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

Łukajtis Rafał, Łęgowska Anna, Wysocka Magdalena, Dębowski Dawid,
Lesner Adam, Rolka Krzysztof

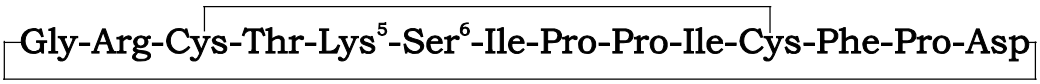
Faculty of Chemistry, University of Gdansk, 80-952 Gdansk, Poland

Summary: 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 conservative in Bownam-Birk inhibitors Ser6 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, Nhse are tolerated to afford potent inhibitors. This is the first evidence that the absolutely conservative Ser present in the inhibitor**'s** P_1' position can be successfully replaced by a synthetic derivative.

Key words: 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 (BBIs) family. Its primary structure is shown below:



Since 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 Lys^5 and Ser^6. 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 [2,3].

Therefore, the P_1 amino acid residue, 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. Introduction of other amino acid residues in this position of canonical inhibitors produced analogues without any affinity towards trypsin. 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, Har) [4]. The affinity of inhibitors with Arg or Lys in P_1 position towards 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 [5]. In our previous paper, 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 [6]. On the other hand, substitution of Lys5 by Phe in the monocyclic SFTI-1 gave a potent chymotrypsin inhibitor [7]. In our previous work [8] we have shown that *N*-substituted glycine derivatives (called peptoid monomers) *N*-(4-aminobutyl)glycine (Nlys) and *N*-benzylglycine (Nphe) that mimic proteinogenic amino acids Lys and Phe, respectively, when introduced in 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 [9] we have also proved that a linear analogue of SFTI-1 with Nphe in 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's worth emphasizing that a linear analogue with Phe in that position was completely inactive and readily hydrolyzed by chymotrypsin [9]. 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 conservative 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 not essential for the inhibitory activity. For instance, Odani and Ikenaka [10] have shown that the substitution of Ser located in 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 [11] 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) four times, but this modification did not destroy structural integrity of the inhibitor. The same substitution introduced into the SFTI-1 heptapeptide transplanted onto a hairpin-induced template yielded 7-fold less trypsin inhibitor as compared with the one containing Ser [12]. Substitution Ser→Ala in the wild SFTI-1 preserved trypsin inhibitory activity, but the K_d value was eleven times lower [13]. The affinity of this analogue towards trypsin was also supported by screening the biosynthesized SFTI-1library [14]. In addition, complete substitutional analysis of monocyclic SFTI-1 using SPOT synthesis proved the affinity towards trypsin analogues modified in P_1' position [15]. All those reports illustrate only few attempts made to obtain synthetic analogues of BBIs modified in this position (see review paper [16]). With SFTI-1, X-ray studies [1] have shown that the hydroxyl group of this amino acid residue is involved in the hydrogen network comprising also Thr4 and Ile10. According to Mc Bride *et al.* [16], the interaction between Ser6 and Thr4 appears to be instrumental in projecting the P_1 side chain outwards 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 hydroxyl group of Ser6 in the inhibitor – enzyme interaction.

Herein we report 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 are as follows (residues that do not occur in the wild SFTI-1 are marked in bold type):

Gly-Arg-**Abu**-Thr-**Nphe**-**Ala**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp ([Abu^{3,11},Nphe^5,Ala^6]SFTI-1) (**1**)

Gly-Arg-**Abu**-Thr-**Phe**-**Ala**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp ([Abu^{3,11}, Phe^5,Ala^6]SFTI-1) (**2**)

Gly-Arg-**Abu**-Thr-**Nphe**-**Sar**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp ([Abu^{3,11},Nphe^5,Sar^6]SFTI-1) (**3**)

Gly-Arg-**Abu**-Thr-**Phe**-**Sar**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp ([Abu^{3,11},Phe^5,Sar^6]SFTI-1) (**4**)

Gly-Arg-**Abu**-Thr-**Nphe**-**Hse**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp ([Abu^{3,11},Nphe^5,Hse^6]SFTI-1) (**5**)

Gly-Arg-**Abu**-Thr-**Nphe**-**Nhse**-Ile-Pro-Pro-Ile-**Abu**-Phe-Pro-Asp ([Abu^{3,11},Nphe^5,Nhse^6]SFTI-1) (**6**)

Gly-Arg-Cys(&)-Thr-**Phe**-**Ala**-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp ([Phe^5,Hse^6]SFTI-1) (**7**)

Gly-Arg-Cys(&)-Thr-**Phe**-**Hse**-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp ([Phe^5,Hse^6]SFTI-1) (**8**)

Gly-Arg-Cys(&)-Thr-**Phe**-**Nhse**-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp ([Phe^5,Nhse^6]SFTI-1) (**9**)

Gly-Arg-Cys(&)-Thr-**Nphe**-H**se**-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp ([Nphe^5,Hse^6]SFTI-1) (**10**)

Gly-Arg-Cys(&)-Thr-**Nphe**-**Nhse**-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp ([Nphe^5,Nhse^6]SFTI-1) (**11**)

As just 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 as compared with that of the wild SFTI-1. Such peptides would provide promising leading 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. *N*-Substituted glycine derivatives (Nphe and Nhse) were introduced into the peptide chain by the submonomeric approach [17]. In the first step, bromoacetic acid (5 equiv.) was attached to the peptidyl-resin using DIPCI/HOBt method, followed by the nucleophilic substitution of bromine 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, disulfide bridge was formed by 0.1 M methanolic iodine solution [19]. 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 systems was 0.1% TFA (A) and 80% acetonitrile in A (B). Either isocratic condition or 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 & 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). Linear gradient from 10 to 90% B for 40 min, flow rate 1 mL/min, 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 matrix.

Determination of association equilibrium constants. Bovine β-trypsin was standardized by burst kinetics with 4-nitrophenyl-4'-guanidinobenzoate (NPGB) at an enzyme concentration of 10^{-6} M. The standardized bovine β-trypsin solution was used for titration of the turkey ovomucoid third domain inhibitor, which in turn served to determine the activity of bovine α-chymotrypsin. The concentration of SFTI-1 analogues was determined by titration of their stock solutions with a standardized bovine α-chymotrypsin, Suc-Ala-Ala-Pro-Leu-4-nitroanilide as substrate. The association constants were measured by the method developed in the laboratory of M. Laskowski, Jr., [20,21]. The interaction between bovine α-chymotrypsin and the inhibitor was determined in 0.1 M Tris-HCl, pH 8.3, buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100 at room temperature. Increasing amounts of the inhibitor, varying from 0 to $3E_0$ (E_0 - the initial enzyme concentration) were added to the constant amount of the enzyme and after 2-h incubation the residual enzyme activity was measured using a turnover substrate on a Cary 3E spectrophotometer (Varian, Australia). The measurements were carried out at initial enzyme concentrations of 8 nM for trypsin and chymotrypsin. The residual enzymatic activity was measured with Ac-Phe-Ala-Thr-Tyr-5-amino-2-nitrobenzoic acid [22] as a chromogenic substrate for chymotrypsin inhibitors. The initial substrate concentration was kept below 0.1 K_M throughout Experimental points were analysed by plotting the residual enzyme concentration [E] versus initial inhibitor concentration [I_0]. The experimental data were fitted to the theoretical values using the GraFit software package [23]. The calculated K_a values of the synthesized compounds are collected in Table 1.

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%) [24]. 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). Linear gradient was from 10 to 90% B for 40 min, flow rate 1 mL/min, monitored at 226 nm.

Results and discussion

Physicochemical properties and kinetic characteristics of the synthesized SFTI-1 analogues are summarized in Table 1. 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 ([Abu^{3,11}, Nphe^5]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 in this position the Ala residue, 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 residue present in 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 towards proteinase. Proteolytic susceptibility studies of these analogues (see Fig. 1) have shown that only **2** behave like a typical substrate and the Phe^5-Ala^6 peptide bond (with the P_1–P_1' reactive site) underwent hydrolysis. Figure 1A 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**s.** 1B and 1C) and its fragments (peaks with m/z 565.3 and 954.5) are present. The remaining three analogues (**3**, **5**, **6**) with the non-proteinogenic amino acid residue in P_1' position and the peptoid monomer in P_1 position were, under experimental condition**s**, completely proteinase resistant. Considering their lack of chymotrypsin inhibitory activity, this means that they did not display affinity towards 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 [Phe^5,Ala^6]SFTI-1 (**7**) with chymotrypsin was 14 times lower than of the mono-substituted monocyclic [Phe^5]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 containing 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 absolutely conservative 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 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 [8, 9]. Therefore we decided to conduct appropriate studies on proteolytic resistance also on these active monocyclic analogues. The results are shown in Fig. 3. Similar to the monosubstituted [Nphe^5]SFTI-1, also 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 (see Fig. 3; peak with m/z 1582.3 corresponding to the analogue with cleaved P_1–P_1' peptide bond). This indicate**s** 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 conservative in inhibitors of the Bownan-Birk family 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 contain**s** a disulfide bridge. Any attempt to modify the P_1' position of the linear SFTI-1 analogue afforded invariantly an inactive compound. This support**s** our previous results that a cyclic fragment, in this case the disulfide bridge, is crucial for the interaction with enzyme.

Acknowledgement

This work was supported by Ministry of Science and Higher Education (grant No. 2889/H03/2008/34).

References

1 Luckett S, Santiago Garcia R, Barker JJ, Konarev AV, Shewry PR, Clarke AR, Brady RL. High-resolution structure of a potent, cyclic proteinase inhibitor from sunflower seeds. *J. Mol. Biol.* 1999; **290**: 525-533.

2 Lu W, Apostol IMA, Qasim N, Warne R, Wynn WL, Zhang S, Anderson W, Chiang E, Ogin I, Rotherg K, Ryan M, Laskowski MJr. Binding of amino acid side-chains to S1 cavities of serine proteinases. *J. Mol. Biol.* 1997; **266**: 441-461.

3 Krowarsch D, Dadlez M, Buczek O, Krokoszynska I, Smalas AO, Otlewski J. Interscaffolding additivity: binding of P_1 variants of bovine pancreatic trypsin inhibitor to four serine proteases. *J. Mol. Biol.* 1999; **289**: 175-186.

4 Jaśkiewicz A, Lesner A, Różycki J, Rodziewicz S, Rolka K, Ragnersson U, Kupryszewski G. Distance between the basic group of the amino acid residue's side chain in position P_1 of trypsin inhibitor CMTI-III and Asp189 in the substrate pocket of trypsin has an essential influence on the inhibitory activity. *Biochem. Biophys. Res. Commun.* 1997; **240**: 869-871.

5 Zabłotna E, Kaźmierczak K, Jaśkiewicz A, Kupryszewski G, Rolka K. Inhibition of bovine α-chymotrypsin by cyclic trypsin inhibitor SFTI-1 isolated from sunflower seeds and its two acyclic analogues. *LIPS* 2002; **9**: 131-134.

6 Zabłotna E, Kaźmierczak K, Jaśkiewicz A, Stawikowski M, Kupryszewski G, Rolka K. Chemical synthesis and kinetic study of the smallest naturally occurring trypsin inhibitor SFTI-1 isolated from sunflower seeds and its analogues. *Biochem. Biophys. Res. Commun.* 2002; **292**: 855-859.

7 Łęgowska A, Dębowski D, Lesner A, Wysocka M, Rolka K. Introduction of non-natural amino acid residues into the substrate-specific P_1 position of trypsin inhibitor SFTI-1 yields potent chymotrypsin and cathepsin G inhibitors. *Bioorg. Med. Chem.* 2009; **17**: 3302-3307.

8 Stawikowski M, Stawikowska R, Jaśkiewicz A, Zabłotna E, Rolka K. Examples of peptide-peptoid hybrid serine protease inhibitors based on the trypsin inhibitor SFTI-1 with complete protease resistance at P_1-P_1' reactive site. *ChemBioChem* 2005; **6**: 1057-1061.

9 Łęgowska A, Bulak E, Wysocka M, Jaśkiewicz A, Lesner A, Dębowski D, Rolka K. Peptomeric analogues of trypsin inhibitor SFTI-1 isolated from sunflower seeds. *Bioorg. Med. Chem.* 2008; **16**: 5644-5652.

10 Odani S, Ikenaka T. Studies on soybean trypsin inhibitors. IV. Change of the inhibitory activity of Bowman-Birk inhibitor upon replacements of the alpha-chymotrypsin reactive site serine residue by other amino acids. *J. Biochem.* 1978; **84**: 1-9.

11 Brauer ABE, Leatherbarrow RJ. A conserved cis peptide bond is necessary for the activity of Bowman-Birk inhibitor protein. *BBA* 2003; **308**: 300-305.

12 Descours A, Moehle K, Renard A, Robinson JA. A new family of beta-hairpin mimetics based on a trypsin inhibitor from sunflower seeds. *ChemBioChem.* 2002; **3**: 318-323.

13 Daly NL, Chen Y-K, Foley FM, Bansal PS, Bharathi R, Clark RJ, Sommerhoff C, Craik DJ. The absolute structural requirement for a proline in the P_3'-position of Bowman-Birk protease inhibitors is surmounted in the minimized SFTI-1 scaffold. *J. Biol. Chem.* 2006; **281**: 23668-23675.

14 Austin J, Kimura RH, Woo Y-H, Camarero J. In vivo biosynthesis of an Ala-scan library based on the cyclic peptide SFTI-1. *Amino Acids*, 2010; **38**: 1313-22.

15 Hilpert K, Hansen G, Wessner H, Volkmer-Engert R, Höhne W. Complete substitutional analysis of a sunflower trypsin inhibitor with different serine proteases. *J. Biochem.* 2005; **138**: 383-390.

16 McBride JD, Watson EM, Brauer ABE, Jaulent AM, Leatherbarrow RJ. Peptide mimics of the Bowman-Birk inhibitor reactive site loop. *Biopolymers* 2002; **66**: 79-92.

17 Zuckermann RN, Kerr JM, Kent SBH, Moos W.H. Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid phase synthesis. *Am. Chem. Soc.* 1992; **114**: 10646-10647.

18 Sole NA, Barany GJ. Optimization of solid-phase synthesis of [Ala^8]-dynorphin. *J. Org. Chem.* 1992; **57**: 5399-5403.

19 Yang Y, Sweeney WV, Schneider K, Chait BT, Tam JP. Two-step selective formation of three disulfide bridges in the synthesis of the C-terminal epidermal growth factor-like domain in human blood coagulation factor IX. *Protein Sci.* 1994; **3**: 1267-1275.

20 Empie MW, Laskowski MJr. Thermodynamics and kinetics of single residue replacements in avian ovomucoid third domains: effect on inhibitor interactions with serine proteinases. *Biochemistry* 1982; **21**: 2274-2284.

21 Park SJ, Effect of Amino Acid Replacement in Ovomucoid Third Domains upon Their Association with Serine Proteinase. PhD thesis, 1985 Purdue University (IN, USA).

22 Wysocka M, Kwiatkowska B, Rzadkiewicz M, Lesner A, Rolka K. Selection of new chromogenic substrates of serine proteinases using combinatorial chemistry methods. *Comb. Chem. High Throughput Screen* 2007; **10**: 171-80.

23 Leatherbarrow RJ. GraFit Version 5. Erithacus Software, Horley, 2005.

24 Jaulent JA, Leatherbarrow RJ. Design, synthesis and analysis of novel bicyclic and bifunctional protease inhibitors. *Protein Eng. Des. Select.* 2004; **17**: 681-687.

Table 1. 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]	MW Calc. (found)[b]	RT[c] [min.]	$K_a[M^{-1}]$
SFTI-1 wild [5]	1513.8 (1513.4)	16.71	$(5.2\pm1.6)\times10^6$
SFTI-1 [7]	1531.8 (1531.2)	18.15	$(5.0\pm1.4)\times10^6$
[Phe^5]SFTI-1 [7]	1550.2 (1550.8)	20.64	$(2.0\pm0.2)\times10^9$
[Nphe^5]SFTI-1 [8]	1550.8 (1550.5)	20.10	$(3.9\pm0.3)\times10^8$
[Abu^{3,11},Nphe^5]SFTI-1 [9]	1516.7 (1516.6)	22.05	$(6.2\pm0.8)\times10^7$
[Abu^{3,11},Nphe^5,Ala^6]SFTI-1 (**1**)	1500.8 (1500.8)	24.45	NA
[Abu^{3,11},Phe^5,Ala^6]SFTI-1 (**2**)	1500.8 (1500.5)	24.81	NA
[Abu^{3,11},Nphe^5,Sar^6]SFTI-1 (**3**)	1500.8 (1501.0)	24.78	NA
[Abu^{3,11},Phe^5,Sar^6]SFTI-1 (**4**)	1500.8 (1501.1)	24.55	NA
[Abu^{3,11},Nphe^5,Hse^6]SFTI-1 (**5**)	1530.0 (1529.9)	24.10	NA
[Abu^{3,11},Nphe^5,Nhse^6]SFTI-1 (**6**)	1530.0 (1529.8)	24.07	$(3.1\pm0.2)\times10^3$
[Phe^5,Ala^6]SFTI-1(**7**)	1534.5 (1534.3)	24.51	$(1.4\pm0.1)\times10^8$
[Phe^5,Hse^6]SFTI-1 (**8**)	1563.9 (1563.7)	25.46	$(2.1\pm0.2)\times10^8$
[Phe^5,Nhse^6]SFTI-1 (**9**)	1563.9 (1564.0)	26.28	$(3.4\pm0.6)\times10^9$
[Nphe^5,Hse^6]SFTI-1 **(10)**	1563.9 (1563.8)	25.05	$(4.0\pm0.4)\times10^7$
[Nphe^5,Nhse^6]SFTI-1 **(11)**	1563.9 (1563.8)	25.75	$(1.1\pm0.1)\times10^8$

[a] wild SFTI-1 (dicyclic), SFTI-1, [Phe^5]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 experimental part. Following linear gradients were applied: 10 – 90% B in 40 min and 20–80% B in 30 min for reference compounds, NA not active

Figures legend:

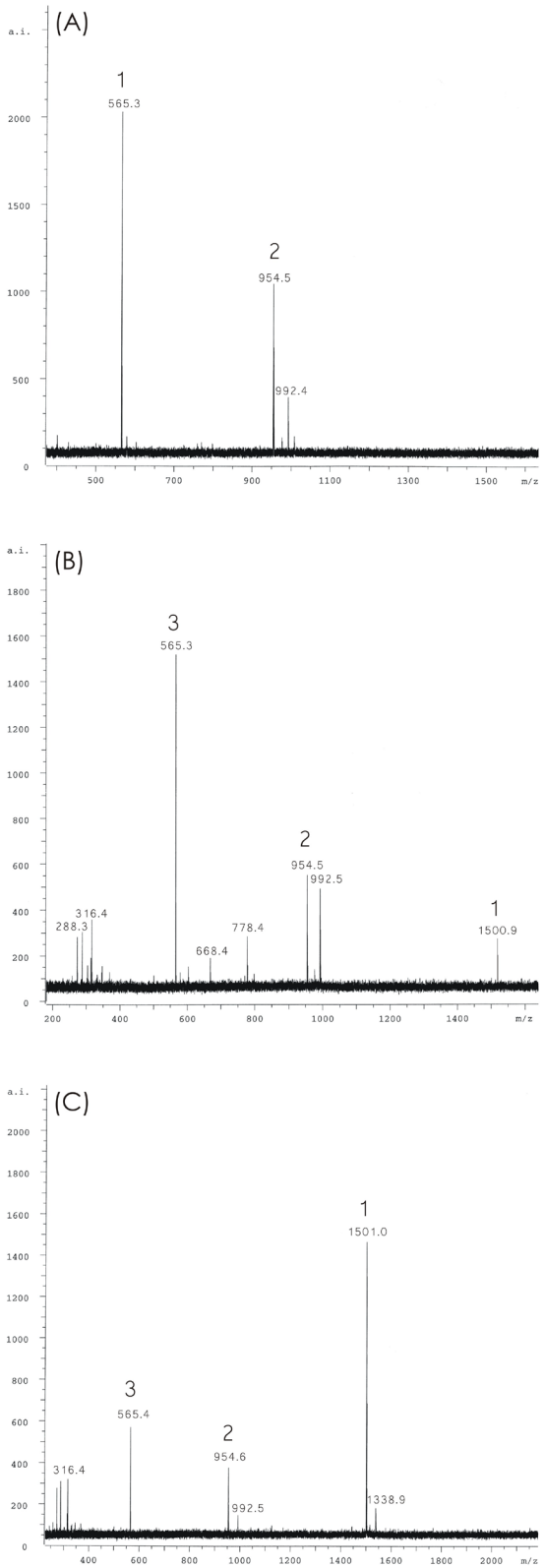


Fig. 1. MS spectra of a mixture of α-chymotrypsin and linear inhibitor: (A) analogue **2**, (B) analogue **1**, (C) analogue **4**; m/z 565.3 corresponds to peptide fragment Gly-Arg-Abu-Thr-Phe, m/z 954.5 corresponds to peptide fragment Ala(or Sar)-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp m/z 1501 – intact peptide

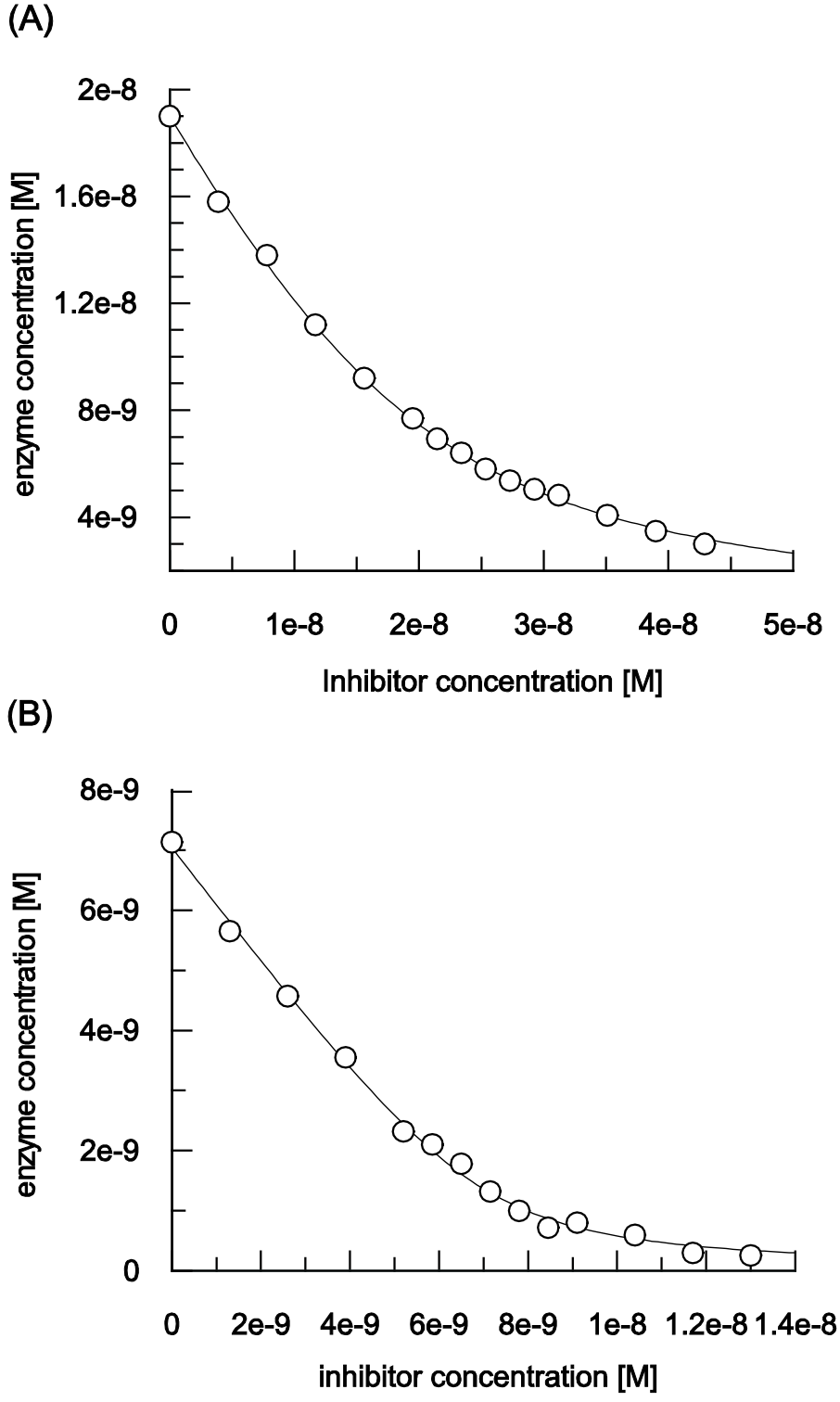


Fig. 2. Inhibition curves of the most potent monocyclic inhibitors of α-chymotrypsin: (A) analogue **8**, (B) analogue **9**

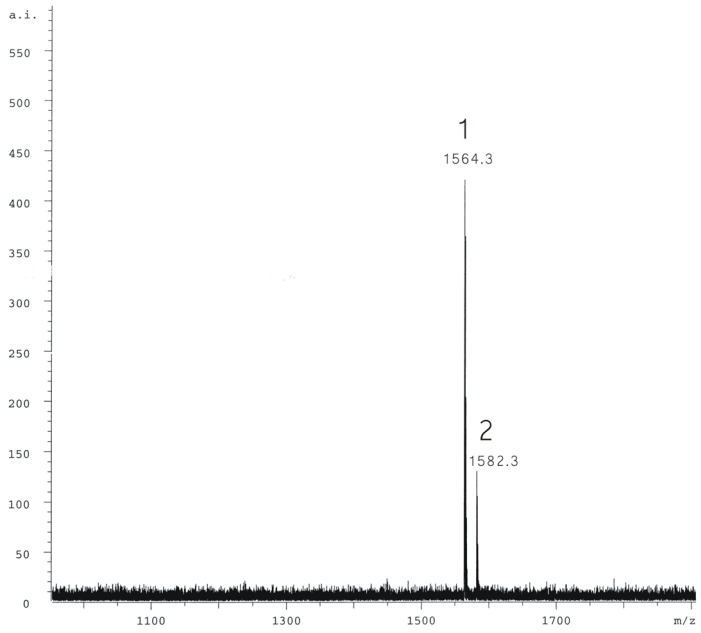


Fig. 3. MS spectrum of a mixture of α-chymotrypsin and analogue **9**; peak 1 **(**m/z 1564.3) – intact peptide, peak 2 (m/z 1582.3) – peptide with cleaved P_1–P_1' peptide bond