

Introduction of Pro and its analogues in the conserved P₁' position of trypsin inhibitor SFTI-1 retains its inhibitory activity

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Summary

A number of monocyclic SFTI-1 analogues modified in the conserved inhibitor P₁' position by Pro, its L-hydroxyproline (Hyp) derivative as well as mimetics with different ring size were synthesized by the solid-phase method. Replacement of Ser⁶ by Pro, Hyp, and a four-member ring, L-azetidine-2-carboxylic acid (Aze), retained trypsin or chymotrypsin inhibitory activity. The determined association equilibrium constants of these analogues with a cognate enzyme were about two orders of magnitude lower than those obtained for ones with conserved Ser⁶. In all analogues, with the exception of one, [Phe⁵,Aze⁶]SFTI-1, the P₁-P₁' reactive site remained intact. The results provide first evidence that the conserved Ser in the P₁' position of Bowman-Birk inhibitors can be successfully replaced by an amino acid with a secondary amine group.

Keywords: peptides, proteinase inhibitors, SFTI-1, mimetics of Pro

Introduction

Sunflower trypsin inhibitor – 1 (SFTI-1) is up to date the smallest known endogenous peptidic protease inhibitor [1]. This bicyclic peptide shares a high sequential homology with the binding loop of inhibitors of the Bowman-Birk (BBI) family. Its amino acid sequence is shown below:



Owing to its small size, high inhibitory activity and a well-defined three-dimensional structure [1,2,3,4], SFTI-1 became an attractive object to study enzyme – inhibitor interaction. In our recent work [5], we focused our attention on the inhibitor's P₁' position which in BBIs is occupied exclusively by a Ser residue. It was first reported by Odani and Ikenaka [6] that introduction in this position of BBIs uncharged amino acid residues resulted in decreased inhibitory properties in the following sequence: Ser > Ala > Thr > Val > Leu > Gly. Interestingly enough, the P₁' inhibition of serine proteinases by other canonical inhibitor,

BPTI, was found to decrease in a different sequence: Ala > Gly > Ser > Arg > Val > Leu > Trp [7]. In the case of SFTI-1, several research teams [8-12] including ours [5] reported that analogues modified in that position by Ala displayed inhibitory activity. We have shown that Ser6 replaced in SFTI-1 not only by the proteinogenic Ala, but also by either synthetic amino acid residue, Hse (L-homoserine), or its peptoid mimetic Nhse [*N*-(2-hydroxyethyl)glycine] retained inhibitory activity. Substitution of Ser by Ala gave a chymotrypsin inhibitor with a 14-fold lower activity as compared with that of the parent compound. Introduction of the peptoid monomer Nhse in the P₁' position produced SFTI-1 analogues almost equipotent with that containing in this position naturally occurring (and highly conserved) Ser, whereas Hse in that position decreased inhibitory activity by one order of magnitude. This is the first evidence that the absolutely conserved Ser6 present in the inhibitor's P₁' position can be successfully replaced by a synthetic derivative.

In order to elucidate the role of the inhibitor P₁', we also synthesized monocyclic (with a disulfide bridge only) SFTI-1 analogues with Pro in position 6. This analogue ([Pro⁶]SFTI-1) displayed the ability to inhibit bovine β-trypsin. This was rather an unexpected result, because this amino acid does not have proton(s) which could be involved in the intra- or intermolecular hydrogen bond network. According to our knowledge, the only report on this modification was published by Hilpert *et al.* [12], who performed complete substitutional analysis of SFTI-1 revealing that substitution of Ser6 by Pro led to an analogue with no affinity towards the investigated proteinases (bovine β-trypsin, porcine pancreatic elastase and proteinase K). This very intriguing finding and also lack of published experimental data for other canonical inhibitors modified in the P₁' by Pro, encouraged us to investigate the problem in more detail. In this paper, we describe chemical synthesis and inhibitory activity of a number of monocyclic SFTI-1 analogues modified in position 6 by Pro and its derivatives and mimetics. The primary structure of these analogues are presented in Table 1.

The synthesized SFTI-1 analogues contained either Lys (peptides with odd numbers) or Phe (those with even numbers) in the substrate specificity P₁ position. For this reason, their inhibitory activity was checked on trypsin and chymotrypsin, respectively. In the P₁' position introduced were: Pro and its derivative L-hydroxyproline and the mimetics: four-member ring – Aze (L-azetidine-2-carboxylic acid), six-member ring – Hpr (L-homoproline) and Oic (L-octahydroindole-2-carboxylic acid). The last-named one can be considered as a disubstituted, conformationally constrained Pro derivative. Chemical formulas of these derivatives are presented in Fig. 1.



Materials and methods

Peptide synthesis

All the peptides were synthesized by the solid-phase method using Fmoc chemistry. The following amino acid derivatives were used: Fmoc-Gly, Fmoc-Abu, Fmoc-Arg(Pbf), Fmoc-Cys(Trt), Fmoc-Thr(*t*Bu), Fmoc-Ser(*t*Bu), Fmoc-Ile, Fmoc-Pro, Fmoc-Phe, Fmoc-Asp(*O**t*Bu), Fmoc-Hyp and three *N*-protected Pro mimetics: Fmoc-Hpr, Fmoc-Aze, Fmoc-Oic, all purchased from Bachem. The C-terminal amino acid residue, Fmoc-Asp(*O**t*Bu), was attached to the 2-chlorotrityl chloride resin (substitution of Cl 1.46 meq/g) (Calbiochem-Novabiochem AG, Switzerland) in the presence of equimolar quantity of DIPEA in anhydrous DCM solution. Peptide chains were elongated in consecutive cycles of deprotection and coupling. Deprotection was performed with a 20% piperidine solution in DMF/NMP (1:1, v/v) with addition of 1% of Triton X-100, whereas the chain elongation was achieved with standard DIC/HOBt chemistry, 3 equivs. of protected amino acid derivatives were used. After completing the synthesis, the peptides were cleaved from the resin simultaneously with the side chain deprotection in a one-step procedure, using a TFA/phenol/triisopropylsilane/water (88:5:2:5, v/v/v/v) mixture [13]. In the last step, the disulfide bridge formation was performed using a 0.1 M methanolic iodine solution and the procedure described elsewhere [14]. The crude peptides were purified by HPLC on a Beckman Gold System (Beckman, USA) using an RP Kromasil-100, C₈, 5 μm column (8 x 250 mm) (Knauer, Germany). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Either isocratic conditions or a linear gradient were applied (flow rate 3.0 mL/min, monitored at 226 nm). The purity of the synthesized peptides was checked on another RP Kromasil 100, C₈, 5 μm column (4.6 x 250 mm) (Knauer, Germany). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Linear gradients from 10 to 90% B in 40 min or from 20 to 80% B in 30 min with a flow rate of 1 mL/min were employed and monitored at 226 nm. The mass spectrometry analysis was carried out on a MALDI MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using an α -cyano-4-hydroxycinnamic acid as a matrix.

Determination of the association equilibrium constants

Bovine α -chymotrypsin and β -trypsin, the chromogenic trypsin burst substrate – nitrophenyl-4'-guanidinobenzonate (NPGb) and ovomukoid from turkey egg whites (OMTKY-3) were purchased from Sigma-Aldrich Co. USA. The chromogenic turnover substrates: *N*^α-benzoyl-DL-Arg-*p*NA (BAPNA), Suc-Ala-Ala-Pro-Phe-*p*NA, Suc-Ala-Ala-Pro-Leu-*p*NA were



purchased from Bachem (Switzerland). All measurements were performed using a Cary 3E Spectrophotometer (Varian, Australia).

The bovine β -trypsin and α -chymotrypsin solutions were prepared by dissolving few milligrams of a lyophilized enzyme, in 1 mL of 1 mM HCl containing 20 mM CaCl_2 . The stock solutions of OMTKY-3 (1.5 mg/mL) and the SFTI-1 analogues (about 2 mg/mL) were prepared with 1 mM HCl. The stock solution of bovine β -trypsin was standardized with NPGGB according to Chase and Shaw [15]. The enzyme concentration was calculated from at least 7 separate experiments whose results differed less than 2%. The standardized trypsin solution was used to titrate OMTKY-3 (used as a mutual inhibitor of both experimental enzymes) with BAPNA as a substrate. Then, OMTKY-3 was used to determine the concentration of α -chymotrypsin (using Suc-Ala-Ala-Pro-Phe-pNA as a substrate). In order to determine the concentration of the inhibitors, standardized solutions of experimental enzymes were used to titrate SFTI-1 analogues which, according to preliminary trials, exhibited tight inhibition. The concentrations of weak inhibitors **5** and **6** were determined by HPLC method. The area corresponding to the inhibitor peak was integrated and compared to that obtained with a strong trypsin inhibitor such as SFTI-1 whose concentration was determined by means of the enzyme. The measured concentrations of both, enzyme and inhibitor stock solutions, were in the range of 10^{-6} M. The association constants, K_a , were determined by a modified method of Green and Work as described by Empie and Laskowski [16] and by Otlewski and Zbyryt [17]. All measurements were carried out in a 100 mM Tris-HCl buffer containing 20 mM CaCl_2 and 0.005% Triton X-100, at a pH of 8.3, at room temperature. The measurements were carried out at total enzymes concentrations over the ranges 6.3×10^{-6} M – 1.3×10^{-8} M. Increasing quantities of the inhibitor (in the range of 0 to $2[E_0]$) were added to a fixed quantity of enzyme. After preliminary estimation of K_a of the inhibitor to be tested (the estimation was done based on our earlier experience and expectations), the total enzyme concentration $[E_0]$ was usually chosen to meet the condition $2 < [E_0] \times K_a < 50$. The enzyme-inhibitor mixtures were incubated for appropriate time, that means for about 10 times half –life ($t_{1/2}$) of the second-order association reaction at room temperature ($22 \pm 2^\circ\text{C}$) according to the equation:

$$t_{1/2} = \frac{1}{k_{\text{on}} \times E_0}$$

where: k_{on} is the second-order association rate constant (usually $6.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ has been assumed [17])



After a suitable incubation time, the residual enzyme activity [E] was measured with Ac-Ile-Arg-Asp-Asn-Lys-ANB-NH₂[18] and Suc-Ala-Ala-Pro-Leu-4-nitroanilide or Z-Phe-Ala-Thr-Tyr-ANB-NH₂ [19] as chromogenic substrates for trypsin and chymotrypsin inhibitors, respectively. The measurements were carried out for about 150 s by monitoring the linear release of *p*-nitroanilide at 405 nm. It is worth noting that prolonged incubation time (4 – 5 times) caused only a negligible deviation (2%) in the monitored absorbance. In order to avoid disturbance of enzyme – inhibitor interaction, the final chromogenic substrate concentration inside the cuvette never exceeded 10% of its K_m. For the determination of low K_a values (<10⁷ M⁻¹), the inhibitors were used at a much higher concentration than the enzyme, to enforce enzyme – inhibitor complex formation.

The K_a value was calculated by a three-parameter algorithm using the non-linear regression analysis program GraFit [20], according to the equation:

$$E = \frac{1}{2} \left([E_0] - F[I_0] - K_a^{-1} + \sqrt{([E_0] + F[I_0] + K_a^{-1})^2 - 4[E_0]F[I_0]} \right)$$

Where [E₀] and [I₀] are the total enzyme and inhibitor concentrations, respectively, [E] is the residual enzyme concentration, and F is the enzyme-inhibitor equimolarity factor. If the experiment does not have any errors (theoretical error, systematic error, and random error), the F value would remain 1 after the three parameter fit. In the case of weak associations (K_a < 10⁷ M⁻¹) only a two-parameter algorithm and the default value of F = 1 was applied. When the F value fell outside the established range of 0.9 < 1 < 1.1, the estimation concerning total enzyme concentration [E₀] was changed and the whole procedure was repeated. The final K_a values collected in Table 2 are average ones calculated from three separate measurements, as described.

Proteolytic susceptibility

The SFTI-1 analogues were incubated in 100 mM Tris-HCl buffer (pH 8.3) containing 20 mM CaCl₂ and 0.005% Triton X-100 using catalytic amounts of the enzymes (1 mole%) [21]. The incubation was carried out at room temperature and aliquots of the mixture were taken out periodically and submitted to RP-HPLC analysis. The analysis was performed using a Vydac Protein & Peptide, C₁₈, 10 μm column (4.6 x 250 mm) (Grace, USA). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). A linear gradient from 10 to 90% B for 40 min with a flow rate of 1 mL/min was employed and monitored at 226 nm. The collected fractions of the peaks were analyzed by MALDI MS (Biflex III MALDI-TOF spectrometer, Bruker



Daltonics, Germany) using the α -cyano-4-hydroxycinnamic acid matrix. Alternatively, the mixture was subjected to MS analysis without product separation on HPLC.

Results and discussion

All the monocyclic SFTI-1 analogues modified in the P_1' position by Pro derivative and the three mimetics were synthesized by the solid-phase method, purified and analysed by RP-HPLC (see Fig. 2). The correctness of molecular masses of the synthesized peptides was confirmed by MALDI-TOF analysis (see Fig. 3). Physicochemical properties of these SFTI-1 analogues, together with their trypsin or chymotrypsin inhibitory activities and proteolytic susceptibility are summarized in Table 2. Inhibition curves of the most potent analogues of this series are shown in Figure 4.

The results clearly indicate that the amino acid residue with the secondary amino group introduced in the discussed position is acceptable by the proteinases. Monocyclic SFTI-1 with Pro in position 6 (peptide **1**) is a potent trypsin inhibitor, although its activity expressed as the association constant (K_a) is by two orders of magnitude lower than that of the reference inhibitors (SFTI-1 wild and monocyclic SFTI-1). The same relation was also noticed for compound **2** with Phe in the P_1 position of substrate specificity. Introduction of the hydroxyl group in position 4 of the Pro ring (analogues **3** and **4**) did not have the influence on association with the enzymes. It's worth emphasizing that shortening of the Pro ring to a four-member size (Aze residue) retained trypsin inhibitory activity and caused even slightly higher inhibition (analogue **8**) of chymotrypsin. On the other hand, substitution of Pro6 by its bigger mimetics (Hpr or Oic) dramatically decreased the affinity towards both enzymes. Analogues **5** and **6** modified by Hpr displayed a three orders of magnitude lower K_a values, whereas analogues **9** and **10** containing Oic were virtually inactive.

The results presenting inhibitory activity of the SFTI-1 analogues with Pro or its mimetics in the P_1' position are rather unexpected. In natural protein inhibitors of serine proteinase, very different amino acid residues occur in that position [7]. They are conserved within families of these canonical inhibitors. Since they are part of binding loop possessing canonical conformation, the requirements of dihedral angles of an amino acid residue present in this position are mandatory, and Pro does not meet these conformational criteria [22]. The probable reason is that there are only very few examples of naturally occurring serine proteinase inhibitors with Pro residue in the P_1' position [7]. In addition, X-Pro peptide bonds are often resistant to the action of endo- and exopeptidases, even those with broad specificity. It is well known, that peptide bonds Lys-Pro and Phe-Pro are proteolytically resistant against

experimental enzymes (bovine β -trypsin and bovine α -chymotrypsin, respectively) [23]. According to Peptidase Database MEROPS (<http://merops.sanger.ac.uk/>) the only two trypsin and chymotrypsin-like proteinases (chymotrypsin A (cattle-type) and trypsin 1, respectively) are able to hydrolyze these peptide bonds. In the case of canonical inhibitors, including SFTI-1, the P_1 - P_1' reactive site is hydrolyzed upon the interaction with proteinase, and the hydrolysis constant is usually close to the unity at a neutral pH [17]. Bearing all this in mind, we decided to check the proteolytic susceptibility of the synthesized SFTI-1 analogues. The results are summarized in Table 2. With the exception of analogues **5**, **8** and **9**, the remaining ones are completely proteolytically resistant under experimental conditions. Also the wild-type SFTI-1 (dicyclic) remained intact even after 72 hours incubation with trypsin. Entirely different results were obtained for SFTI-1 monocyclic (with a disulfide bridge only) inhibitors, SFTI-1 and [Phe⁵]SFTI-1 with the native sequence. Under the same conditions they underwent enzymatic proteolysis. In both cases, the P_1 - P_1' reactive site (Lys/Phe⁵-Ser⁶) was partially hydrolyzed. Additional Arg²-Cys³ cleavage was noticed for the first inhibitor. The HPLC analysis of analogues **5**, and **9** revealed the presence of additional peaks assigned to peptides with a truncated *N*-terminal dipeptide Gly¹-Arg² (see Fig. 5). Under assumption that these two analogues display low inhibitory activity or even its lack (analogue **9**), one could speculate that this dipeptide is essential for inhibitory activity. In our previous paper [6, 24] we have shown that SFTI-1 analogues truncated at their *N*-termini retained inhibitory activity. This is in line with the trypsin inhibitory activity displayed by the reference analogue [desGly¹,desArg²]SFTI-1 (see Table 2). Interestingly, substitution of Ser⁶ in this analogue by Pro completely abolished the ability of such peptide to inhibit trypsin. This suggests that contribution of the *N*-terminal dipeptide of SFTI-1 analogues modified in position P_1' by Pro or its derivatives is important for their association with trypsin (e.g. the P_1 - P_1' reactive site is displaced from position 5-6 to 2-3). In order to verify this assumption we also synthesized analogue [Ala²,Pro⁶]SFTI-1 with basic Arg² replaced by Ala. A moderate inhibitory activity of this analogue (K_a around 10^5 M⁻¹) indicate that Arg² can't be located in inhibitor's P_1 position. Nevertheless, its positively charged side chain helps to interact with bovine β -trypsin. It should be emphasized that in the active analogue **8**, the peptide Phe⁵-Aze⁶ bond is partially hydrolyzed by chymotrypsin (Fig. 6) and is less proteolytically resistant than the peptide bond formed by the proteinogenic Pro.

Conclusion

The results presented here demonstrate that absolutely conservative in the family BBI Ser residue located in the inhibitor's P_1' position, can be replaced by Pro and its derivatives,



retaining inhibitory activity of the modified SFTI-1 analogues. Bearing in mind that Pro (and its derivatives discussed herein) display distinctly different physico-chemical properties from those of other amino acids, its accommodation in the inhibitor's binding loop possessing very rigid conformation is rather unusual. Another interesting result is provided by proteolytic susceptibility of peptide bond Phe-Aza form by a four-member ring of the Pro derivative. This synthetic amino acid is much better recognizable by chymotrypsin than the proteinogenic Pro.

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Table legend

Table 1. Primary structure of monocyclic analogues of SFTI-1 modified in position 6.

Analogue	Primary structure
[Pro ⁶]SFTI-1 (1)	Gly-Arg-Cys-Thr-Lys-Pro-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Phe ⁵ ,Pro ⁶]SFTI-1 (2)	Gly-Arg-Cys-Thr-Phe-Pro-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Hyp ⁶]SFTI-1 (3)	Gly-Arg-Cys-Thr-Lys-Hyp-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Phe ⁵ ,Hyp ⁶]SFTI-1 (4)	Gly-Arg-Cys-Thr-Phe-Hyp-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Hpr ⁶]SFTI-1 (5)	Gly-Arg-Cys-Thr-Lys-Hpr-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Phe ⁵ ,Hpr ⁶]SFTI-1 (6)	Gly-Arg-Cys-Thr-Phe-Hpr-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Aze ⁶]SFTI-1 (7)	Gly-Arg-Cys-Thr-Lys-Aze-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Phe ⁵ ,Aze ⁶]SFTI-1 (8)	Gly-Arg-Cys-Thr-Phe-Aze-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Oic ⁶]SFTI-1 (9)	Gly-Arg-Cys-Thr-Lys-Oic-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp
[Phe ⁵ ,Oic ⁶]SFTI-1 (10)	Gly-Arg-Cys-Thr-Phe-Oic-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp

Table 2. Physicochemical properties and association equilibrium constants (K_a) with bovine β -trypsin or bovine α -chymotrypsin of SFTI-1 analogues modified in the P₁ or/and P_{1'} position.

Analogue ^a	MW Calc. (found) ^b	RT ^c [min.]	K_a [M ⁻¹] ^d	Proteolytic susceptibility ^d
SFTI-1 wild [3]	1513.8 (1513.4)	16.71	$(1.1\pm 0.2)\times 10^{10}$	R
SFTI-1 [3]	1531.8 (1531.2)	18.15	$(9.9\pm 1.1)\times 10^9$	Lys ⁵ -Ser ⁶ , Arg ² -Cys ³ partially cleaved
[Phe ⁵]SFTI-1 [25]	1550.2 (1550.8)	20.64	$(2.0\pm 0.2)\times 10^9$	Phe ⁵ -Ser ⁶ partially cleaved
[Ala ² ,Pro ⁶]SFTI-1	1456.8 (1457.4)	18.31	$(3.5\pm 0.2)\times 10^5$	R
[desGly ¹ ,desArg ²]SFTI-1	1318.6 (1319.0)	21.15	$(7.3\pm 0.3)\times 10^7$	Lys ⁵ -Ser ⁶ partially cleaved
[desGly ¹ ,desArg ² ,Pro ⁶]SFTI-1	1328.6 (1329.0)	21.84	NA	R
[Pro ⁶]SFTI-1 (1)	1541.9 (1541.3)	17.55	$(9.3\pm 0.5)\times 10^7$	R
[Phe ⁵ ,Pro ⁶]SFTI-1 (2)	1560.9 (1560.5)	24.15	$(5.4\pm 0.6)\times 10^7$	R
[Hyp ⁶]SFTI-1 (3)	1557.9 (1557.3)	16.84	$(6.4\pm 0.2)\times 10^7$	R
[Phe ⁵ ,Hyp ⁶]SFTI-1 (4)	1576.9 (1576.3)	22.59	$(9.5\pm 0.3)\times 10^7$	R
[Hpr ⁶]SFTI-1 (5)	1555.9 (1555.7)	20.74	$(9.8\pm 0.8)\times 10^4$	Arg ² -Cys ³ partially cleaved
[Phe ⁵ ,Hpr ⁶]SFTI-1 (6)	1574.9 (1574.9)	26.85	$(7.9\pm 0.3)\times 10^4$	R
[Aze ⁶]SFTI-1 (7)	1527.8 (1527.5)	17.36	$(8.4\pm 0.3)\times 10^7$	R
[Phe ⁵ ,Aze ⁶]SFTI-1 (8)	1546.8 (1546.5)	25.49	$(1.5\pm 0.1)\times 10^8$	Phe ⁵ -Aze ⁶ partially cleaved
[Oic ⁶]SFTI-1 (9)	1595.9 (1595.5)	19.65	NA	Arg ² -Cys ³ completely cleaved
[Phe ⁵ ,Oic ⁶]SFTI-1 (10)	1614.9 (1614.2)	27.26	NA	R

NA - not active

R - fully proteolytically resistant

^aexcept wild SFTI-1, all peptides are monocyclic with a disulfide bridge only

^bmolecular weights of the peptides were determined on a Bruker Biflex III MALDI-TOF spectrometer (Bruker, Germany). The average values are given.

^cHPLC was performed as described in experimental section. Following linear gradients were applied: 10 – 90% B in 40 min and 20–80% B in 30 min for reference compounds, RT – retention time.

^ddetermined with trypsin or chymotrypsin for peptides with Lys5 or Phe5, respectively



Figures legend

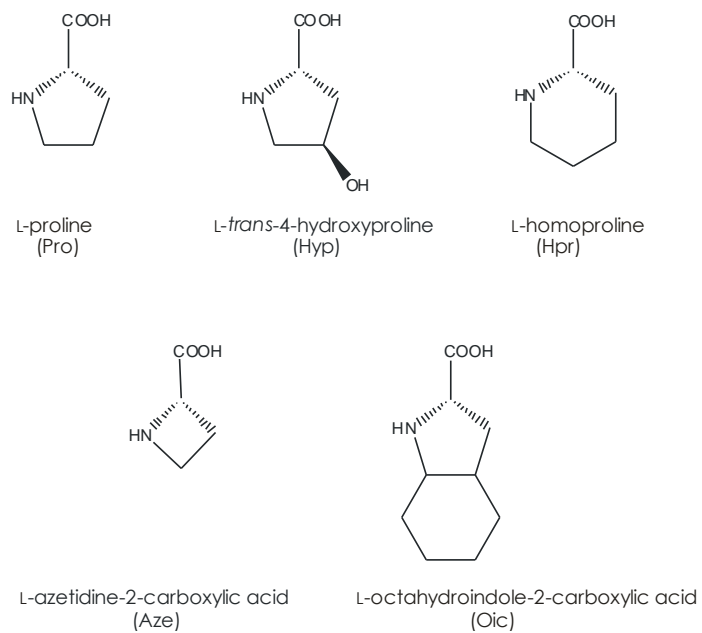


Fig. 1. Chemical formulas of Pro and its derivatives and mimetics.

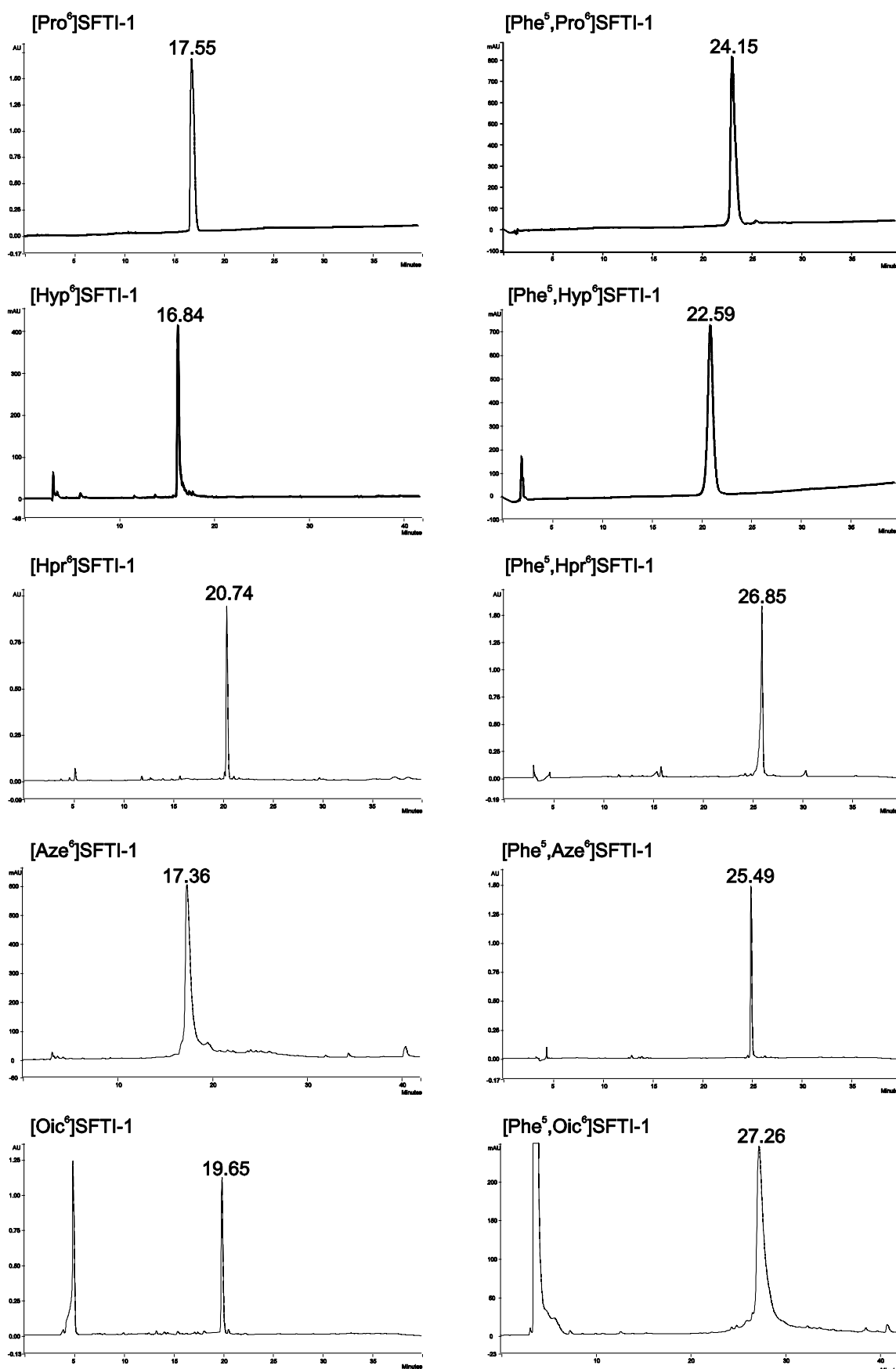


Fig. 2. HPLC analysis of new synthesised analogues of SFTI-1.

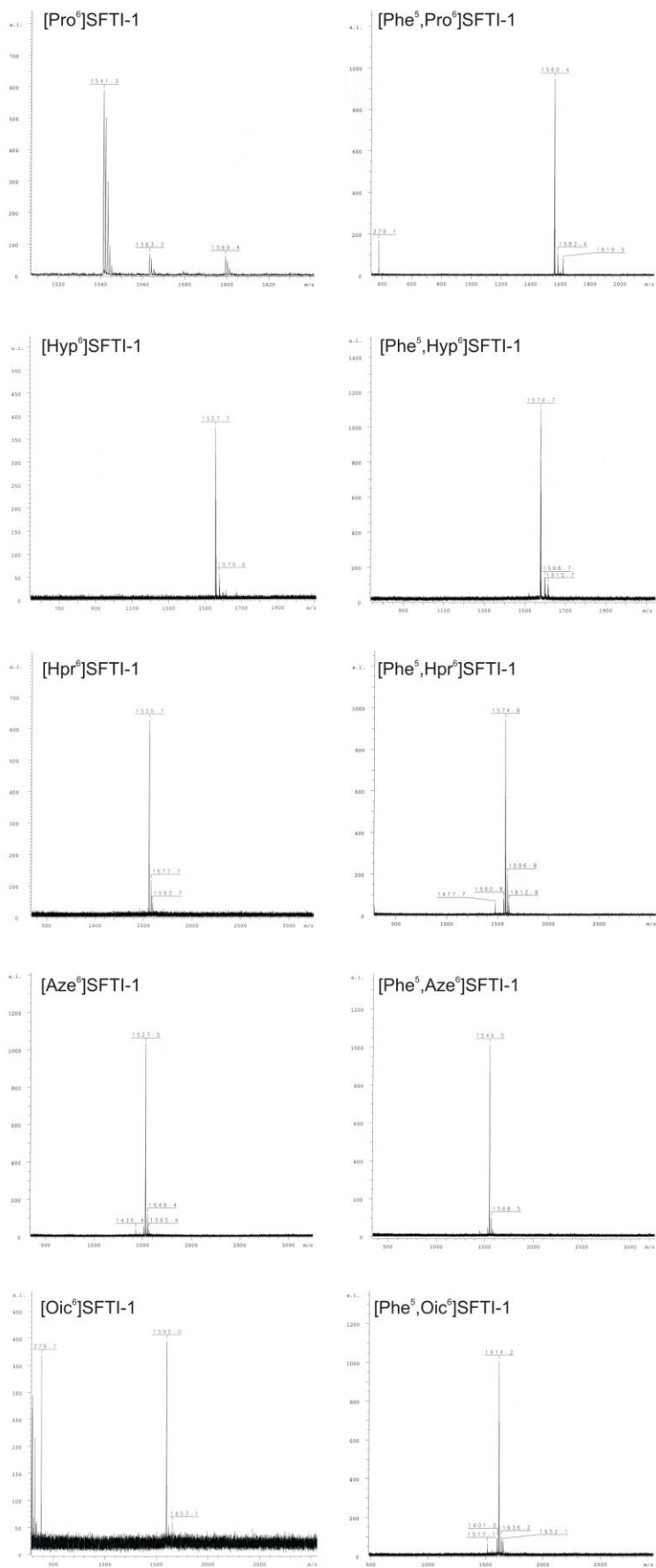


Fig. 3. MS spectra of new synthesised analogues of SFTI-1.

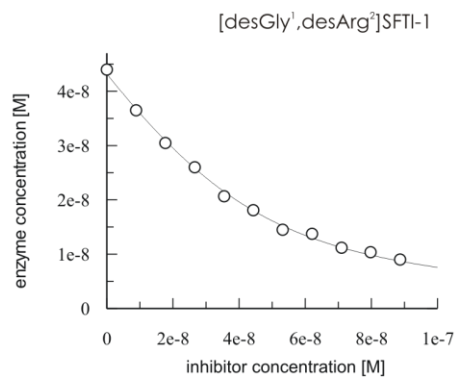
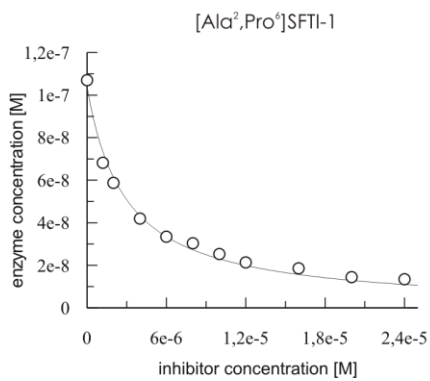
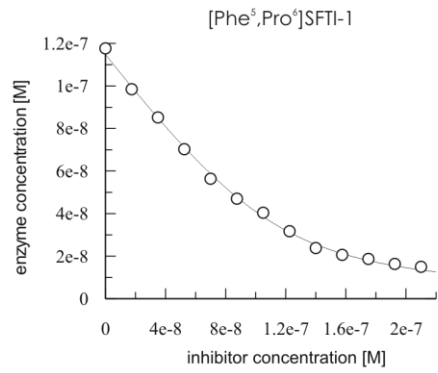
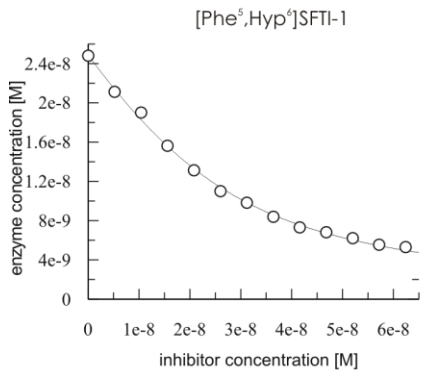
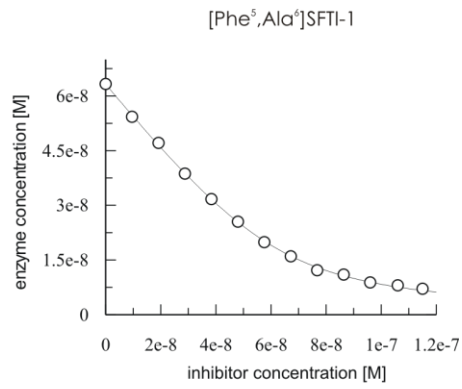
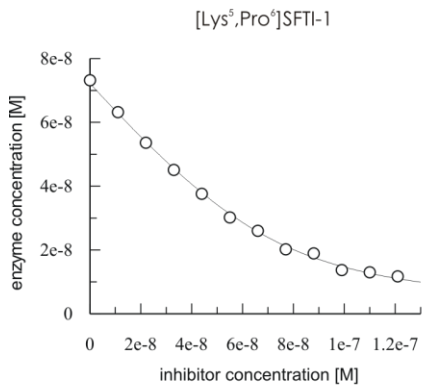
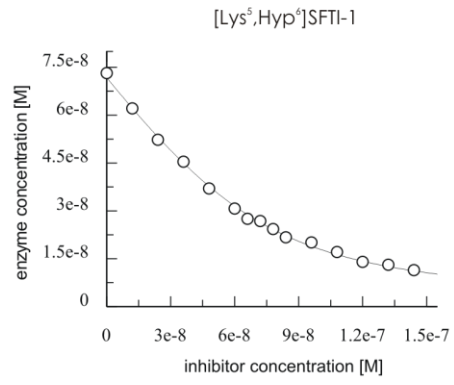
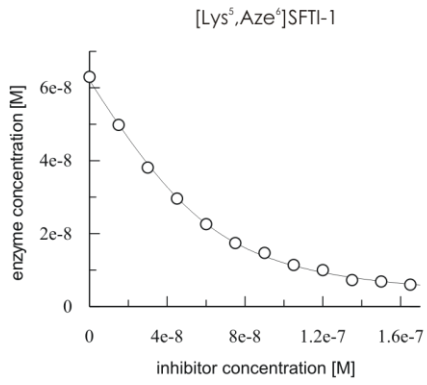


Fig. 4. Inhibition curves of the most potent analogues in this series.

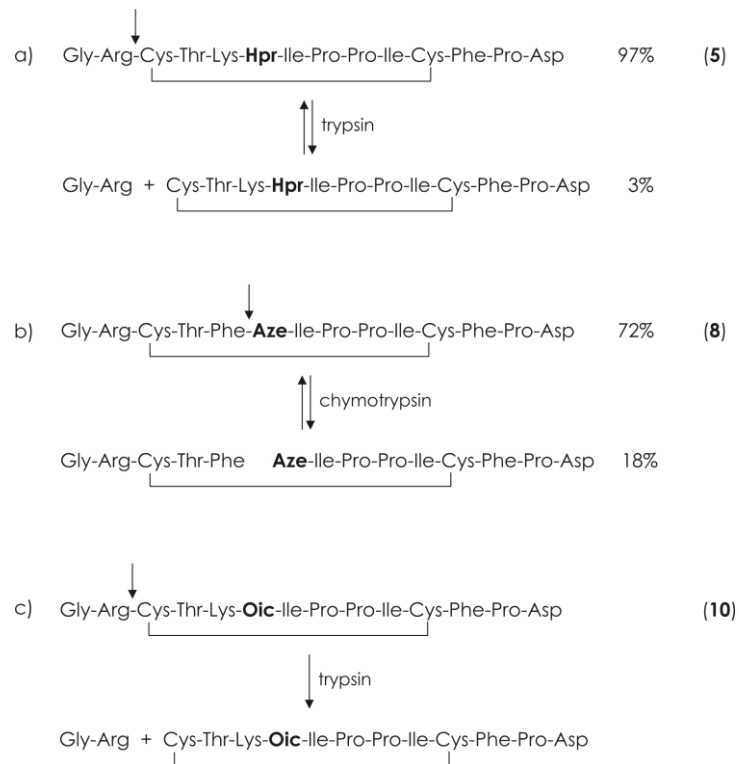
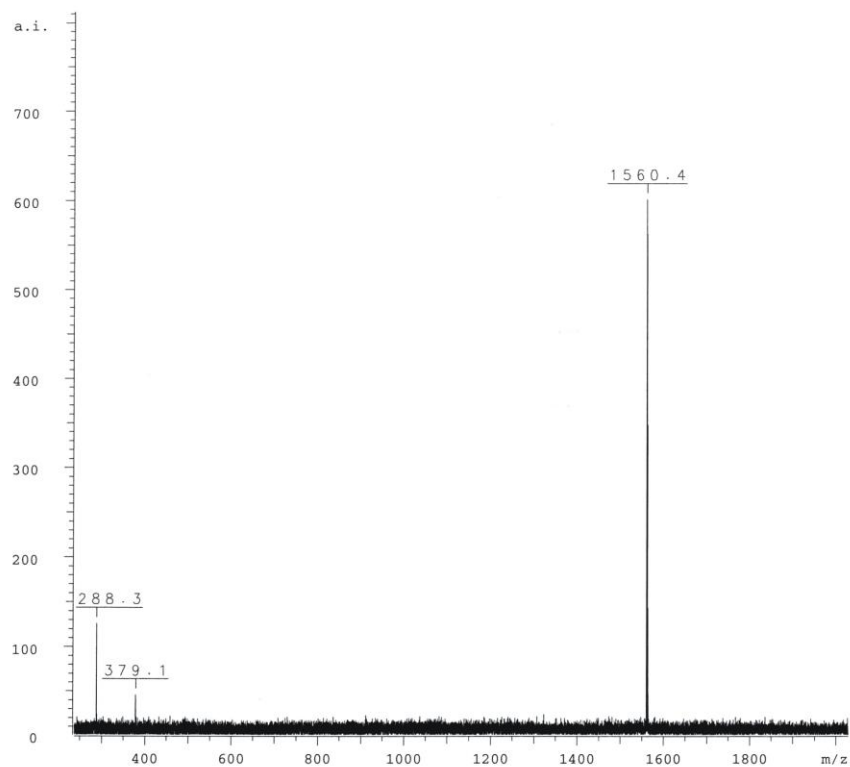


Fig. 5. Enzymatic hydrolysis of analogues **5**, **8**, and **9**.

A) [Phe⁵,Pro⁶]SFTI-1



B) [Phe⁵,Aze⁶]SFTI-1

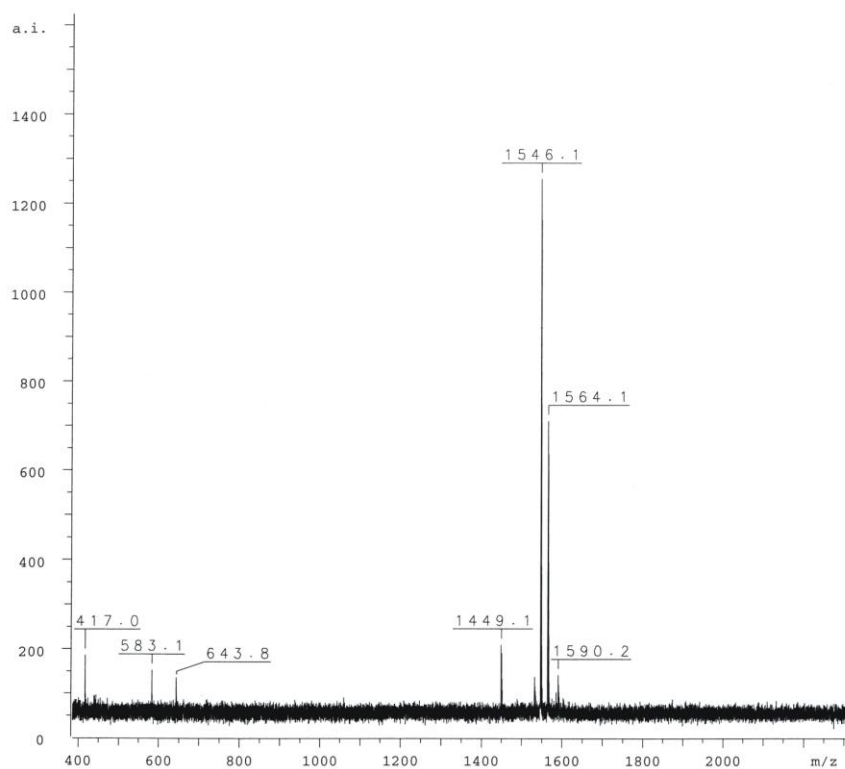


Fig. 6. MS spectra of a mixture of α -chymotrypsin and (A) analogue **2** (m/z 1560.4 intact peptide); (B) analogue **8**, m/z 1546.1 intact peptide and m/z 1564.1 peptide with hydrolyzed P₁-P₁' bond.

