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Analogues of trypsin inhibitor SFTI-1 modified in the conserved P_1' position by synthetic or non-proteinogenic amino acids retain their inhibitory activity

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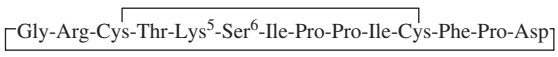
A series of linear and monocyclic (with a disulfide bridge only) analogues of trypsin inhibitor SFTI-1 modified in the P_1 and/or P_1' positions were synthesized by the solid-phase method. In the substrate specificity P_1 position, Phe or *N*-benzylglycine (Nphe) were introduced, whereas the conserved Ser6 in Bownam-Birk (BBI) inhibitors was replaced by Hse (L-homoserine), Nhse [*N*-(2-hydroxyethyl)glycine], Sar, and Ala. Kinetic studies of interaction of the analogues with bovine α-chymotrypsin have shown that in monocyclic (but not linear) analogues, Hse and Nhse are tolerated to afford potent inhibitors. This is the first evidence that the absolutely conserved Ser present in the inhibitor's P_1' position can be successfully replaced by a synthetic derivative.

Keywords: peptides; proteinase inhibitors; chemical synthesis; peptomers; SFTI-1

Introduction

Serine proteinases are widely distributed in nature and are responsible for many physiological processes. Their uncontrolled activity may be detrimental to the organism, evoking a series of critical pathological conditions. Therefore, serine proteinase inhibitors controlling the activity of these enzymes constitute a promising class of therapeutic agents. In 1999, Luckett *et al*. [1] isolated from sunflower seeds a trypsin inhibitor SFTI-1, the smallest one among the most potent inhibitors of the Bowman-Birk (BBI) family. Its primary structure is shown below:



Because of small size, high inhibitory activity and well-defined three-dimensional structure [1–4], SFTI-1 became for several groups an attractive object to study enzyme–inhibitor interaction. As SFTI-1 interacts with an enzyme according to the substrate-like manner, it has been classified as a canonical inhibitor. The reactive site P_1-P_1' is an important part of the primary contact region called the binding loop of the inhibitor, which is located between residues Lys5 and Ser6. In the case of canonical inhibitors, the P_1' position is responsible for the majority (up to 50%) of the total association energy released during the complex formation with the target enzyme [5,6].

Therefore, the P_1 amino acid that deeply penetrates into the S_1 specificity pocket of the enzyme in substrates and inhibitors is often referred to as the primary specificity residue. In naturally occurring trypsin inhibitors, this position is exclusively occupied by either Arg or Lys residue. The introduction of other amino acids in this position of canonical inhibitors produced analogues without any affinity toward trypsin. A few years ago, we were able to demonstrate that this effect could be observed even when the amino acid residues differed from the proteinogenic ones by one methylene group in their side chains (Orn, Hly and Har) [7]. The affinity of inhibitors with Arg or Lys in the P_1 position toward other than trypsin-like enzymes is significantly lower. The association equilibrium constants for interaction of both the wild SFTI-1 and its monocyclic analogue (with the disulfide bridge only) with bovine α-chymotrypsin are more than three orders of magnitude lower [8]. In our previous article, we have shown that monocyclic analogues (except for the linear one) with one of the naturally occurring cyclic fragments (the disulfide bridge or a head-to-tail cyclization) retained their inhibitory activity [3]. On the other hand, substitution of Lys5 by Phe in the monocyclic SFTI-1 gave a potent chymotrypsin inhibitor [9]. In our previous work [10], we have shown that *N*-substituted glycine derivatives (called peptoid monomers) *N*-(4-aminobutyl)glycine (Nlys) and *N*-benzylglycine (Nphe) that mimic the proteinogenic amino acids Lys and Phe, respectively, when introduced in the P_1 position of the monocyclic SFTI-1 are recognized by the enzyme and do not affect the inhibitory activity and are proteolytically resistant, in contrast to the P_1–P_1' reactive site formed by proteinogenic amino acids. Recently [11], we have also proved that a linear analogue of SFTI-1 with Nphe in the P_1 position inhibits α-chymotrypsin, but the peptide bond formed by this derivative was prone to proteolysis. However, the rate of this cleavage was slower than that of the 'regular' peptide bond. It is worth emphasizing that a linear analogue with Phe in that position

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was completely inactive and readily hydrolyzed by chymotrypsin [11]. The high chymotrypsin inhibitory activity of the linear peptomeric SFTI-1 analogue indicates that this compound might be a convenient model for further structure-activity studies and it is reasonable to assume that a larger number of peptoid monomers could successfully be introduced into the proteinase inhibitors.

The amino acid sequences of BBIs indicate that Ser at P_1' and *cis*-Pro at P_3' are absolutely conserved in this family of inhibitors. In the case of SFTI-1, these amino acid residues are located in positions 6 and 8, respectively. Unlike Pro8, Ser6 seems to be non-essential for the inhibitory activity. For instance, Odani and Ikenaka [12] have shown that the substitution of Ser located in the P_1' position of soybean BBI by other uncharged, proteinogenic amino acids preserved chymotrypsin's inhibitory activity. These results are compatible with more recent reports. Thus, Brauer and Leatherbarrow [13] reported that substitution of this amino acid residue by Ala in the BBI-binding loop suppressed trypsin inhibitory activity (as expressed by dissociation constant, K_d) fourfold, but this modification did not destroy the structural integrity of the inhibitor. The same substitution introduced into the SFTI-1 heptapeptide transplanted onto a hairpin-induced template yielded sevenfold less trypsin inhibiton as compared to the one containing Ser [14]. Substitution of Ser → Ala in the wild SFTI-1 preserved trypsin inhibitory activity but the K_d value was 11 times lower [15]. The affinity of this analogue toward trypsin was also supported by screening the biosynthesized SFTI-1library [16]. In addition, complete substitutional analysis of monocyclic SFTI-1 using SPOT synthesis proved the affinity toward trypsin analogues modified in the P_1' position [17]. All those reports illustrate only the few attempts made to obtain synthetic analogues of BBIs modified in this position (see Review paper [4]). With SFTI-1, X-ray studies [1] have shown that the hydroxyl group of this amino acid residue is involved in the hydrogen network involving Thr4 and Ile10. According to Mc Bride *et al*. [4], the interaction between Ser6 and Thr4 appears to be instrumental in projecting the P_1 side chain outward for the interaction with the enzyme S_1 pocket. Bearing in mind the results of our previous studies on peptomeric SFTI-1 analogues and the limited experimental evidence supporting the statement about the role played by the inhibitor's P_1' position, we decided to focus our attention on the role of the hydroxyl group of Ser6 in the inhibitor–enzyme interaction.

Herein, we report the chemical synthesis and determination of α-chymotrypsin inhibitory activity of a series of linear and monocyclic analogues of SFTI-1 modified in the P_1' position by Ala, Sar, Hse (L-homoserine), and Nhse [*N*-(2-hydroxyethyl)glycine]. Consequently, either Phe or Nphe were introduced in the substrate specificity P_1 position. The primary structure of these analogues is presented in Table 1 (residues that do not occur in the wild SFTI-1 are marked in bold type).

As mentioned, peptide bonds formed by peptoid monomers are more proteolytically resistant; therefore, one of the goals of this study was also to obtain serine proteinase inhibitors with a simplified structure when compared with that of the wild SFTI-1. Such peptides would provide promising lead structures to design inhibitors combining properties of both natural and synthetic compounds.

Materials and Methods

Peptide synthesis

All compounds were synthesized by the solid-phase method using Fmoc chemistry [18]. *N*-substituted glycine derivatives (Nphe and Nhse) were introduced into the peptide chain by the submonomeric approach [19]. In the first step, bromoacetic acid (5 equiv.) was attached to the peptidyl-resin using DIPCI/HOBt method, followed by the nucleophilic substitution of diisopropylcarbodiimide by primary amines, benzylamine and 2-aminoethanol, respectively. After completing the syntheses, the peptides were cleaved from the resin simultaneously with the side chain deprotection in a one-step procedure, using a mixture of TFA/phenol/triisopropylsilane/H_2O (88 : 5 : 2 : 5 v/v/v/v) [18]. In the case of cyclic analogues, the disulfide bridge was formed by 0.1 M methanolic iodine solution [20]. The crude peptides were purified by HPLC on a Beckman Gold System (Beckman, USA) using an RP Kromasil-100, C_8, 5 µm column (8 × 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 a Vydac protein and peptide, C_{18}, 10 µm column (4.6 × 250 mm) (W. R. Grace and Co., 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 mass spectrometric analysis was carried out on a MALDI MS (a Biflex III MALDI–TOF spectrometer, Bruker Daltonics, Germany) using α-CCA (alpha-cyanocinnamic acid) matrix.

Determination of Association Equilibrium Constants

Materials

The 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-D,L-arginine 4-nitroanilide (BAPNA), Suc-Ala-Ala-Pro-Phe-*p*NA, and Suc-Ala-Ala-Pro-Leu-*p*NA were purchased from Bachem (Switzerland), while Z-Phe-Ala-Thr-Tyr-ANB-NH_2 was synthesized according to Wysocka *et al*. [21]. All measurements were performed using a Cary 3E Spectrophotometer (Varian, Australia).

Determination of enzymes and inhibitors concentrations

Bovine α-chymotrypsin solution was prepared by dissolving 1 mg of 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 NPGB according to Chase and Shaw [22]. The enzyme concentration was calculated from at least seven separate experiments, which values differ by less than 2%. The standardized trypsin solution was used to titrate OMTKY-3 (used as a mutual inhibitor of trypsin and chymotrypsin) with BAPNA as a substrate. Then, OMTKY-3 was used to determine the concentration of the α-chymotrypsin stock solution using Suc-Ala-Ala-Pro-Leu-*p*NA as a substrate. The measured concentration of both, enzyme and inhibitor stock solutions were around 10^{-5} M. It is worth noticing that the concentration of β-trypsin solution determined by weight was usually approximately 40% higher than the measured one, while for α-chymotrypsin both concentrations were very similar. Standardized solutions of experimental enzymes were used to titrate SFTI-1 analogues which, according to the initial trials, exhibited tight inhibition. The concentration of weak inhibitors were determined by HPLC method (the area corresponding to

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Table 1. The primary structure of synthesized SFTI-1 analogues

Analogue		Primary structure
[Abu3,11,Nphe5,Ala6]SFTI-1	**(1)**	Gly-Arg-**Abu**-Thr-**Nphe**-**Ala**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp
[Abu3,11, Phe5,Ala6]SFTI-1	**(2)**	Gly-Arg-**Abu**-Thr-**Phe**-**Ala**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp
[Abu3,11,Nphe5,Sar6]SFTI-1	**(3)**	Gly-Arg-**Abu**-Thr-**Nphe**-**Sar**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp
[Abu3,11,Phe5,Sar6]SFTI-1	**(4)**	Gly-Arg-**Abu**-Thr-**Phe**-**Sar**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp
[Abu3,11,Nphe5,Hse6]SFTI-1	**(5)**	Gly-Arg-**Abu**-Thr-**Nphe**-**Hse**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp
[Abu3,11,Nphe5,Nhse6]SFTI-1	**(6)**	Gly-Arg-**Abu**-Thr-**Nphe**-**Nhse**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp
[Phe5,Ala6]SFTI-1	**(7)**	Gly-Arg-Cys(and)-Thr-**Phe**-**Ala**-Ile-Pro-Pro-Ile-Cys(and)-Phe-Pro-Asp
[Phe5,Hse6]SFTI-1	**(8)**	Gly-Arg-Cys(and)-Thr-**Phe**-**Hse**-Ile-Pro-Pro-Ile-Cys(and)-Phe-Pro-Asp
[Phe5,Nhse6]SFTI-1	**(9)**	Gly-Arg-Cys(and)-Thr-**Phe**-**Nhse**-Ile-Pro-Pro-Ile-Cys(and)-Phe-Pro-Asp
[Nphe5,Hse6]SFTI-1	**(10)**	Gly-Arg-Cys(and)-Thr-**Nphe**-H**se**-Ile-Pro-Pro-Ile-Cys(and)-Phe-Pro-Asp
[Nphe5,Nhse6]SFTI-1	**(11)**	Gly-Arg-Cys(and)-Thr-**Nphe**-**Nhse**-Ile-Pro-Pro-Ile-Cys(and)-Phe-Pro-Asp

Table 2. Physicochemical properties and association equilibrium constants (K_a) with bovine α-chymotrypsin of SFTI-1 analogues modified in P_1 or/and P_1' position

Analogue[a]	Molecular weight Calc. (found)[b]	RT[c] (min.)	$K_a(M^{-1})$	$K_i(M)$
SFTI-1 wild [8]	1513.8 (1513.4)	16.71	$(5.2 \pm 1.6) \times 10^6$	$(7.4 \pm 1.5) \times 10^{-6}$ [1]
SFTI-1 [8]	1531.8 (1531.2)	18.15	$(5.0 \pm 1.4) \times 10^6$	
[Phe5]SFTI-1 [26]	1550.2 (1550.8)	20.64	$(2.0 \pm 0.2) \times 10^9$	
[Nphe5]SFTI-1 [10]	1550.8 (1550.5)	20.10	$(3.9 \pm 0.3) \times 10^8$	
[Abu3,11,Nphe5]SFTI-1 [11]	1516.7 (1516.6)	22.05	$(6.2 \pm 0.8) \times 10^7$	
[Abu3,11,Nphe5,Ala6]SFTI-1 **(1)**	1500.8 (1500.8)	24.45	NA	NA
[Abu3,11,Phe5,Ala6]SFTI-1 **(2)**	1500.8 (1500.5)	24.81	NA	NA
[Abu3,11,Nphe5,Sar6]SFTI-1 **(3)**	1500.8 (1501.0)	24.78	NA	NA
[Abu3,11,Phe5,Sar6]SFTI-1 **(4)**	1500.8 (1501.1)	24.55	NA	NA
[Abu3,11,Nphe5,Hse6]SFTI-1 **(5)**	1530.0 (1529.9)	24.10	NA	NA
[Abu3,11,Nphe5,Nhse6]SFTI-1 **(6)**	1530.0 (1529.8)	24.07	$(3.1 \pm 0.2) \times 10^3$	$(5.0 \pm 0.1) \times 10^{-4}$
[Phe5,Ala6]SFTI-1**(7)**	1534.5 (1534.3)	24.51	$(1.4 \pm 0.1) \times 10^8$	$(9.1 \pm 0.4) \times 10^{-8}$
[Phe5,Hse6]SFTI-1 **(8)**	1563.9 (1563.7)	25.46	$(2.1 \pm 0.2) \times 10^8$	$(1.6 \pm 0.1) \times 10^{-8}$
[Phe5,Nhse6]SFTI-1 **(9)**	1563.9 (1564.0)	26.28	$(3.4 \pm 0.6) \times 10^9$	$(8.8 \pm 0.5) \times 10^{-9}$
[Nphe5,Hse6]SFTI-1 **(10)**	1563.9 (1563.8)	25.05	$(4.0 \pm 0.4) \times 10^7$	$(2.7 \pm 0.3) \times 10^{-7}$
[Nphe5,Nhse6]SFTI-1 **(11)**	1563.9 (1563.8)	25.75	$(1.1 \pm 0.1) \times 10^8$	$(3.2 \pm 0.5) \times 10^{-8}$

[a] Wild SFTI-1 (dicyclic), SFTI-1, [Phe5]SFTI-1, [Nphe]SFTI-1, and analogues **7**–**11** (monocyclic with disulfide bridge), the remaining ones are linear.
[b] Molecular weights of the peptides were determined on a Bruker Biflex III MALDI–TOF spectrometer (Bruker, Germany). The average values are given.
[c] HPLC was performed as described in the experimental part. The following linear gradients were applied: 10–90% B in 40 min and 20–80% B in 30 min for reference compounds, RT-retention time, NA-not active.

the inhibitor peak was integrated and compared with the area obtained with a strong trypsin inhibitor such as SFTI-1 whose concentration was determined by the means of the enzyme).

Determination of association equilibrium constants

The association constant (K_a) values were determined by a modified method of Green and Work as described by Empie and Laskowski [23] and Otlewski and Zbyryt [24]. All measurements were carried out in 100 mM Tris–HCl buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100, pH 8.3, at room temperature. After making an initial 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 fulfill the condition $2 < (E_0) \times K_a < 50$. To the constant amount of enzyme injected into about 30–36 plastic cuvettes (2.5 ml), an increasing volumes of the inhibitor were added. Briefly, the inhibitor was usually added in about 10–12 different volumes, each particular volume was injected into three cuvettes that contained buffer solution and enzyme. The inhibitor concentrations (I_0) inside the last three cuvettes were about two times higher than the total enzyme concentration (I_0) = 0-2 (E_0). The enzyme–inhibitor mixtures were incubated for the appropriate time, that means for about 10 times half-life ($t_{1/2}$) of the second-order association reaction at room temperature (22 °C ± 2), according the equation:

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

where k_{on} is the second-order association rate constant (usually, the value 6.7×10^6 $m^{-1}s^{-1}$ has been assumed [24]), (E_0) is a total enzyme concentration.

After a suitable incubation time, the residual enzyme activity (E) was measured with chromogenic substrate for about 150 s by monitoring the linear release of *p*-nitroanilide at a wavelength

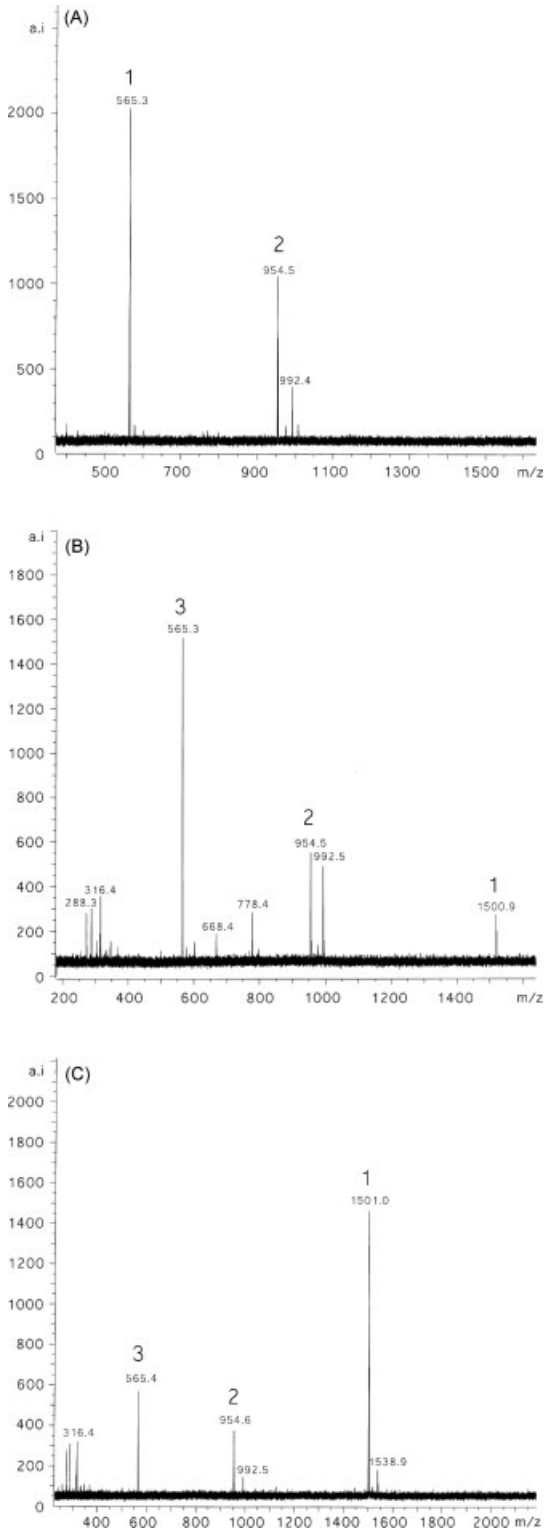

Figure 1. MS spectra of a mixture of α-chymotrypsin and linear inhibitor: (A) analogue **2**, (B) analogue **1**, and (C) analogue **4**; *m/z* 565.3 corresponds to peptide fragment Gly-Arg-Abu-Thr-Phe, *m/z* 954.5 corresponds to peptide fragment Ala(or Sar)-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp *m/z* 1501 – intact peptide.

405 nm. It is worth noticing that prolonged incubation time (four to five times) caused the inconsiderable deviation (2%) in monitored absorbance.

To avoid the disturbance of the enzyme–inhibitor interaction, the final chromogenic substrate concentration inside the cuvette never exceeds 10% of its K_M. For the determination of the low K_a values ($<10^7$ M^{-1}), the inhibitor was used at a much higher concentration than the enzyme, to force enzyme–inhibitor complex formation.

The value of K_a was calculated by a three-parameter algorithm using the non-linear regression analysis program GraFit [25], according to 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 (E_0) and (I_0) 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 1.0 after the three-parameter fit. In the case of weak associations ($K_a < 10^7$ M^{-1}), only the two-parameter algorithm and the default value of $F = 1$ was applied. When the F value was out of the established range $0.9 < 1 < 1.1$, the estimation concerning total enzyme concentration (E_0) was changed and all the above procedures were repeated. The final K_a values presented in Table 2 are the average values calculated from three separate measurements, as described above. Inhibition curves of analogues of this series are shown in Figure 2.

Proteolytic Susceptibility

The SFTI-1 analogues were incubated in a 100 mM Tris–HCl buffer (pH 8.3) containing 20 mM $CaCl_2$ and 0.005% Triton X-100 using catalytic amounts of the enzymes (1 mole%) [27]. 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 on an HPLC Pro Star system (Varian, Australia) equipped with a Kromasil 100 C_8 column (8 × 250 mm) (Knauer, Germany) and a UV–VIS detector. The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). A linear gradient was from 10 to 90% B for 40 min, flow rate 1 ml/min was employed and monitored at 226 nm.

Results and Discussion

Physicochemical properties and kinetic characteristics of the synthesized SFTI-1 analogues are summarized in Table 2. Unfortunately, all linear analogues, **1**–**6,** appeared to be inactive (or displayed very low activity as did analogue **6**). Bearing in mind that the K_a value determined for interaction of a linear analogue with Nphe in position P_1 ([Abu3,11, Nphe5]SFTI-1) with bovine α-chymotrypsin was high, the obtained results can be considered as rather surprising ones. They differ from those of the reference compound in P_1'. The first two contained the Ala residue in this position, the second pair Sar and the remaining two Hse (L-homoserine) and its peptoid mimetic Nhse. These results clearly show that in linear SFTI-1 analogues, the conservative Ser

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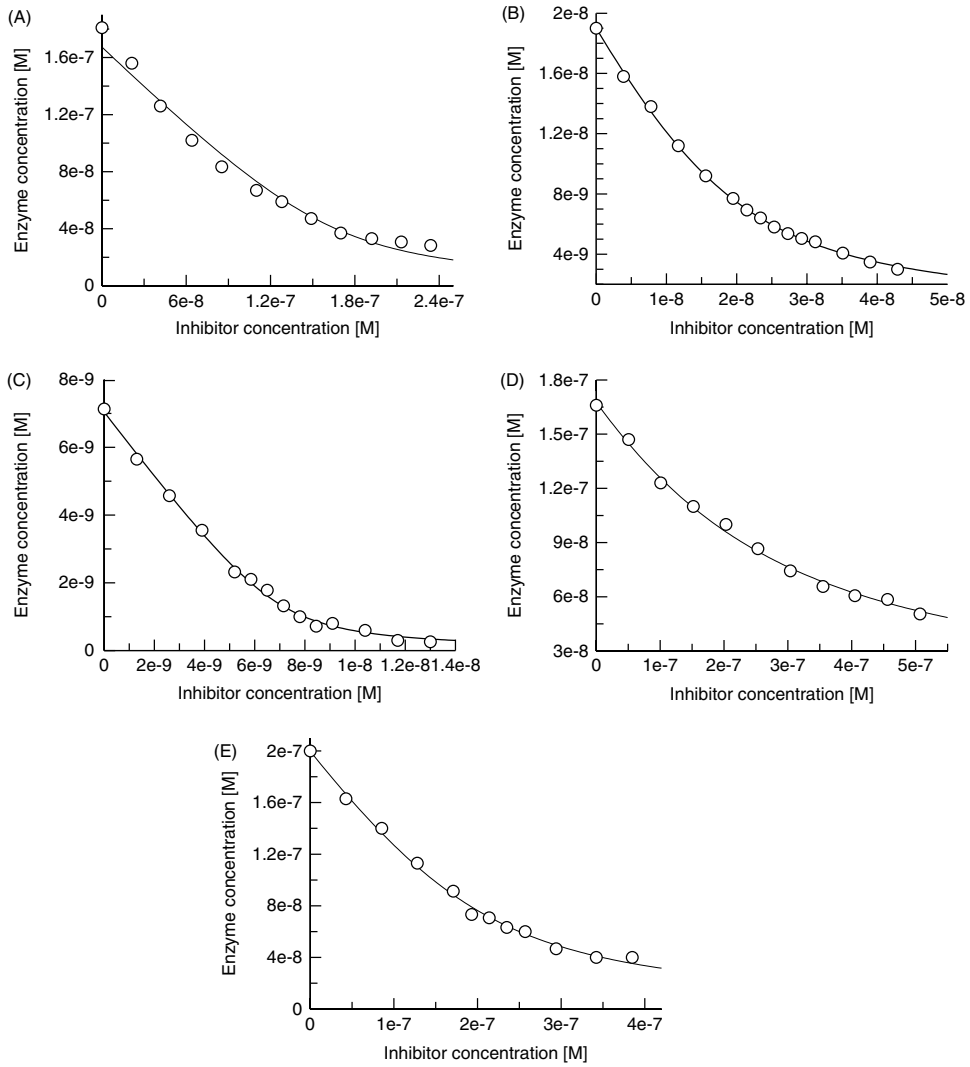

Figure 2. Inhibition curves of all the monocyclic inhibitors of α-chymotrypsin: (A) analogue **7**, (B) analogue **8**, **(C)** analogue **9**, **(D)** analogue **10**, and **(E)** analogue **11.** The equation used for calculation the association constant (K_a) is: $(E) = 1/2 \times \left[(E_0) - F \times (I_0) - 10^{-\log K_a} + \sqrt{[(E_0) + F \times (I_0) + 10^{-\log K_a}]^2 - 4 \times F \times (I_0) \times (E_0)}\right]$.

residue present in the P_1' position of BBI inhibitors is essential for preserving chymotrypsin inhibitory activity.

In the case of canonical inhibitors displaying a substrate-like mechanism, two factors are important: the hydrolysis rate of the P_1–P_1' reactive bond and the affinity toward proteinase. Proteolytic susceptibility studies of these analogues (Fig. 1) have shown that only **2** behave like a typical substrate and the Phe5–Ala6 peptide bond (with the P_1–P_1' reactive site) underwent hydrolysis. Figure 1(A) represents a mass spectrum recorded after incubation of analogue **2** with α-chymotrypsin. The peaks with *m/z* 565.3 and 954.5 correspond to peptide fragments Gly-Arg-Abu-Thr-Phe and Ala-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp, respectively, resulting from P_1–P_1' bond cleavage. Substitution of Phe by its peptoid mimetic Nphe (analogue **1**) and Ala by Sar (analogue **4**) increased proteolytic resistance although both the intact peptide (*m/z* 1500.9 in Fig. 1(B) and (C)) and its fragments (peaks with *m/z* 565.3 and 954.5) are present. The remaining three analogues (**3,5**, and **6**) with the non-proteinogenic amino acid residue in the P_1' position and the peptoid monomer in P_1 position were, under experimental conditions, completely proteinase resistant. Considering their lack of chymotrypsin inhibitory activity, this means that they did not display affinity toward the experimental enzyme.

The remaining SFTI-1 analogues (**7**–**11**) containing the disulfide bridge dramatically improved the chymotrypsin inhibitory activity. All appeared to be potent chymotrypsin inhibitors. Inhibition curves of the most potent inhibitors are shown in Fig. 2. The determined K_a value of [Phe5,Ala6]SFTI-1 (**7**) with chymotrypsin was 14 times lower than of the mono-substituted monocyclic [Phe5]SFTI-1 used as a reference peptide for these monocyclic analogues. This is compatible with the literature data and those mentioned above. Interestingly enough, introduction of the peptoid monomer Nhse in position P_1' produced analogues **9** and **11** almost equipotent with that contained in this position naturally occurring (and highly conserved) Ser. The chymotrypsin inhibitory activity of **8** and **10** with Hse in the discussed position was only one order of magnitude lower. This is the first evidence that the absolutely conserved Ser present in the inhibitor P_1' position can be successfully replaced by a synthetic derivative. Two pairs of analogues: **8**, **9** and **10**, **11** differ in the substrate

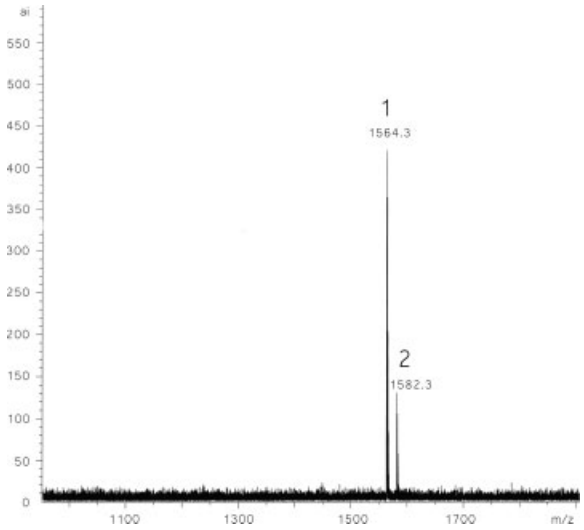

Figure 3. MS spectrum of a mixture of α-chymotrypsin and analogue **9**; peak 1(*m/z* 1564.3) – intact peptide, peak 2 (*m/z* 1582.3) – peptide with a cleaved P_1-P_1' peptide bond.

specificity P_1 position. The activity of analogues with Phe is one order of magnitude higher than that determined for ones with its mimetic Nphe.

As already mentioned, peptoid monomers introduced into the inhibitor's peptide chain significantly suppressed proteolytic susceptibilities of the peptide bonds formed by such derivatives [10,11]. Therefore, we decided to conduct appropriate studies on proteolytic resistance on these active monocyclic analogues also. The results are shown in Fig. 3. Similar to the mono-substituted [Nphe5]SFTI-1, analogues **10** and **11** containing Nphe displayed full proteolytic resistance, whereas analogues **8** and **9** with Phe5 and the non-proteinogenic Hse and Nhse in position 6 were slowly hydrolyzed by the cognate enzyme (Fig. 3; peak with *m/z* 1582.3 corresponding to the analogue with cleaved P_1-P_1' peptide bond). This indicates that the amino acid residue in P_1' has a lesser impact on the proteolytic resistance of the P_1-P_1' reactive site.

Conclusions

The results presented herein clearly show that Ser6, being absolutely conserved in the SFTI-1 molecule, can be successfully replaced not only by the proteinogenic Ala residue but also by a synthetic mimetic of Ser. However, this can only be done in an inhibitor that contains a disulfide bridge. Any attempt to modify the P_1' position of the linear SFTI-1 analogue afforded invariantly an inactive compound. This supports our previous results that a cyclic fragment, in this case the disulfide bridge, is crucial for the interaction with enzyme.

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