Examples of Peptide–Peptoid Hybrid Serine Protease Inhibitors Based on the Trypsin Inhibitor SFTI-1 with Complete Protease Resistance at the P1–P1′ Reactive Site

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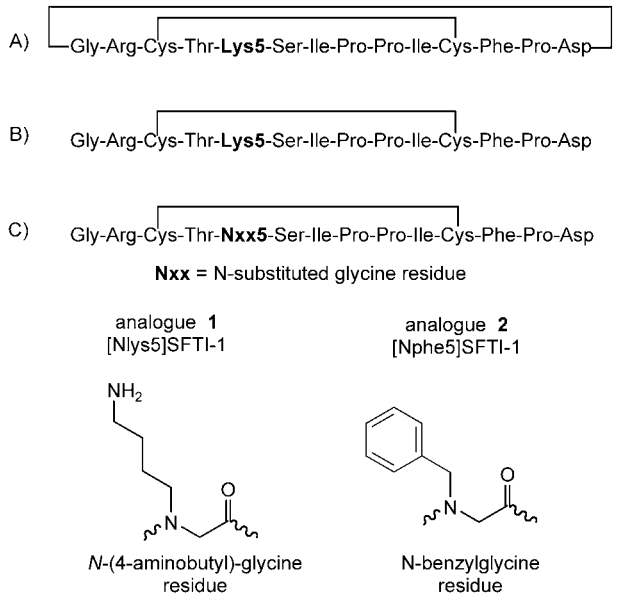
Research in the field of protease inhibitors is focused on obtaining potent, specific and protease-resistant inhibitors. To our knowledge, there are no reports in the literature that consider the application of N-substituted glycine residues (peptoid monomers) for the design of peptidomimetic protease inhibitors. We hereby present the chemical synthesis and kinetic properties of two new analogues of the trypsin inhibitor SFTI-1 modified at the P1 position. Substitution of Lys5 in SFTI-1 by N-*(4-aminobutyl)-glycine and* N*-benzylglycine, which mimic Lys and Phe, respectively, made these analogues completely protease-resistant at their P1–P1′ reactive sites. The analogues synthesised appeared to be potent inhibitors of bovine β-trypsin and α-chymotrypsin. These noncovalent, competitive and selective peptide–peptoid hybrid (peptomeric) inhibitors might open the way to targeting unwanted proteolysis.*

Introduction

Protease inhibitors are widely distributed in nature; their main role is to regulate the activity of proteolytic enzymes.[1,2] Among these, inhibitors of serine proteases are being studied the most. Very often they also serve as model objects in the study of protein–protein interactions. All the so-called canonical inhibitors have been grouped into at least 18 inhibitor families.[2,3] The Bowman–Birk protease inhibitors (BBI) are a class of protease inhibitors occurring in plants. The first inhibitor of that family was isolated from leguminous seeds by Bowman,[4] and its properties were characterised by Birk and co-workers in 1963.[5] In 1999, Luckett and co-workers isolated a 14-amino-acid-residue, cyclic, homodetic peptide from sunflower seeds whose structure is additionally stabilised by a disulfide bridge[6] (see Scheme 1 A). This inhibitor, named SFTI-1, is the smallest naturally occurring serine protease inhibitor isolated up to date. The reactive site of the SFTI-1 inhibitor (the P1–P1′ peptide bond according to notation of Schechter and Berger[7]) is located between residues Lys5 and Ser6. Sequential and conformational analysis has shown its similarity to the reactive site loop of the Bowman–Birk inhibitors.



Scheme 1. Chemical formulas of: A) native SFTI-1, B) acyclic variant of SFTI-1, C) peptomeric analogues of SFTI-1.

Since the late 1970s, there has been growing interest in the research of new analogues based on the BBI reactive canonical loop,[8] and up to now over one hundred such inhibitors have been described in the literature.[9] The small size and high trypsin inhibitory activity of SFTI-1 make that inhibitor an attractive template for the design of new protease inhibitors with the potential to be used as therapeutic agents. Several papers describing the potent inhibitory activity of SFTI-1 and its analogues have been published recently.[10–14]

The goals of the research into protease inhibitors are to obtain specific, potent and protease-resistant inhibitors. Continuing this type of investigations, we report the use of N-sub-

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stituted glycine residues (peptoid monomers) as proteinogenic amino acid mimetics, which can be used to design completely protease-resistant inhibitors. The hybrid combinations of peptides and peptoids, which we prefer to call "peptomers" (*pep*tide-pep*t*oid hybrid poly*mers*) after Ostergaard and Holm,[15] might reveal new, interesting properties from the structural and functional point of view.

Peptoids are composed of N-substituted glycine building blocks that differ from peptides in that their side chains are shifted from the C^{α} to the N^{α} atom of monomer unit. Peptoids exhibit many interesting properties including backbone achirality, lack of hydrogen bonds due to the lack of an amide hydrogen atom and thus lack of secondary structures, and stability against proteolysis.[16]

We decided to incorporate the N-substituted glycines at the P1 position of SFTI-1, thus yielding analogues **1** and **2** (Scheme 1). It is well documented that this position is responsible for the specificity of the inhibitor and is involved in many contacts between the inhibitor and the cognate enzyme.

Results and Discussion

Overview

There are many examples in the literature that considering the application of poly(N-substituted glycines) as peptidomimetics,[17] but to our knowledge none of them relates to the application of N-substituted glycine residues (peptoid monomers) as the mimetics of proteinogenic amino acids, which are used to replace them in the native peptidic protease inhibitors. In this paper, we report the synthesis and kinetic properties of two novel inhibitors based on the acyclic analogue of trypsin inhibitor SFTI-1. Although native, cyclic SFTI-1 is a very potent inhibitor, in our previous studies we have shown that the elimination of head-to-tail cyclisation in this inhibitor did not influence the inhibitory activity expressed as the association equilibrium constant (K_a).[11] For this reason, the acyclic SFTI-1 (Scheme 1 B) was chosen as a starting structure in this study. By replacing Lys5 in the SFTI-1 sequence by *N*-(4-aminobutyl)glycine ([Nlys5]SFTI-1, analogue **1**) and *N*-benzylglycine ([Nphe5]SFTI-1, analogue **2**), which mimic L-lysine and L-phenylalanine, respectively (Scheme 1 C), we have obtained potent inhibitors of trypsin and chymotrypsin that are completely protease-resistant. These inhibitors belong to a novel class of serine protease inhibitors—noncovalent and competitive peptide–peptoid hybrid (peptomeric) inhibitors—that might be good drug candidates, since the latest research on the Bowman–Birk type of inhibitors has revealed that inhibitors of that type can be used as chemopreventive agents with anticarcinogenic activity.[18]

Peptomer synthesis

The synthesis of two peptomeric analogues of SFTI-1 with Nlys and Nphe introduced at the P1 position was achieved by a solid-phase technique, as described in the Experimental Section. The physicochemical properties of the synthesised analogues are given in Table 1. During the synthesis of [Nlys5]SFTI-1, the amino side-chain group of *N*-(4-aminobutyl)glycine was protected by a trityl moiety, which was applied directly to the N^{ε} atom after attaching 1,4-diaminobutane to the peptidyl-resin.

Table 1. Physicochemical properties and association equilibrium constants (K_a) of analogues of SFTI-1.

Inhibitor	t_R (HPLC) [min]	M_W calcd	M_W found $[M+H]^+$	K_a [M^{-1}] bovine β-trypsin	K_a [M^{-1}] bovine α-chymotrypsin
SFTI-1 acyclic	15.00	1531.2	1531.8	9.9×10^{10}	4.9×10^{6}
[Nlys5]SFTI-1	15.50	1530.7	1531.5	1.0×10^{8}	N/A
[Nphe5]SFTI-1	20.15	1549.7	1550.5	N/A	3.8×10^{8}

Errors in determination of K_a values never exceeded 10%; N/A = not active. The values for acyclic SFTI-1 have been published elsewhere.[11,14]

Enzyme kinetics

Figure 1 shows the Lineweaver–Burk plot for [Nphe5]SFTI-1. The straight lines obtained at different inhibitor concentrations, all intersecting in one point (within the regression error) that corresponds to $1/V_{max}$, indicate that the studied compound acts as a purely competitive inhibitor. The same type of inhibition was also observed in experiments performed with analogue **1** (not shown).

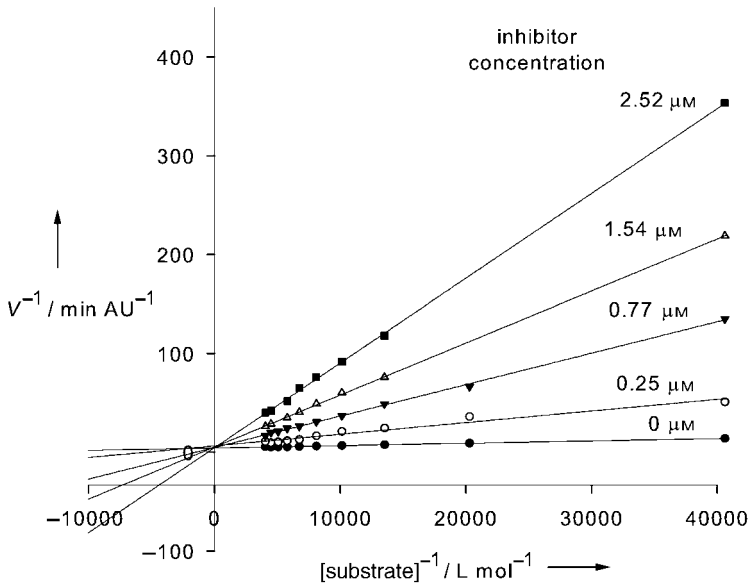

Figure 1. The Lineweaver–Burk plot, showing competitive inhibition of bovine α-chymotrypsin by [Nphe5]SFTI-1. V = reaction velocity measured at $\lambda = 410$ nm.

The inhibition curves obtained for the analogues studied are shown in Figure 2. The determined value of the association equilibrium constant (K_a) for analogue **1** with bovine β-trypsin

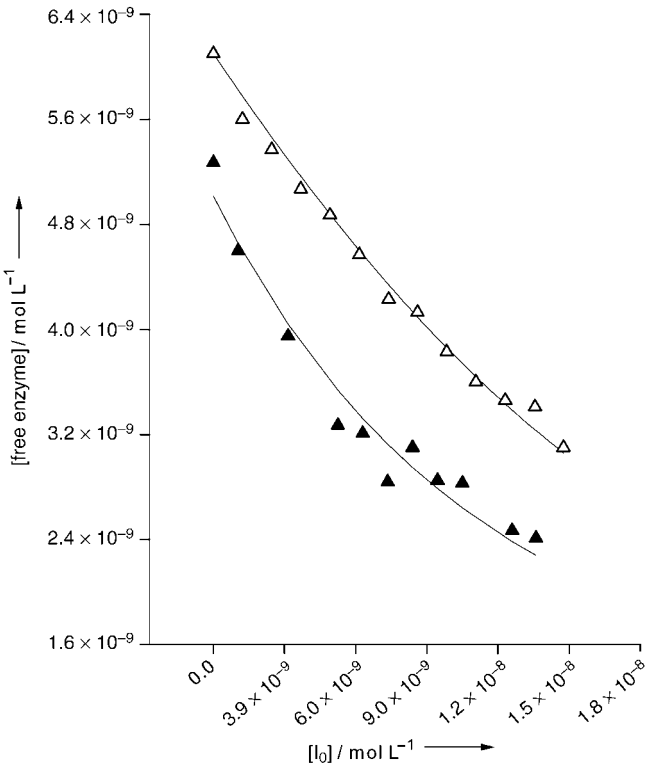

Figure 2. The inhibition curves of analogues **1** (▲) and **2** (△) against bovine β-trypsin and α-chymotrypsin, respectively. I_0 = initial inhibitor concentration. The experimental data were fitted according to the procedure described in text.

is $1.0\times10^8\,\text{M}^{-1}$, whereas analogue **2** inhibits bovine α-chymotrypsin with a calculated K_a of $3.8\times10^8\,\text{M}^{-1}$ (see Table 1). Although the trypsin inhibitory activity of analogue **1** is two orders of magnitude lower than that determined for the native SFTI-1, the result obtained is very promising. In our previous work on the trypsin inhibitor CMTI-III,[19] we have shown that requirements for the P1 position are very strict. In fact only analogues with Arg and Lys at this position exhibited a strong interaction with trypsin. The introduction of additional methylene group into the side chain (L-homoarginine) decreased trypsin inhibitory activity by almost two orders of magnitude, whereas shortening the side chain even by one methylene group (Orn residue in position P1) completely cancelled inhibitory activity. From the chemical point of view, the changes introduced in [Nlys5]SFTI-1 and [Nphe5]SFTI-1 are substantial: the side chain in position 5 is shifted from C^α to N^α, and, as a consequence, that moiety is achiral. The changes applied might also cause further peptide bond isomerisation between P1–P1′ residues and/or other residues. Our preliminary NMR study of [Nphe5]SFTI-1 in [D_6]DMSO suggests that there are no significant changes in the overall structure compared with acyclic SFTI-1. The presence of more than one set of residual proton resonances in all spectra might reflect *cis*/*trans* isomerisation of the X-Pro peptide bond(s). Detailed results of that study should be available in the near future.

Analogue **2** displays a strong chymotrypsin-inhibiting activity, and in fact it seems to be one of the most potent synthetic inhibitors of bovine α-chymotrypsin described so far that has a structure based on the BBI reactive-site loop.[9] Since the surroundings of the reactive sites in trypsin and chymotrypsin are different, simple substitution of a basic amino acid residue in trypsin inhibitors by a hydrophobic residue does not produce strong chymotrypsin inhibitors. In our previous work,[20] we showed that the substitution of Arg5, present at the P1 position of the trypsin inhibitor CMTI-III, by Phe yielded an analogue with moderate chymotrypsin-inhibiting activity. Additional modifications introduced in the binding loop increased chymotrypsin inhibitory activity by five orders of magnitude. Moreover, a common problem with short peptidic inhibitors is that they are usually hydrolysed quickly, and the observed inhibition is temporary. During the determination of K_a values of the inhibitors studied, we observed practically no difference in the inhibition ratio of the enzyme when the incubation time was increased up to 24 h. Although the P1–P1′ bond is intact in the determined crystallographic structure of SFTI-1,[6] it is well known that this bond is cleaved by the enzyme. In experiments by Marx and co-workers,[21] the cleaved P1–P1′ bond of acyclic SFTI-1 was enzymatically resynthesised by bovine β-trypsin. In the case of the SFTI-1 analogues described here, the P1–P1′ bond is intact even after 72 h of incubation in the presence of the enzyme. The proteolytic susceptibilities of analogues **1** and **2** are presented in Figure 3. As can be seen in the case of analogue **1**, during incubation, the peak with a retention time of about 15.5 min vanishes, and a peak with retention time of 17.4 min appears. MALDI MS analysis of the fractions corresponding to these peaks revealed that the peak migration is a result of cleavage of the N-terminal dipeptide Gly-Arg fragment from [Nlys5]SFTI-1 by bovine β-trypsin. In the case of analogue **2** there is no migration of peaks, but within the incubation time, a small, unidentified peak with a retention time of around 21.2 min appears. In both cases the P1–P1′ bond remains intact and no $[M+H]^+$ ions corresponding to the cleavage of the P1–P1′ bond are observed in MALDI spectra. These results are in agreement with previous experiments showing the resistance of poly(N-substituted glycines) to enzymatic hydrolysis.[15,22] The results obtained for acyclic SFTI-1 revealed that this inhibitor is readily hydrolysed under such conditions. The half-life of that reaction was calculated to be approximately 2.7 h (data not shown).

The novel, peptide–peptoid hybrid class of inhibitors of serine proteases described above contain a reactive-site P1–P1′ that is completely resistant to proteases. It may be deduced easily that inhibitors of this type could be designed for other proteases as well. Therefore, by modifying the key P1 position of peptide inhibitors by using N-substituted glycine residues new, potent and protease resistant inhibitors might be discovered and, in the future, applied as therapeutic agents.

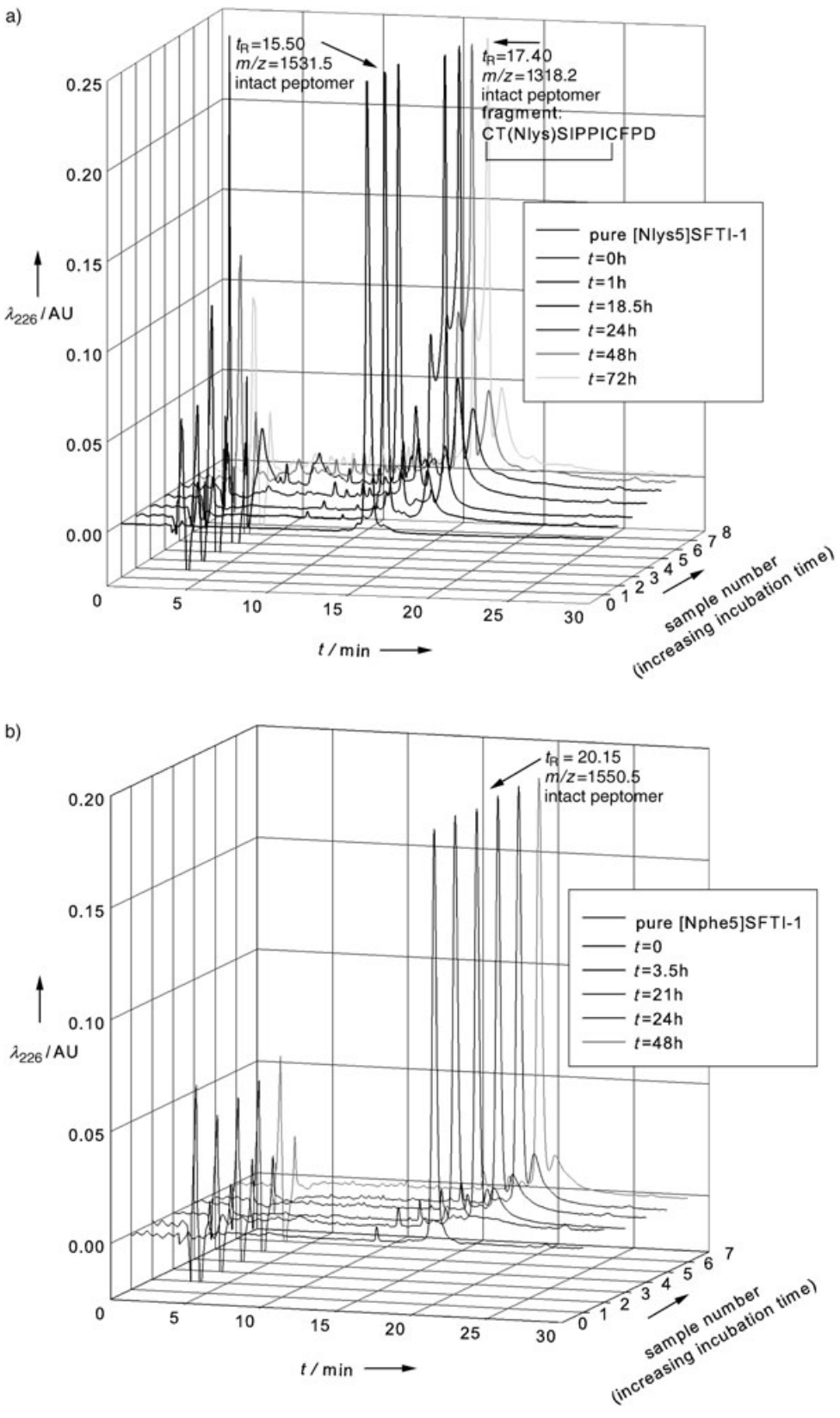

Figure 3. HPLC chromatograms showing proteolytic susceptibility assays of A) analogue **1** in the presence of bovine β-trypsin and B) analogue **2** in the presence of bovine α-chymotrypsin. The experiments were performed at room temperature and pH 3.5 in 50 mM sodium acetate and 20 mM $CaCl_2$ and with catalytic amounts of enzymes (1 mol%).

Experimental Section

Peptomer synthesis: Both peptomeric SFTI-1 analogues were synthesised manually by a solid-phase method by using Fmoc chemistry, as described previously.[23] The amino acids derivatives used for the syntheses were as follows: Fmoc-Gly, Fmoc-Arg(Pbf), Fmoc-Cys(Trt), Fmoc-Thr, Fmoc-Ser(*t*Bu), Fmoc-Ile, Fmoc-Pro, Fmoc-Phe and Fmoc-Asp(O*t*Bu). The C-terminal amino acid residue was attached to chlorotrityl polystyrene resin (Rapp Polymere, Germany) by using 50% molar excess of Fmoc-Asp(O*t*Bu) in the presence of an equimolar amount of *N*,*N*-diisopropylethylamine (DIPEA). N-substituted glycine derivatives were introduced into the peptide chain by the submonomeric approach[22] by using bromoacetic acid and primary amines—-1,4-diaminobutane and benzylamine, for Nlys and Nphe, respectively. In the case of [Nlys5]SFTI-1, the ε-amino group of Nlys was protected with a trityl moiety. The protecting group was introduced directly onto the peptidyl resin. Briefly, trityl chloride (1.1 equiv with respect to the resin loading capacity) together with DIPEA (1 equiv) was dissolved in DMF. This solution was added to the peptidyl resin (with attached 1,4-diaminobutane), and the reaction was run for 3 h at room temperature. The progress of the reaction was monitored by MALDI-TOF analysis as well as by chloranil and Kaiser tests. The negative results of both the chloranil (in the presence of acetaldehyde) and Kaiser tests, positive chloranil test (in the presence of acetone) and appropriate $[M+H]^+$ signals found in MALDI spectra were the evidence that the Trt moiety was introduced onto the ε-amino group of Nlys.

After completion of the synthesis, the peptomers were cleaved from the resin, and the protecting groups were removed in one step by using TFA/phenol/triisopropylsilane/H_2O (88:5:2:5, *v*/*v*/*v*/*v*). Crude linear SFTI-1 analogues were cyclised in a mixture of 5% acetic acid and 20% DMSO, pH 6 (adjusted with ammonium carbonate) with gentle stirring for 24 h[24] and desalted by solid-phase extraction on C_{18} LiChrolut cartridges (Merck,

Germany). Finally, both analogues were purified by HPLC on a Beckman Gold System chromatograph (Beckman, USA) by using an RP Kromasil-100, C_8, 5 μm column (8×250 mm) (Knauer, Germany). The solvent system was A) 0.1% TFA and b) 80% acetonitrile in A. A linear gradient from 20 to 80% B for 30 min was employed, the flow rate was 1.5 mL min^{-1} monitored at 226 nm.

Determination of the type of enzyme inhibition: Simple experiments were performed to identify the type of inhibition for analogues **1** and **2** as new serine protease inhibitors. In the case of analogue **1**, the samples of inhibitor (0–50 μL, 77.4 mM) were added to the set of cuvettes (1.5 mL) containing buffer solution (0.1 M Tris-HCl, pH 8.3, 20 mM $CaCl_2$ and 0.005% Triton X-100) and enzyme (25 μM), and then reaction mixture was incubated at room temperature for 1 h. After that, the chromogenic substrate solution (Bz-D,L-Arg-4-nitroanilide, 200 mM; 0–10 μL) was added, and the absorbance was measured for 2 min at 410 nm. In the case of inhibitor **2**, all conditions were similar except the substrate (Suc-Ala-Ala-Pro-Phe-4-nitroanilide, 35 mM). The slope values obtained at various inhibitor and substrate concentrations were used to draw the Lineweaver–Burk plot (Figure 1).

Determination of association equilibrium constants: Bovine β-trypsin was standardised by burst kinetics with 4-nitrophenyl-4′-guanidinebenzoate (NPGB)[25] at an enzyme concentration of 1 μM. The standardised bovine β-trypsin solution was used for the titration of turkey ovomucoid third domain inhibitor (OMTKY), which in turn served to determine the activity of bovine α-chymotrypsin. The concentration of both inhibitors was determined by titration of their stock solutions with standardised bovine β-trypsin or bovine α-chymotrypsin.

The association equilibrium constants (K_a) were determined by the Green–Work method modified in the laboratory of M. Laskowski.[26,27] Increasing amounts of inhibitor were added to a constant amount of enzyme, and, after suitable incubation time, the residual enzyme activity was measured on a Cary 3E spectrophotometer (Varian, Australia) by using a turnover substrate. Enzyme–inhibitor interactions were determined in Tris-HCl buffer (0.1 M, pH 8.3) containing $CaCl_2$ (20 mM) and 0.005% Triton X-100 at 22 °C. The measurements were carried out at initial enzyme concentrations of 5.3 nM and 6 nM for trypsin and chymotrypsin, respectively. After proper incubation time, the residual enzyme activity was measured with Phe-Val-Pro-Arg-Anb^{5,2}-NH_2[28] for analogue **1** and Suc-Ala-Ala-Pro-Leu-4-nitroanilide for analogue **2**. The experimental points were analysed based on the plot of [enzyme] versus [I_o]. The experimental data were fitted to the theoretical values by the utilising program of A. Liwo (University of Gdansk) and the Marquardt method.[29] The K_a values calculated are given in Table 1, whereas inhibition curves are shown in Figure 2.

Proteolytic susceptibility assays: Analogues **1** and **2** and acyclic SFTI-1 were incubated in sodium acetate (50 mM) and $CaCl_2$ (20 mM, pH 3.5) with catalytic amounts of enzymes (1 mol%).[30] The incubation was carried out at room temperature, and sample aliquots of the mixture were taken out periodically and submitted to RP-HPLC analysis. This analysis was performed on HPLC Gold System chromatograph (Beckman, USA) by using an RP Kromasil-100, C_8 column (4.6×250 mm×5 μm; Knauer, Germany). The solvent system was A) 0.1% TFA and B) 80% acetonitrile in A; linear gradient from 20 to 80% B for 30 min, flow rate 1 mL min^{-1}, monitored at 226 nm. The collected fractions of the peaks were analysed by MALDI MS (Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) by using a α-CCA matrix.

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[1] M. Laskowski, Jr., I. Kato, *Annu. Rev. Biochem.* **1980**, *49*, 593.
[2] M. Laskowski, Jr., M. A. Qasim, *Biochim. Biophys. Acta* **2000**, *1477*, 324.
[3] D. Krowarsch, T. Cierpicki, F. Jelen, J. Otlewski, *Cell. Mol. Life Sci.* **2003**, *60*, 2427.
[4] D. E. Bowman, *Proc. Soc. Exp. Biol. Med.* **1946**, *63*, 547.
[5] Y. Birk, A. Gertler, S. Khalef, *Biochem. J.* **1963**, *87*, 281.
[6] S. Luckett, R. S. Garcia, J. J. Barker, A. V. Konarev, P. R. Shewry, A. R. Clarke, R. L. Brady, *J. Mol. Biol.* **1999**, *290*, 525.
[7] I. Schechter, A. Berger, *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
[8] N. Nishino, H. Aoyagi, T. Kato, N. Izumiya, *J. Biochem.* **1977**, *82*, 901.
[9] J. D. McBride, E. M. Watson, A. B. E. Brauer, A. M. Jaulent, R. J. Leatherbarrow, *Biopolymers* **2002**, *66*, 79.
[10] Y. Q. Long, S. L. Lee, C. Y. Lin, I. J. Enyedy, S. Wang, P. Li, R. B. Dickson, P. P. Roller, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2515.
[11] E. Zabłotna, K. Kaźmierczak, A. Jaśkiewicz, M. Stawikowski, G. Kupryszewski, K. Rolka, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 855.
[12] A. Descours, K. Moehle, A. Renard, J. A. Robinson, *ChemBioChem* **2002**, *3*, 318.
[13] S. M. Miles, R. J. Leatherbarrow, S. P. Marsden, W. J. Coates, *Org. Biomol. Chem.* **2004**, *2*, 281.
[14] E. Zabłotna, K. Kaźmierczak, A. Jaśkiewicz, G. Kupryszewski, K. Rolka, *Lett. Pept. Sci.* **2002**, *9*, 131.
[15] S. Ostergaard, A. Holm, *Mol. Divers.* **1997**, *3*, 17.
[16] S. M. Miller, R. J. Simon, S. Ng, R. N. Zuckermann, J. M. Kerr, W. H. Moos, *Drug Dev. Res.* **1995**, *35*, 20.
[17] J. A. Patch, K. Kirshenbaum, S. Seurynck, R. Zuckermann, A. E. Barron in *Pseudo-peptides in Drug Development*. (Ed: Peter E. Neilsen), Wiley-VCH, Weinheim, **2004**, pp. 1–32.
[18] F. L. Meyskens, Jr., *Ann. N.Y. Acad. Sci.* **2001**, *952*, 116.
[19] A. Jaśkiewicz, A. Lesner, J. Różycki, S. Rodziewicz, K. Rolka, U. Ragnarsson, G. Kupryszewski, *Biochem. Biophys. Res. Commun.* **1997**, *240*, 869.
[20] K. Rolka, G. Kupryszewski, J. Otlewski, I. Krokoszyńska, T. Wilusz, *Biol. Chem. Hoppe-Seyler* **1991**, *372*, 63.
[21] U. C. Marx, M. L. J. Korsinczky, H. J. Schirra, A. Jones, B. Condie, L. Otvos, Jr., D. J. Craik, *J. Biol. Chem.* **2003**, *278*, 21782.
[22] R. J. Simon, R. S. Kania, R. N. Zuckermann, V. D. Huebner, D. A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C. K. Marlowe, D. C. Spellmeyer, R. Tan, A. D. Frankel, D. V. Santi, F. E. Cohen, P. A. Bartlett, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 9367.
[23] S. Rodziewicz-Motowidło, K. Brzozowski, A. Łęgowska, A. Liwo, J. Silberring, M. Smoluch, K. Rolka, *J. Pept. Sci.* **2002**, *8*, 211.
[24] J. P. Tam, C. R. Wu, W. Liu, J. W. Zhang, *J. Am. Chem. Soc.* **1991**, *113*, 6657.
[25] T. Chase, E. Schaw, *Methods Enzymol.* **1970**, *19*, 20.
[26] M. W. Empie, M. Laskowski Jr., *Biochemistry* **1982**, *21*, 2274.
[27] S. J. Park, PhD thesis, Purdue University, (IN, USA), **1985**.
[28] E. Zabłotna, H. Dysasz, A. Lesner, A. Jaśkiewicz, K. Kaźmierczak, H. Mieczkowska, K. Rolka, *Biochem. Biophys. Res. Commun.* **2004**, *319*, 185.
[29] D. W. Marquardt, *J. Soc. Indust. Appl. Math.* **1963**, *11*, 431.
[30] A. M. Jaulent, R. J. Leatherbarrow, *Protein Eng. Des. Sel.* **2004**, 17, 681.

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