A New Family of β-Hairpin Mimetics Based on a Trypsin Inhibitor from Sunflower Seeds

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*The ability of proteases to regulate many aspects of cell function and defense accounts for the considerable interest in the design of novel protease inhibitors. There are many naturally occurring proteinaceous serine protease inhibitors, one of which is a 14 amino acid cyclic peptide from sunflower seeds that shows both sequence and conformational similarity with the trypsin-reactive loop of the Bowman–Birk family of serine protease inhibitors. This inhibitor adopts a β-hairpin conformation when bound at the active site of bovine β-trypsin. We illustrate here an approach to inhibitor design in which the β hairpin from the naturally occurring peptide is transplanted onto a hairpin-inducing template. Two mimetics with the sequences RC*TKSIPPIC*F (where C*C* is a disulfide) and TKSIPPI are studied, each mounted onto a* D-Pro–L-*Pro template. NMR studies revealed a well-defined β-hairpin conformation for each mimetic in aqueous solution; this conformation is closely related to the trypsin-bound conformation of the natural inhibitor and includes a* cis*-Ile–Pro peptide bond. Both mimetics inhibit trypsin in the mid nanomolar range. An alanine scan revealed the importance for inhibitory activity of the specificity-determining Lys residue and of the first but not the second Pro residue in the IPPI motif. Since these hairpin mimetics can be prepared by parallel combinatorial synthesis, this family of molecules may be a useful starting point for the discovery of other biologically or medicinally useful serine protease inhibitors.*

KEYWORDS:

conformation analysis · inhibitors · NMR spectroscopy · peptides · peptidomimetics

Introduction

Methods that allow the parallel and combinatorial synthesis of conformationally defined peptidomimetics that are derived from surface loops in biologically active peptides and proteins may be of great value for ligand and inhibitor design in chemical biology and medicinal chemistry. One approach involves transplanting β-hairpin loop sequences from the protein onto a template that fixes the N and C termini of the loop into a β-hairpin geometry.[1–3] In this way, the loop is preorganized to adopt a regular hairpin structure. Here we demonstrate how this strategy can be used to prepare a new family of β-hairpin-mimetic trypsin inhibitors.

The starting point for this work was the crystal structure of a novel 14 amino acid cyclic peptide trypsin inhibitor from sunflower seeds in a complex with trypsin.[4] The bound conformation of the natural product revealed a well-defined β-hairpin loop. Its sequence and conformation are similar to those of the reactive site loop of the Bowman–Birk family of serine protease inhibitors, which contains multiple disulfide bridges and has a mass of typically 6–9 kDa.[5, 6] Synthetic peptides that incorporate the reactive site loop of the Bowman–Birk inhibitors retain inhibitory activity provided they possess a disulfide bridge to link the ends of the loop.[7–13]

Our aim was to produce conformational mimics of the natural sunflower seed inhibitor that retain the β-hairpin geometry, as well as an unusual *cis*-Ile–Pro peptide bond at the tip of the hairpin loop. The mimetics were made by transplanting either 11 or 7 residues of the hairpin loop onto a D-Pro–L-Pro template.[1, 2] NMR studies of the resulting peptidomimetics in aqueous solution revealed a well-defined hairpin conformation, essentially identical to that seen in the crystal structure of the natural product bound to trypsin.[4] Both peptidomimetics are good inhibitors of bovine trypsin (apparent dissociation constant of the protease–inhibitor complex, $K_i = 11$ and 103 nM, respectively). An alanine scan also revealed which side chains in the mimetics are important for binding to trypsin.

Results

Design and synthesis of mimetics

A comparison of the structure of the natural inhibitor **1** and the D-Pro–L-Pro template reveals that when either 7 or 11 residues are transplanted, mimetics with the desired β-hairpin conformation should be afforded (see above). This is illustrated in Figure 1. The synthetic targets are, therefore, the cyclic peptides **2** and **3**.

The synthesis of **2** and **3** was performed by a two-stage solid-phase peptide chain assembly and solution-phase macrocyclization procedure similar to that described in previous work.[1, 2] The linear peptide precursors were assembled by using Fmoc chemistry (Fmoc = 9-fluorenylmethoxycarbonyl) with serine as

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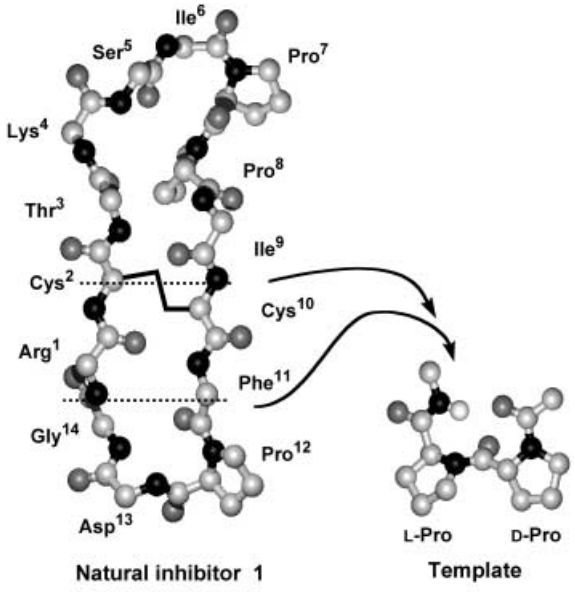

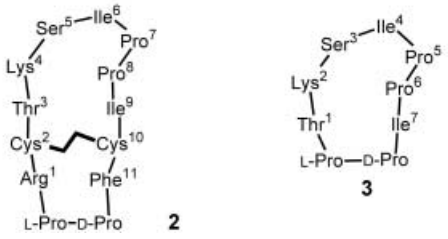

Figure 1. *Backbone conformation of the natural inhibitor from the Protein Data Bank (PDB) structure file 1SFI.[4] The dotted lines show the portions of the hairpin loop transplanted onto the* D*-Pro–*L*-Pro template in the mimetics* ***2*** *and* ***3****.*

Table 1. *^3J(HN,H–C(α)) coupling constants [Hz],[a] amide proton temperature coefficients [ppb K^{-1}],[b] and relative exchange rates[c] for peptide amide protons measured for* ***1*** *–* ***3****.*

Peptide residue	**1** ^3J	$\delta\Delta$/T	HN	**2** ^3J	$\delta\Delta$/T	HN	**3** ^3J	$\delta\Delta$/T	HN
Arg-1	6.9	5.1	●	9.0	1.1	○	–		
Cys-2	9.8	7.4	●	10.2	9.1	●	–		
Thr-3	7.0	6.2	⊙	7.0	4.4	⊙	7.5	2.7	⊙
Lys-4	8.4	6.6	●	8.4	5.9	●	7.4	9.3	●
Ser-5	7.8	1.6	●	7.6	0.5	●	8.0	1.3	●
Ile-6	9.7	9.7	●	9.7	9.9	●	9.5	11.5	●
Ile-9	9.6	3.3	○	9.8	5.4	○	9.7	2.0	○
Cys-10	10.1	8.3	●	10.2	9.8	●	–		
Phe-11	9.1	2.0	○	9.7	4.2	○	–		
Asp-13	7.0	2.4	⊙	–			–		
Gly-14	8.4	4.4	●	–			–		
	4.5								

[a] Measured from 1-D spectra. [b] $-\delta\Delta/T$, measured over the temperature range 279–314 K. [c] Measured from residual peak intensities after dissolution in D_2O at pH 4.0, ● = fast, ⊙ = medium, ○ = slow.

the first residue. After cleavage of the linear precursors from the resin, cyclization in solution, and full deprotection, the required products **2** and **3** were purified by HPLC. The natural product **1** was also prepared by essentially the same approach.

Solution conformations of 1, 2, and 3

The solution conformations of **1** – **3** were investigated by ^{1}H NMR spectroscopy at 300 K and pH 5 in aqueous solution (10% D_2O in H_2O). The ^{1}H NMR spectra revealed a single (> 95%) conformer for each peptide and were assigned unambiguously from homonuclear DQF-COSY, TOCSY, and NOESY or ROESY spectra (see the Supporting Information) by standard methods.[14]

The large coupling constants (^3J(HN, H–C(α)) = 8.5 Hz) observed for several amino acids in **1** – **3**, are indicative of β structure (Table 1). This conclusion is strengthened by a network of short- and long-range NOEs (see the Supporting Information), in particular very strong $d_{\alpha,N}$ (i, $i+1$) NOEs in the sequence RCTK and PICF in **1** and **2**, medium strength d_{NN} NOEs across the β hairpin that connects Arg with Phe as well as Thr^3 with Ile^9 in **1** and **2**, and d_{NN} NOEs between Thr^1 and Ile^7 in **3**. Strong $d_{\alpha\alpha}$ NOEs are also seen between the C(α)–H protons in Cys^2 and Cys^{10} in **1** and **2**, and between Ile^6 (Ile^4 in **3**) and Pro^7 (Pro^5 in **3**) in all three peptides. The latter NOEs reveal a *cis* geometry for the Ile – Pro peptide bond in all three peptides in solution, as observed in the crystal structure of **1** bound to trypsin.

From the NOE data for each of the peptides **1** – **3**, a set of interproton distance restraints was derived and used for structure calculations in a dynamic simulated annealing (SA) protocol with the DISCOVER program (MSI, San Diego). The resulting structures were further refined by restrained molecular dynamics (MD) simulations with time-averaged distance restraints (TA-DR) by using the GROMOS96 software.[15] The set of SA structures showed a well-defined β-hairpin backbone conformation for each peptide, with no significant distance restraint violations. This is illustrated by the superimposition of the SA structures shown in Figure 2. The corresponding average pairwise root mean square deviation values for superimposition of the backbone N, C(α), and C atoms in the set of structures for each of **1** – **3** were 0.46 ± 0.15, 0.63 ± 0.34 and 0.35 ± 0.17 Å, respectively. In Figure 3, the set of solution structures deduced for each molecule are superimposed on the crystal structure of **1** taken from the complex with trypsin (PDB file 1SFI).

Trypsin inhibitory activity and alanine scan

The inhibitory activity of the peptidomimetics **1** – **3** was measured against bovine trypsin. The K_i values are shown in Table 2. An alanine scan was performed on **3** to assess which side chains in the hairpin loop are important for inhibitory activity. Various loop mimetics were prepared in a method similar to that used for **3**. The sequences of the mimetics and their inhibitory acivities against trypsin are also shown in Table 2. Several additional mutants were also prepared to test their inhibitory activity against chymotrypsin (see Table 2).

The solution conformation of mimetic **9**, with Pro^6 replaced by Ala, was studied by ^{1}H NMR spectroscopy. As with **3**, a network of NOEs was observed in ROESY spectra between residues across the β hairpin, as well as between the Ile^4 and Pro^5 C(α)–H

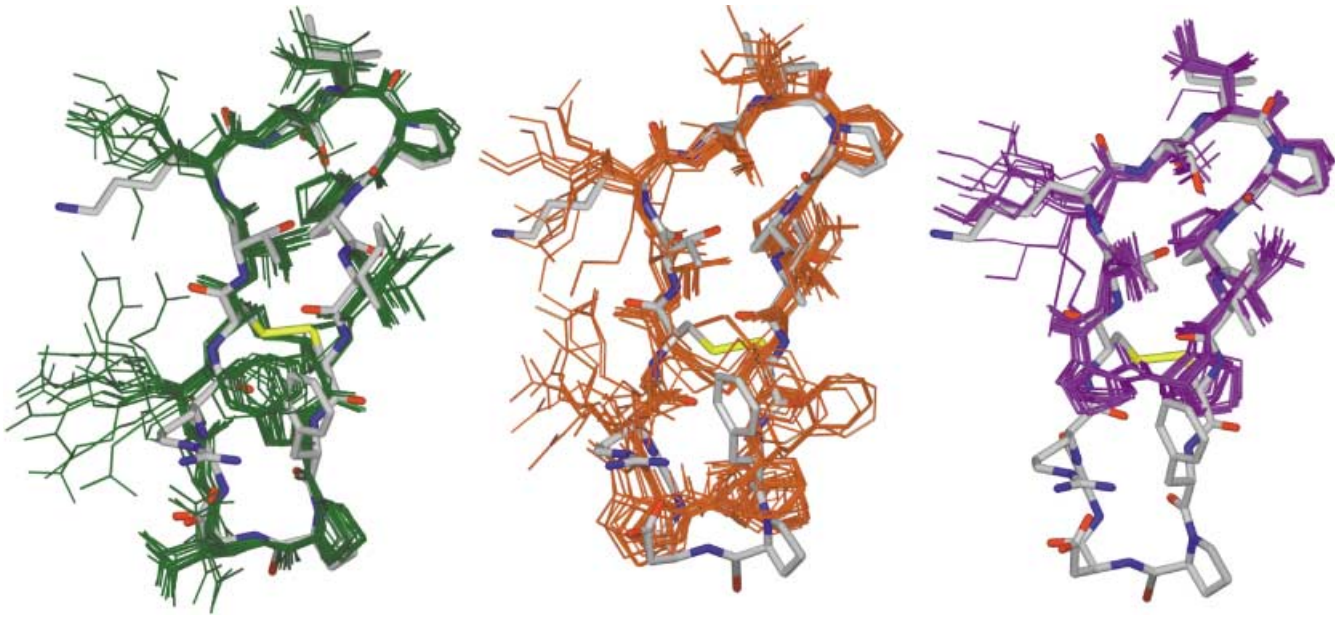

Figure 2. *The backbone N, C(α), and C atoms of the solution NMR structures deduced for **1** (green lines), **2** (orange lines), and **3** (purple lines), each superimposed on the crystal structure of **1** from PDB 1SFI (carbon skeleton: grey sticks; N atoms: blue; O atoms: red; disulfide bridge: yellow).*

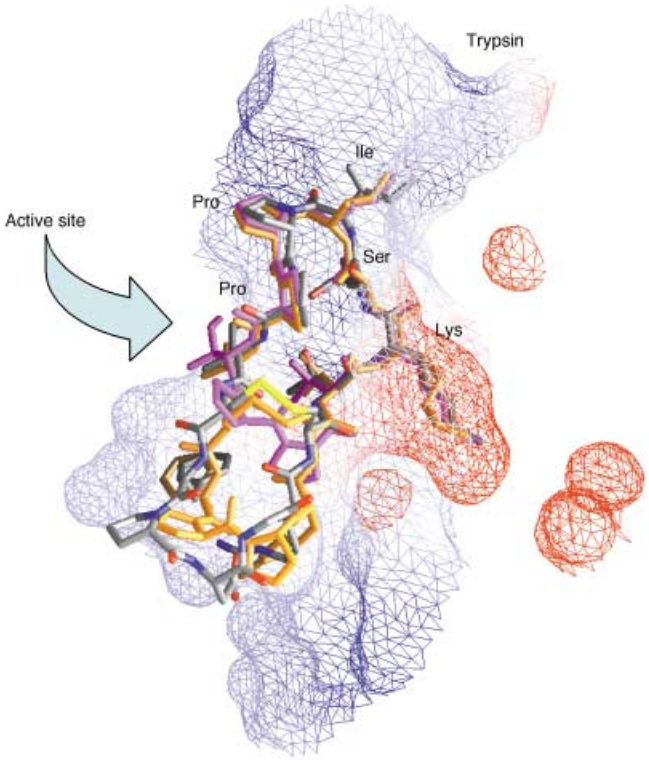

Figure 3. *Superimposition of the **1**:trypsin crystal structure from PDB 1SFI (stick diagram in grey/blue/red) onto the models of **2** (orange sticks) and **3** (purple sticks) bound to trypsin (see text). The KSIPP motif in the inhibitors is labeled. The net shows the surface of trypsin lying within 8 Å of the inhibitor. The Lys side chain of each inhibitor is clearly seen in the negatively charged P1 specificity pocket. This figure was prepared with the GRASP program.[31]*

protons. These results show that the backbone conformation of the inhibitor, including the *cis*-Ile^4–Pro^5 peptide bond, is essentially identical to that deduced for **3** (see above).

Table 2. *Inhibitory activity (K_i) of the peptidomimetics[a] against trypsin and chymotrypsin.*

Peptide	Sequence	K_i [nm] Trypsin	Chymotrypsin
1	GRCTKSIPPICFPD	13 ± 5	2300 ± 100
2	RCTKSIPPICF	11 ± 3	6600 ± 240
3	TKSIPPI	103 ± 7	> 100000
4	AKSIPPI	1000 ± 80	nd[b]
5	TASIPPI	> 10000	nd[b]
6	TKAIPPI	730 ± 40	nd[b]
7	TKSAPPI	1920 ± 40	nd[b]
8	TKSIAPI	> 25000	nd[b]
9	TKSIPAI	114 ± 6	nd[b]
10	TKSIPPA	740 ± 40	nd[b]
11	TYSIPPI	> 25000	4500 ± 200
12	TWSIPPI	> 20000	1400 ± 150
13	TFSIPPI	> 20000	4800 ± 270

[a] The sequence of each mimetic (**2**–**13**) attached to the D-Pro–L-Pro template (not shown) is given. The sequence of the natural product **1** is also given. [b] nd = not determined.

Discussion

With the emergence of genomics and proteomics, an increasing number of peptides and proteins which interact with their biological targets through exposed β-hairpin loops are becoming known. One example is the cyclic serine protease inhibitor **1**, isolated from sunflower seeds, and characterized crystallographically in a complex with trypsin.[4] These loop regions of biologically active peptides and proteins are, therefore, interesting targets for mimetic design. One approach to mimetic design, developed in earlier work,[1, 2, 16] involves transplanting the reactive loop onto a hairpin-stabilizing D-Pro–L-Pro template to afford a family of hairpin mimetic peptides amenable to parallel combinatorial synthesis.[2]

The conformation of **1** in the crystalline complex with trypsin includes an unusual *cis*-Ile – Pro peptide bond within an IPPI motif in a type VI β turn at the tip of the hairpin loop.[4] Similar *cis*-Xaa – Pro (where Xaa = an amino acid) peptide bonds have also been found near the tips of hairpin loops in other peptides and proteins such as canonical conformations of antibody light-chain hypervariable-3 loops,[17] and a peptide derived from the V3 loop of HIV-1IIIB in a complex with a neutralizing anti-gp120 antibody.[18]

It is important to maintain the directional register of the peptide bonds upon transfer of the loop sequence to the D-Pro – L-Pro template (Figure 1). Therefore, two mimetics seemed viable synthetic targets, one (**2**) with 11 residues and the other (**3**) with 7 residues from the inhibitor attached to the template. The mimetic **3** is considerably smaller than the natural product **1** and contains no disulfide bridges. During the synthesis of these mimetics, a two-step solid-phase assembly and solution-phase cyclization strategy was followed, as in earlier work,[2] such that the template residues are incorporated near the center of the linear sequence so as to favour backbone conformations ideal for the subsequent macrocyclization reaction.

Structural studies of the natural product **1** and the two mimetics **2** and **3** in aqueous solution by NMR spectroscopy revealed a well-defined β-hairpin conformation in all three (Figure 2). The average solution conformation deduced for **1** is essentially identical to the crystal structure of **1** bound to trypsin.[4] Also, the average solution structures of both mimetics **2** and **3** are very similar to that of the natural product (Figure 2), thus the design strategy is validated. Very recently, the solution structure of a disulfide-cyclized 11-residue peptide derived from the Bowman – Birk inhibitor reactive site loop was reported.[11] This structure is essentially identical to those deduced here for **1** – **3** in all the parts it has in common with them. Also, during preparation of this paper, the solution NMR structure of **1** was reported by another group to be similar to the crystal structure of the inhibitor in complex with trypsin.[19]

Assays of inhibitory activity against bovine trypsin show that **1** and **2** are of similar potency and that **3** is only about ninefold less active than **2** (Table 2). The alanine scan confirmed the expected energetically important role of the Lys side chain at the P1 position (P1 indicates the first amino acid residue on the N-terminal side of the scissile bond in the substrate; mimetic **5**, Table 2). In addition, this scan revealed that the first Pro (**8**, Table 2), but not the second (**9**, Table 2), in the IPPI motif is important for activity. NMR studies of mutant **9** (Table 2) also revealed the same network of long-range NOEs as seen for **3**, which indicates that the conformation of the macrocycle is largely maintained, including the *cis*-Ile – Pro peptide bond. This result is also of interest in a more general context since considerable effort has been devoted to devising methods to lock Xaa – Pro peptide bonds into the *cis* geometry and such methods include substitution of the proline with a non-natural analogue such as 5-*tert*-butylproline,[20] 5,5-dimethylproline,[21] lactam moieties,[22, 23] and various pseudoproline derivatives.[24, 25] The potential value of such derivatives in synthetic vaccine design has also been highlighted.[26] Here, we show that a *cis*-Xaa – Pro geometry can be locked without modification of the proline residue through its incorporation into a designed cyclic peptide.

Models of the two inhibitors (**2** and **3**) bound to bovine trypsin were constructed by appropriate modification of the **1**:trypsin crystal structure (PDB file 1SFI), followed by short MD simulations (1 ns) of each to relax any high-energy van der Waals contacts. The two resulting models are superimposed on the crystal structure of the **1**:trypsin complex in Figure 3. These superimpositions reveal a closely conserved backbone conformation around the key P1 active-site Lys residue in the bound conformations. The model of **3** bound to trypsin shows most of the same contacts to trypsin as are made by **1** except for the contacts to the (solvent-exposed) side chains of Arg and Asp in the inhibitor, and a buried backbone – backbone hydrogen bond between the Cys^3 (inhibitor) O atom and Gly^{216} (trypsin) N atom.[4] Since the solution structures of **1**, **2**, and **3** are so similar to the crystal structure of **1**, it seems likely that the inhibitors are preorganized in solution for binding to trypsin by a lock and key mechanism.

It was also of interest to determine whether alteration of the residue at the P1 position could change the specificity of the inhibitor. As shown in Table 2, when the P1 Lys residue in **3** was changed to an aromatic one (for example, Tyr (**11**), Trp (**12**), or Phe (**13**)) a significant improvement in selectivity towards chymotrypsin resulted, although the potency is not as high as seen for **3** against trypsin. It is conceivable, however, that through combinatorial changes to the sequence of the macrocycle the affinity of the inhibitor could be improved.

These results may be useful in the design of related cyclic peptide inhibitors targeted against other medicinally or biologically interesting serine proteases, in particular since these molecules can be produced by parallel combinatorial synthesis methods.[2] For example, **1** was shown recently to be a potent inhibitor of the type II transmembrane serine protease matriptase.[27] Finally, the design strategy may also prove amenable to the discovery of peptidomimetics derived from other biologically interesting β-hairpin-containing peptides and proteins.

Experimental Section

Synthesis of mimetics:

Cyclo(-Arg-Cys*-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys*-Phe-Pro-Asp-Gly-) (**1**): Fmoc-Ser(*t*Bu)-OH (1.2 equiv) was coupled to 2-chlorotrityl chloride resin (1.08 mmol g^{-1}) in the presence of diisopropylethylamine (4 equiv) in CH_2Cl_2. The solid-phase synthesis was performed by using an ABI 433A peptide synthesizer on a 0.25-mmol scale by chain elongation with Fmoc-Lys(Boc)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Ile-OH (1 mmol each), and 20% piperidine/dimethylformamide (DMF) for Fmoc deprotection, 1-hydroxy-1*H*-benzotriazole/*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HOBt/HBTU) for activation, diisopropylethylamine as a base, and *N*-methylpyrrolidone as solvent (Boc = *tert*-butoxycarbonyl, Trt = triphenylmethyl, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). After completion of the synthesis, the resin was washed with CH_2Cl_2 and MeOH and then treated three times with CF_3COOH (1%) in CH_2Cl_2. The filtrate was neutralized with pyridine (150 μL) and then evaporated to give the

linear protected peptide. The crude product (typically 1 mg ml^{-1}) was then cyclized overnight at RT in 1% diisopropylethylamine/DMF with 7-aza-1-hydroxy-1*H*-benzotriazole/*N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate (HOAt/HATU; 3 equiv). After evaporation of the DMF, the product was dissolved in CH_2Cl_2 and extracted with water. Purification by HPLC gave the cyclic protected peptide (220 mg, 34%). This peptide (30 mg) was treated at RT for 2 h with a mixture that contained CF_3COOH/thioanisol/phenol/H_2O/ethanedithiol/triisopropylsilane (82.5/5/5/2.5/2.5/2.5; 6 mL). Evaporation, precipitation with diethylether, and purification by HPLC gave the cyclic deprotected reduced peptide (5 mg, 28%). The reduced peptide (10 mg) was stirred for 30 h in ammonium acetate buffer (100 mL, 0.1 M, pH 8) and purified by HPLC (C18 column, 5% MeCN in H_2O with 0.1% CF_3COOH for 2 min, 5–50% MeCN in H_2O with 0.1% CF_3COOH over 15 min, 50–100% MeCN in H_2O with 0.1% CF_3COOH over 4 min) to give the oxidized product **1** (8.6 mg, 86%). Electrospray ionization mass spectrometry (ESI-MS): *m/z*: 1515 (*M*+H). ^{1}H NMR (H_2O/D_2O (9:1), pH 5.0, 300 K): for assignments see the Supporting Information.

Cyclo(-Arg-Cys*-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys*-Phe-DPro-Pro-) (**2**): **2** was prepared by the method used for **1** (see above). ESI-MS: *m/z*: 1439 [*M*+H]. ^{1}H NMR (H_2O/D_2O (9:1), pH 5.0, 300 K): for assignments see the Supporting Information.

Cyclo(-Thr-Lys-Ser-Ile-Pro-Pro-Ile-DPro-Pro-) (**3**): **3** was prepared by the method used for **1** (see above). ESI-MS: *m/z*: 932 [*M*+H]. ^{1}H NMR (H_2O/D_2O (9:1), pH 5.0, 300 K): for assignments, see the Supporting Information.

Parallel synthesis of the library: The library was prepared by the method used for **1** (see above) and each product was purified by HPLC and characterized by ESI-MS (See Table 3).

NMR spectroscopy and structure calculations: ^{1}H one- and two-dimensional NMR spectra were recorded at 600 MHz, 300 K (Bruker DRX600 spectrometer), typically a peptide concentration of approximately 20 mg mL^{-1}, in H_2O/D_2O (9:1), pH 5. The water signal was presaturated. Two-dimensional spectra were analyzed by using Felix software (MSI, San Diego).

For structure calculations, NOEs were determined from NOESY spectra measured with mixing times of 40, 80, 120, and 250 ms, with 1024×256 complex data points zero-filled prior to Fourier transformation to 2048×1024, and transformed with a cosine-bell weighting function. Cross-peak volumes were determined by integration and build-up curves were checked to ensure a smooth exponential increase in peak intensity for NOEs used to derive distance restraints. SA calculations were performed by using the DISCOVER program (MSI, San Diego) and methods described in detail earlier.[28]

The GROMOS96 suite of programs[15] was used for MD simulations with and without TA-DR with the 43A1 force field at 300 K, 1 atm pressure, and with periodic boundary conditions. Arg, Asp, and Lys residues were simulated with charged side chains and Na^+ or Cl^- counterions for electrical neutrality. The upper distance restraints were the exact values obtained from NOE build-up curves, where necessary with pseudoatom corrections, a memory decay time $t_{dr} = 50$ ps, and a force constant $K_{dr} = 1000$ kJ mol^{-1} nm^{-2}. The initial structure was one of the lowest energy SA structures embedded in a truncated octahedral box filled with simple point-charge water molecules. The temperature was held constant by weak coupling ($t_T = 0.1$ ps) to an external bath at 300 K. The SHAKE algorithm was used to maintain bond lengths with a relative precision of 10^{-4} and the integrator time step was 0.002 ps. Nonbonded interactions evaluated at every step were within a short-range cut-off of 8 Å. For long-range interactions, calculated every 5 steps, the cut-off was 14 Å. Structures were saved for analysis every 100 steps (0.2 ps). After short simulations to relax the solute and solvent, simulations with and without TA-DR were each run for 2 ns.

Enzyme assays: The active enzyme concentrations were calculated by using the equation described by Henderson.[29] The inhibitor concentrations were determined by quantitative amino acid analysis. All assays were repeated in quadruplicate.

Determination of antitrypsin activity: A solution of bovine trypsin (Boehringer) was incubated for 5 min with inhibitor. The assays were carried out at 20 °C in tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 7.8, 100 mM) that contained 10 mM $CaCl_2$. The substrate was *N*-α-benzoyl-L-arginin-4-nitroanilide (0.32 mM) and the initial reaction rate was monitored over 30 min at 405 nm.

Determination of anti-chymotrypsin activity: As above, except the substrate was *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanin-4-nitroanilide (55 μM).

Apparent K_i values were calculated by fitting initial rate data to Equation (1), which assumes competitive tight-binding inhibition[30] and in which v is the steady state rate, v_o is the rate in the absence of inhibitor, E_t is the total protein concentration that can bind inhibitor, I_t is the total inhibitor concentration, and K_i is the apparent dissociation constant of the protease–inhibitor complex.

$$v = \frac{v_o}{2E_t}\left[E_t - I_t - K_i + \sqrt{(I_t + K_i - E_t)^2 + 4K_i I_t}\right] \quad (1)$$

For inhibitors assayed under Michaelis–Menten conditions, the Michaelis–Menten equation was used.

Table 3. *HPLC retention times and peaks observed (m/z) for each mimetic by electrospray mass spectrometry.*

Peptide	Formula	Calculated mass	Obtained mass [*M*+H]$^+$	HPLC retention time [min]
3	$C_{45}H_{74}N_{10}O_{11}$	931.15	931.6	13.39
4	$C_{44}H_{72}N_{10}O_{10}$	901.13	901.8	14.57
5	$C_{42}H_{67}N_9O_{11}$	874.06	874.8	14.28
6	$C_{45}H_{74}N_{10}O_{10}$	915.15	915.8	13.97
7	$C_{42}H_{68}N_{10}O_{11}$	889.07	889.6	12.71
8	$C_{43}H_{72}N_{10}O_{11}$	905.11	905.8	14.40
9	$C_{43}H_{72}N_{10}O_{11}$	905.11	905.7	13.44
10	$C_{42}H_{68}N_{10}O_{11}$	889.07	889.7	11.83
11	$C_{48}H_{71}N_9O_{12}$	966.15	966.7	15.99
12	$C_{50}H_{72}N_{10}O_{11}$	989.19	989.7	17.32
13	$C_{48}H_{71}N_9O_{11}$	950.15	950.7	17.02

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