plasmin substrate CH₃-SO₂-d-Arg-Phe-Arg-AMC (47) is shown. The final substrates were purified by preparative HPLC, obtained as TFA salts and freeze-dried. The analytical data of the synthesized substrates are summarized in Table S3.

**Scheme S2.** (a) stepwise addition of 4 equiv mesyl chloride and 4 equiv DIPEA in a mixture of MeCN and water (1:1 ratio, v/v) at 0 °C over 60 min, the pH was maintained at 8-9 by the addition of DIPEA. (b) 1 equiv of Boc-Phe-OSu, 3 equiv of DIPEA in DMF, 1 h at 0 °C, rt overnight. (c) 1 M HCl in acetic acid, 1 h at rt, precipitation in diethyl ether, purification by preparative HPLC. (d) 3.1 equiv DIPEA, 1.1 equiv HBTU, 1.1 equiv HOBt, DMF, 15 min at 0 °C, rt overnight. (e) TFA/H₂O/TIS (95:2.5:2.5 ratio, v/v/v), precipitation in diethyl ether, purification by preparative HPLC.
Table S3: Analytical data of synthesized AMC-substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HPLC start 10% B [min]</th>
<th>HPLC Purity [%]</th>
<th>MS calculated as free base [M+H]+</th>
<th>MS found [M+H]+</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>37.1</td>
<td>&gt; 99.9</td>
<td>733.29</td>
<td>734.43</td>
</tr>
<tr>
<td>45</td>
<td>38.2</td>
<td>&gt; 99.9</td>
<td>717.29</td>
<td>718.38</td>
</tr>
<tr>
<td>46</td>
<td>38.1</td>
<td>&gt; 99.9</td>
<td>762.28</td>
<td>763.33</td>
</tr>
<tr>
<td>47</td>
<td>23.1</td>
<td>&gt; 99.9</td>
<td>712.32</td>
<td>357.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>25.8</td>
<td>97.8</td>
<td>726.33</td>
<td>364.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>(M+2H+)/2
5. Crystallographic data of the trypsin/inhibitor 31 complex

**Table S4:** Crystallographic Data: Data collection and refinement statistics.

<table>
<thead>
<tr>
<th>Data collection and processing</th>
<th>5EG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB code</td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.91841</td>
</tr>
<tr>
<td>Space group</td>
<td>P4₁2₁2</td>
</tr>
<tr>
<td><strong>Unit-cell parameters</strong></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>61.5 , 61.5, 102.2</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>20.0 - 1.32 (1.35 - 1.32)</td>
</tr>
<tr>
<td>Wilson B factor (Å²)</td>
<td>14.0</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>43989 (3051)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.2 (4.0)</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt; (%)</td>
<td>4.9 (48.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>93.8 (99.7)</td>
</tr>
<tr>
<td>&lt;I/σ(I)&gt;</td>
<td>25.6 (2.6)</td>
</tr>
</tbody>
</table>

| Refinement                    |      |
| Resolution range              | 19.7 - 1.32 |
| No. of reflections used for refinement | 43753 |
| R<sub>cryst</sub> (%)         | 18.0 |
| R<sub>free</sub> (%)          | 21.9 |
| No. of refined residues       | 223 |
| No. of ligand atoms           | 59 |
| No. of other ligand atoms     | 10 |
| No. of water molecules        | 163 |
| RMSD bond length (Å)          | 0.007 |
| RMSD bond angles (˚)          | 0.97 |

| Ramachandran plot (%)         |      |
| Most favoured                 | 89.4 |
| Additionally allowed          | 10.6 |
| Generously allowed            | 0 |
| Disallowed                    | 0 |

| Mean B-values (Å²)            |      |
| All atoms                     | 15.8 |
| Main chain                    | 14.9 |
| Side chain                    | 16.7 |
| Ligand atoms                  | 18.2 |
| Other ligand atoms            | 22.7 |
| Waters                        | 22.3 |
Figure S1. Schematic 2D-representation of the interactions between bovine trypsin and inhibitor 31 (PDB: 5EG4). The inhibitor is shown with bonds in green, the figure was prepared using the program LigPlot+. ⁷
7. Abbreviations

Boc, tert-butyloxycarbonyl; Bzls, benzylsulfonyl; Cbz, benzyloxycarbonyl; Chas, cyclohexylsulfamoyl; DIPEA, N,N-diisopropylethylamine; DCM, dichloromethane; DMF, N,N-dimethylformamide; EtOAc, ethyl acetate; Me, methyl; MeCN, acetonitrile; MS, mass spectrometry; NMM, 4-methylmorpholine; Phe(4-NO2), 4-nitrophenylalanine; OSu, succinimide ester, Phs, phenylsulfonyl; PK, plasma kallikrein; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; tBu, tert-butyl; TFA, trifluoroacetic acid; TMS-Cl, trimethylsilyl chloride.

8. References


