Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 5644–5652
Bioorganic & Medicinal Chemistry

Peptomeric analogues of trypsin inhibitor SFTI-1 isolated from sunflower seeds

Anna Łęgowska,* Elżbieta Bulak, Magdalena Wysocka, Anna Jaśkiewicz, Adam Lesner, Dawid Dębowski and Krzysztof Rolka

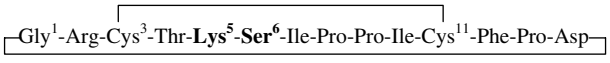
Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland

Received 22 November 2007; revised 20 March 2008; accepted 28 March 2008
Available online 1 April 2008

Abstract—A series of linear and monocyclic analogues of trypsin inhibitor SFTI-1 isolated from sunflower seeds, modified by *N*-(4-aminobutyl)glycine (Nlys) and *N*-benzylglycine (Nphe), were obtained by the solid-phase method. Some of these peptomers displayed trypsin or chymotrypsin inhibitory activity. In contradiction to the literature data, in most analogues peptide bonds formed by these peptoid monomers were at least partially hydrolyzed by the experimental enzymes at two different pH (3.5 and 8.3). Nevertheless, the replacement of Phe present in the P_1 substrate specificity of linear inactive SFTI-1 analogue with Nphe, yielded a potent chymotrypsin inhibitor. The introduction of one cyclic element (a disulfide bridge or head-to-tail cyclization) to the analogues synthesized significantly increased their proteinase resistance.

1. Introduction

Serine proteinases are widely distributed in nature and are responsible for many physiological processes. Their uncontrolled activity may be dangerous to the organism and evoke a series of critical pathological conditions. Therefore, serine proteinase inhibitors which help to control activity of these enzymes are a promising class of therapeutic agents.[1] In 1999, Luckett et al.[2] isolated from sunflower seeds a trypsin inhibitor SFTI-1, the smallest one among the most potent inhibitors of the Bowman-Birk family. SFTI-1 is a bicyclic peptide comprising a head-to-tail cyclization and a disulfide bridge formed by two Cys residues. Its primary structure is shown below.



The reactive site $P_1 - P_1'$ of SFTI-1 is located between residues Lys5 and Ser6. In the case of canonical inhibitors,[3] also called standard mechanism inhibitors,[4] position P_1 is responsible for up to 50% of contacts with the target enzyme.[5,6] Therefore, the P_1 amino acid residue, deeply penetrating the S_1 specificity pocket of the enzyme, in substrates and inhibitors is often referred to as the primary specificity residue. Owing to its very small size, strong inhibitory activity, well-defined and optimized 3D structure for the interaction with proteinase,[2,7] a lot of attention has been focused on this small peptide during last years. Recent studies on SFTI-1 have been summarized in three review papers.[8–10] In our previous work, we have shown that the two cycles are not necessary for retaining inhibitory activity of SFTI-1.[11] The monocyclic analogue of this inhibitor (containing a disulfide bridge only) displays almost the same trypsin inhibitory activity and proteolytic stability as the parent compound. The analogue without the disulfide bridge (two Cys residues were replaced with Abu residues) also inhibited bovine β-trypsin, although the determined value of the association equilibrium constant (K_a) was 2.5-fold lower than that for wild SFTI-1. This was ascribed to lower proteolytic resistance of this analogue.[11]

In one of our recent papers[12], we described a chemical synthesis and kinetic studies of two SFTI-1 analogues modified by *N*-(4-aminobutyl)glycine (Nlys) and *N*-benzylglycine (Nphe). These N-substituted glycines (named peptoid monomers[13]) mimicking proteinogenic Lys and Phe, respectively, introduced in position P_1 yielded analogues ([Nlys[5]]SFTI-1 and [Nphe[5]]SFTI-1) with trypsin and chymotrypsin inhibitory activity. Moreover, the $P_1 - P_1'$ reactive sites formed by these derivatives

Keywords: Peptides; Peptomers; Proteinase inhibitors; Chemical synthesis; Kinetic investigation; Proteolytic resistance.
* Corresponding author. Tel.: +48 58 5235359; fax: +48 58 5235472; e-mail: legowska@chem.univ.gda.pl

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.03.075

appeared to be completely protease resistant at both pH 3.5 and 8.3. Our findings are in a good agreement with results reported in the literature that peptoids are proteolysis-resistant to the major class of proteases, including papain, chymotrypsin, thermolysin, trypsin, elastase, pepsin and carboxypeptidase A.[13,14] Since in the standard mechanism of the inhibitory activity the reactive site $P_1 - P_1'$ is selectively hydrolyzed by the cognate enzyme and the equilibrium constant between the virgin and the modified inhibitor is usually close to unity at neutral pH,[15,16] we postulated that peptomeric SFTI-1 analogues mentioned above may represent a new class of proteinase inhibitors. Both inhibitors, [Nlys^5]SFTI-1 and [Nphe^5]-SFTI-1, were monocyclic and contained a disulfide bridge only.

Continuing our investigations on peptomeric analogues of SFTI-1 we have designed and synthesized a series of linear and monocyclic analogues of SFTI-1 with Nlys or Nphe in position P_1. We expected that the peptoid monomers introduced should increase proteolytic stability of these analogues. The following peptomers were synthesized.

[Nlys^5,Abu^{3,11}]SFTI-1(1,14) (**1**)
[desGly^1,Lys^2,Nlys^5,Abu^{3,11}]SFTI-1(2,14) (**2**)
[desGly^1,Nlys^2,Nlys^5,Abu^{3,11}]SFTI-1(2,14) (**3**)
[desGly^1,Lys^2,Nlys^5]SFTI-1(2,14) (**4**)
[Nlys^5,Abu^3,11]SFTI-1 (**5**)
[Nphe^5,Abu^{3,11}]SFTI-1(1,14) (**6**)
[Nphe^5,Abu^{3,11}]SFTI-1 (**7**)
[Phe^5,Abu^{3,11}]SFTI-1(1,14) (**8**)
[Phe^5,Abu^{3,11}]SFTI-1 (**9**)

The numbers in brackets indicate that the peptide possesses free α-amino and α-carboxyl groups at the N- and C-terminus, respectively. Among all analogues synthesized **1**, **2**, **3**, **6** and **8** are linear peptides, **4** contains a disulfide bridge, **5**, **7** and **9** contain a head-to-tail cyclization. The primary structures of these analogues are presented in Figure 1. All the peptides were synthesized by the solid-phase method, N-substituted glycines were introduced into the peptide chain by the submonomeric approach on the solid support. Kinetic studies of the peptides synthesized were carried out to determine their trypsin or chymotrypsin inhibitory activity and proteolytic susceptibility.

Figure 1. The primary structures of the synthesized SFTI-1 analogues.

2. Results and discussion

One of the goals of this study was to obtain serine proteinase inhibitors with simplified structures (regarding to the number of cyclic elements) as compared with wild SFTI-1. The introduction of peptoid monomers Nlys and Nphe that mimic proteinogenic Lys and Phe in position P_1 of monocyclic SFTI-1 retained the affinity of such modified analogues towards trypsin and chymotrypsin, respectively.[12] Taking into consideration our previous results, also reported by other groups,[13,14] that peptide bonds formed by peptoids are resistant to enzymatic proteolysis, we hoped that even linear peptomers based on the amino acid sequence of SFTI-1 may display inhibitory activity. Kinetic studies of peptomeric analogues, summarized in Table 1, confirmed our expectation only in the case of analogue **6**, with Nphe in the substrate specificity P_1 position. This linear peptomer appeared to be a potent chymotrypsin inhibitor. Additional head-to-tail cyclization (analogue **7**) yielded an inhibitor with a very similar activity. In the case of both analogues, the determined values of the association equilibrium constant were 5–6 times lower than that for the previously obtained monocyclic [Nphe^5]SFTI-1, whereas linear analogue **8** with proteinogenic Phe residue in position P_1 appeared to be completely inactive. On the other hand, the introduction of disulfide bridge into the analogue with this amino acid in position P_1 yielded potent chymotrypsin inhibitor.[17] Also the introduction of the head-to-tail cyclization (analogue **9**) gave chymotrypsin inhibitor one order of magnitude higher than that obtained for [Phe^5]SFTI-1(1,14). Different results were obtained for peptomers modified in the discussed position by Nlys. Linear analogue **1** containing full amino acid sequence of SFTI-1, which differs from analogue **6** only in the substrate specificity P_1 position, did not inhibit bovine β-trypsin. Also linear analogues **2** and **3** devoid of N-terminal Gly and with Arg2 replaced with Lys or Nlys, were inactive. The introduction of the disulfide bridge into the structure of inactive peptide **2** gave a potent trypsin inhibitor **4**. Similarly, the head-to-tail cyclization of peptide **1** produced analogue **5** which was able to inhibit the trypsin activity. Interestingly, the K_a values determined for these two monocyclic analogues (see Table 1) were similar to that measured for previously described monocyclic, monosubstituted [Nlys^5]SFTI-1. Considering high homology between trypsin and chymotrypsin, dramatic changes observed in the inhibitory activity of linear peptomeric SFTI-1 analogues with Nphe and Nlys introduced in position P_1 were rather surprising. Since one can expect that these peptides should exhibit similar affinity towards experimental enzymes, it seems reasonable to assume that their proteolytic susceptibilities might be responsible for such differences in the inhibitory activity of the

Table 1. Physicochemical properties and association equilibrium constants (K_a) of peptomeric SFTI-1 analogues with bovine β-trypsin and bovine α-chymotrypsin

Analogue	MW calcd (found)[a]	RT (min)[b]	K_a (M^{-1})[c]		Proteolytic susceptibility		
			Trypsin	Chymotrypsin	k_{hydr} [mol_{inh} mol_{enz}^{-1} s^{-1}] × 10^4		$t_{1/2}$ (min)
					pH 3.5	pH 8.3	
SFTI-1[11]			9.9×10^{10}	4.9×10^6	0.265		435 ± 23
SFTI-1(1,14)[11]	1531.8 (1531.2)	15.0	9.9×10^9	4.9×10^6	0. 70		165 ± 23
[Phe^5]SFTI-1(1,14)[22]	1550.8 (1551.0)	18.9		2.0×10^9	0.67		182 ± 28
[$Nlys^5$]SFTI-1(1,14)[12]	1531.8 (1531.5)	15.5	1.0×10^8		N/A	N/A	
[$Nphe^5$]SFTI-1(1,14)[12]	1550.8 (1550.5)	20.1		3.8×10^8	N/A	N/A	
[$Nlys^5$,$Abu^{3,11}$]SFTI-1(1,14) (**1**)	1497.8 (1497.6)	15.5	N/A		1.75[d]	+	66 ± 12[d]
[desGly^1,Lys^2,$Nlys^5$,$Abu^{3,11}$]SFTI-1(2,14) (**2**)	1412.7 (1414.1)	18.0	N/A		N/A	+	
[desGly^1,$Nlys^{2,5}$,$Abu^{3,11}$]SFTI-1(2,14) (**3**)	1412.7 (1412.9)	18.1	N/A		N/A	+	
[desGly^1,Lys^2,$Nlys^5$]SFTI-1(2,14) (**4**)	1446.7 (1446.9)	19.8	8.0×10^7		N/A	N/A	
[$Nlys^5$,$Abu^{3,11}$]SFTI-1 (**5**)	1479.8 (1479.8)	17.7	1.9×10^8		1.41[d]	+	82 ± 15[d]
[$Nphe^5$,$Abu^{3,11}$]SFTI-1(1,14) (**6**)	1516.8 (1516.6)	18.3		6.2×10^7	0.36	+	352 ± 38
[$Nphe^5$,$Abu^{3,11}$]SFTI-1 (**7**)	1498.8 (1498.5)	21.3		7.0×10^7	N/A	7%	
[Phe^5,$Abu^{3,11}$]SFTI-1(1,14) (**8**)	1516.8 (1516.7)	22.2		N/A	1.54	+	75 ± 16
[Phe^5,$Abu^{3,11}$]SFTI-1 (**9**)	1498.8 (1498.9)	19.7		1.1×10^{10}	0.36	30%	354 ± 32

[a] Molecular weights of the peptides were determined on a Bruker Biflex III MALDI-TOF spectrometer (Bruker, Germany).

[b] HPLC was performed as described in Section 4.

[c] Errors in determination of K_a values never exceeded 10%.

[d] Peptide bond between Arg^2-Abu^3 was hydrolyzed, N/A not active or not hydrolyzed, + full hydrolysis is observed; in the case of incomplete proteolysis the percent (%) of hydrolyzed form after 24 h is given.

peptides studied. Therefore, we decided to conduct appropriate studies of their proteolytic resistance. It is well documented that at pH 3.5 the catalytic amount of the enzyme cleaves selectively reactive site $P_1 - P'_1$ of the canonical inhibitor. In our previous paper, we have shown that under such condition monocyclic SFTI-1 was readily hydrolyzed.[12] The calculated half life of trypsin hydrolysis ($t_{1/2}$) for this peptide amounted to 165 min and was approximately 2.5 times shorter than that determined for wild SFTI-1.[17] Under the same conditions, monocyclic analogues SFTI-1 with Nlys or Nphe in the position discussed displayed full proteolytic resistance.[12] Both SFTI-1 and its monocyclic analogue remained intact when incubated with trypsin at pH 8.3. Also [Nlys^5]SFTI-1 and [Nphe^5]SFTI-1 showed resistance to hydrolysis by experimental enzymes at this pH. Nevertheless, in the case of the first analogue, a new peak corresponding to the fragment truncated by N-terminal Gly-Arg appeared in the chromatogram after 18 h of incubation with trypsin.

The experiments carried out at pH 3.5 indicated that in the case of all new SFTI-1 analogues with Nlys in position 5, the peptide bond Nlys^5-Ser^6, corresponding to the inhibitor $P_1 - P'_1$, displayed full proteolytic resistance. Unlike analogues **2**, **3** and **4** without N-terminal Gly, the ones with the full length (**1** and **5**) were relatively fast hydrolyzed. HPLC analysis of these peptides is shown in Figure 2. In the case of analogues **1** and **5**, the peaks of the intact peptides (peak 1 in Fig. 2A and peak 1 in Fig. 2B, respectively) are vanishing with the progress of the incubation, and two new peaks (peak 2 in both figures) appear. MALDI-MS analysis of the fractions corresponding to these peaks revealed that the peak migration results from the cleavage of the peptide bond Arg^2-Abu^3 by bovine β-trypsin in the peptides studied. It is worth mentioning that for both analogues the calculated values of $t_{1/2}$ are similar, rather low and are not correlated with their ability to inhibit trypsin. This indicates that the N-terminal part of SFTI-1 analogues plays a minor role in the inhibition of trypsin.

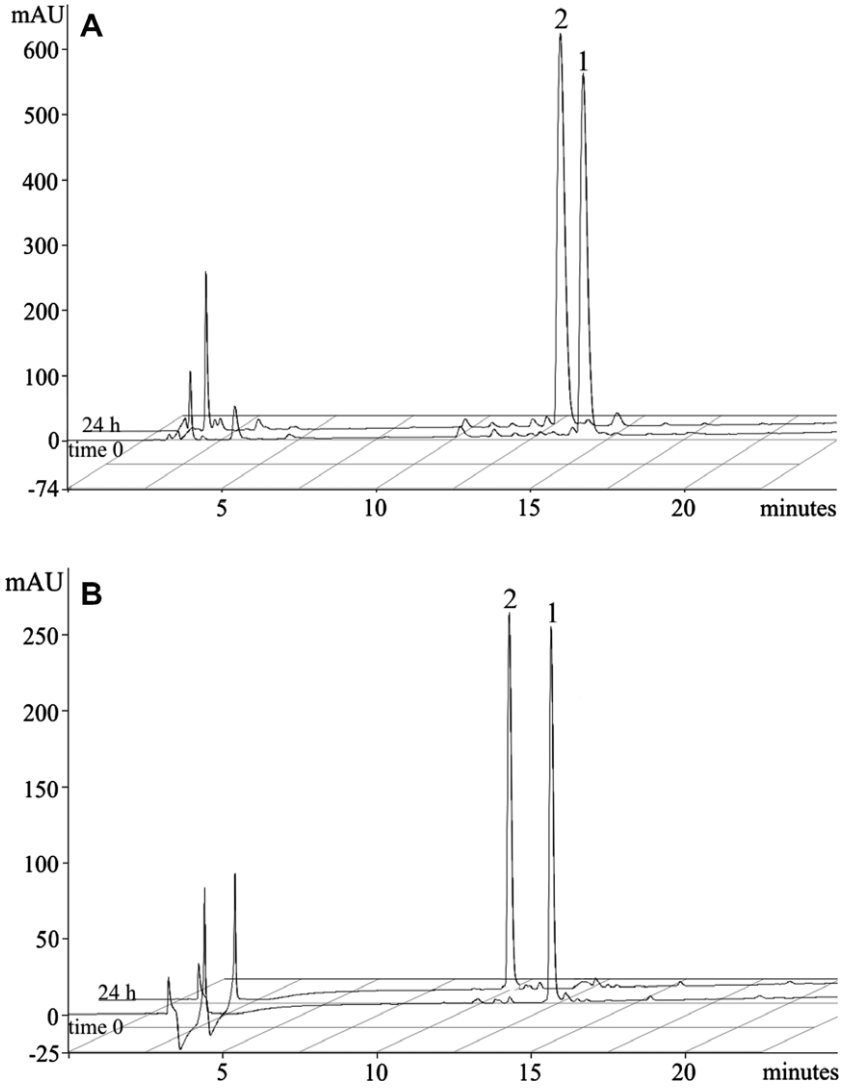


Figure 2. Proteolytic susceptibility of analogues **1** and **5** in the presence of β-trypsin, at pH 3.5. (A) Gly^1-Arg-Abu-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp^{14} (**1**): peak 1—intact peptide, peak 2—fragment 3–14. (B) &Gly-Arg-Abu-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp& (**5**) (nomenclature of cyclic peptides as recommend by Spengler et al.[18]): peak 1—intact peptide, peak 2—peptide with hydrolyzed Arg^2-Abu^3 peptide bond.

The remaining analogues **6**–**9** were incubated with bovine α-chymotrypsin. As expected, in the case of linear analogue **8**, that served as a reference compound, the peptide bond Phe^5-Ser^6 was fast hydrolyzed. Under the same conditions, cyclic analogue **7** was proteolysis-resistant (Fig. 3B). The reactive site $P_1 - P_1'$ in linear inhibitor **6** was slowly hydrolyzed by the enzyme (Fig. 3A) with the calculated value of $t_{1/2}$ very similar to that obtained for cyclic analogue **9** with Phe in position P_1. It should be stressed that among all SFTI-1 analogues studied herein, it was the only case (analogue **6**) when proteolysis of the peptomeric peptide bond at pH 3.5 was observed. As discussed above, canonical inhibitors are rather slowly hydrolyzed at neutral pH. Therefore, low proteolytic stability of the peptides studied at pH 8.3 is rather unexpected. Except analogue **4**, at least partial hydrolysis of the remaining analogues was observed. In linear analogues **2** and **3**, the peptide bonds following Lys2 (for **2** only) and Nlys5 were hydrolyzed (Fig. 4). Interestingly, the replacement of Lys2 in analogue **3** with Nlys makes the first peptide bond proteolysis-resistant (Fig. 4B). MS analysis of the products of proteolysis of **2** is shown in Figure 5.

Both active analogues (**4** and **5**) appeared to be significantly more stable. Peptomer **4** with the disulfide bridge remained intact even after 48 h of incubation with trypsin (Fig. 6). Under the same conditions, analogue **5**, with the head-to tail cyclization, was hydrolyzed (Fig. 7). In this case, two peptide bonds were susceptible to enzymatic action. After 24 h of incubation, two peaks with retention times 11.2 and 12.5 min appeared. As confirmed by MS analysis (Fig. 8), the first peak (M+18) corresponds to the cleavage of the Arg^2-Abu^3 peptide bond. This was followed by the hydrolysis of the $P_1 - P_1'$ reactive site, which yielded the second peak (*m*/*z* equal 1183.9) that match to the truncated peptide (lack of Abu-Thr-Nlys).

Also cyclic peptides **7** and **9**, designed to interact with chymotrypsin, displayed significantly high stability, but it is worth noting that peptomers **6** and **7** with

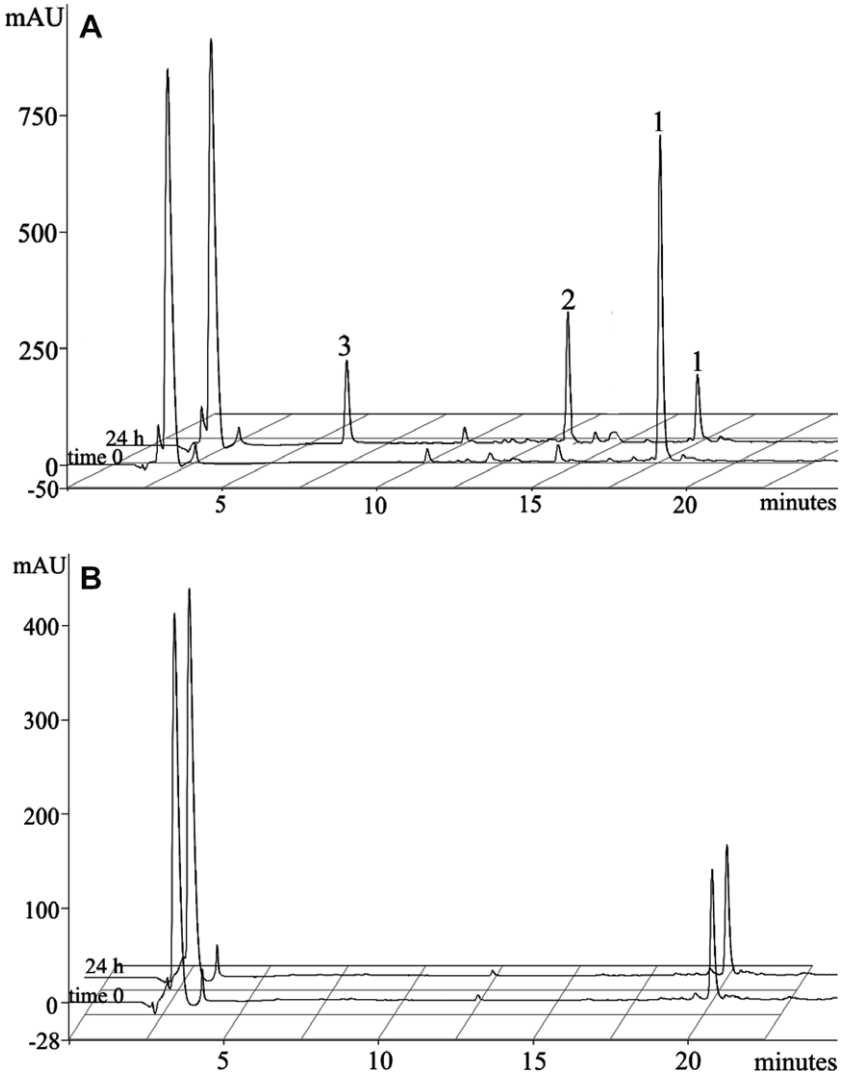


Figure 3. Proteolytic susceptibility of analogues **6** and **7** in the presence of α-chymotrypsin, at pH 3.5. (A) Gly^1-Arg-Abu-Thr-Nphe^5-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp^{14} (**6**): peak 1—intact peptide, peak 2—fragment 6–14, peak 3—fragment 1–5. (B) &Gly-Arg-Abu-Thr-Nphe-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp& (**7**).

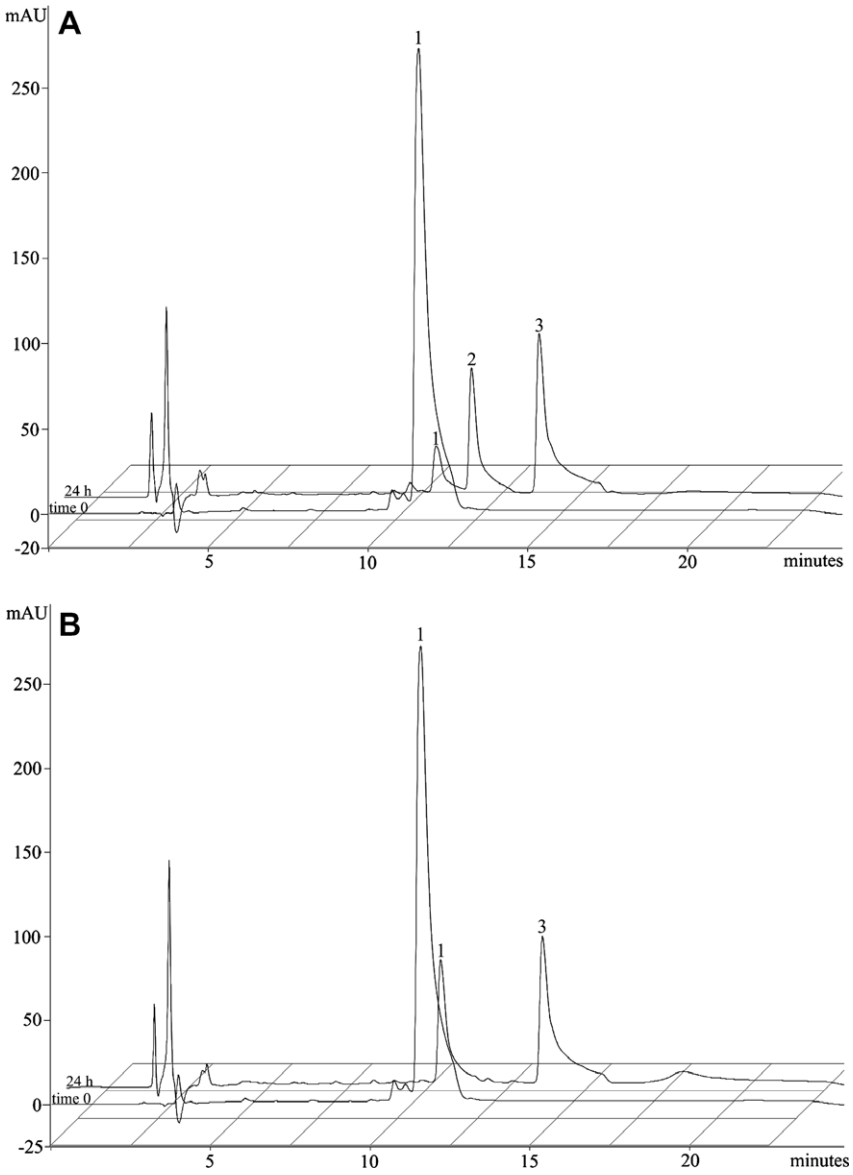


Figure 4. Proteolytic susceptibility of analogues **2** and **3** in the presence of β-trypsin, at pH 8.3. (A) Lys-Abu-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp (**2**): peak 1—intact peptide, peak 2—fragment Abu-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp, peak 3—fragment Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp. (B) Nlys-Abu-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp (**3**): peak 1—intact peptide, peak 3—fragment Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp.

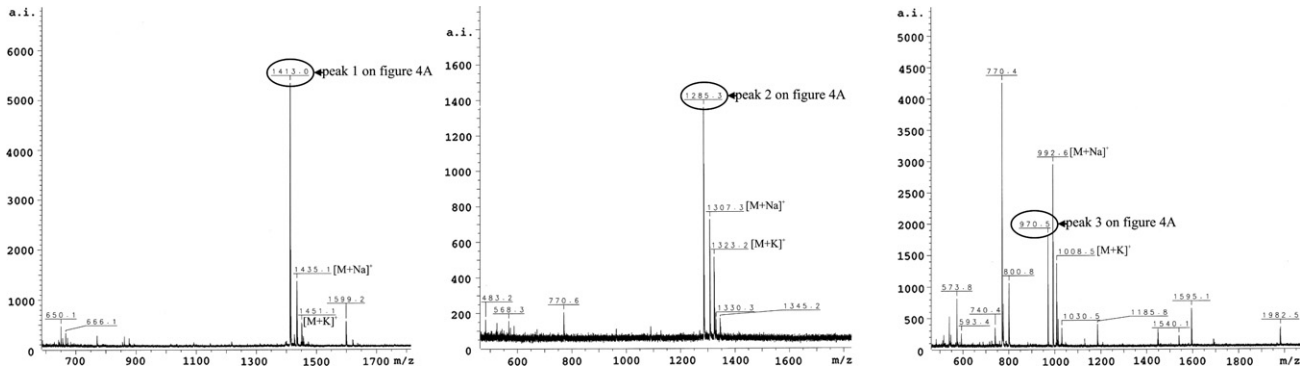


Figure 5. MS spectrum of peptide's fragments resulted from incubation of analogue **2** in presence of β-trypsin, pH 8.3, incubation time 24 h.

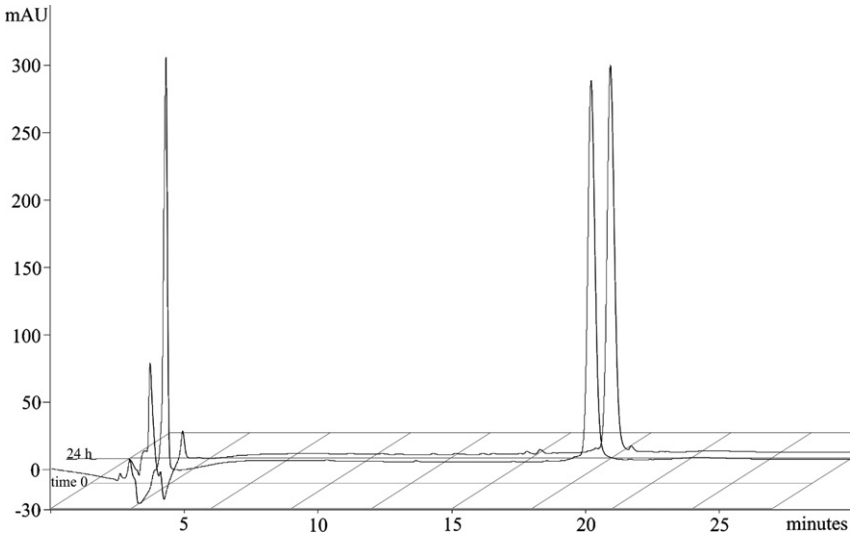


Figure 6. HPLC analysis of Lys-Cys(&)-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Cys(&)-Phe-Pro-Asp (**4**), after incubation with β-trypsin, at pH 8.3.

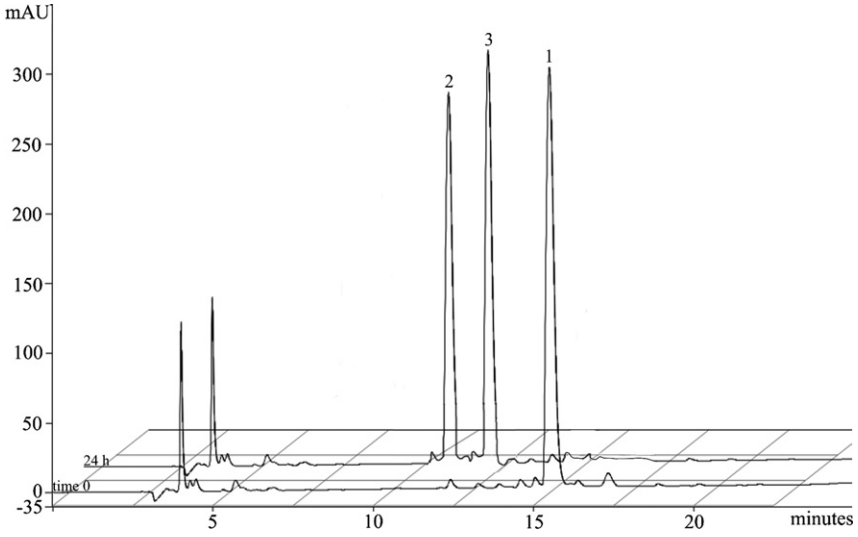


Figure 7. Cleavage pattern of &Gly-Arg-Abu-Thr-Nlys-Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp& (**5**) by β-trypsin, pH 8.3: peak 1—intact peptide, peak 2—peptide with hydrolyzed Arg^2-Abu^3 peptide bond, peak 3—fragment Ser-Ile-Pro-Pro-Ile-Abu-Phe-Pro-Asp-Gly-Arg.

very similar chymotrypsin inhibitory activity exhibited distinctly different proteolytic resistance. In this particular case, high affinity (rather than stability of the $P_1 - P_1'$ reactive site) of linear analogue **6** seems to be a decisive factor for its activity. Similarly to SFTI-1 built of proteinogenic amino acids, the introduction of one cyclic element (a disulfide bridge or head-to-tail cyclization) to the synthesized peptomers significantly increased their proteolytic resistance. Among for all analogues with Phe or Nphe in position P_1, the highest inhibitory activity displayed analogue **9**. To our knowledge, it is one of the most potent peptidic chymotrypsin inhibitor reported to day. Proteolytic resistance of **9** was slightly lower than its peptomer analogue **7**. Nevertheless, it was completely intact under conditions (incubation time 3 h) used for determination of K_a value.

3. Conclusions

The results presented herein clearly show that peptoid monomers which mimic proteinogenic amino acids are recognized by the enzymes. Unfortunately, peptide bonds formed by these derivatives are not proteolysis-resistant, as it was reported by several research groups. When peptoid monomers are introduced into sequences with high affinity towards serine proteinase, as in the case of SFTI-1 analogues, such bonds are cleaved by the enzyme. The rate of this cleavage is lower as compared with the 'regular' peptide bond. High chymotrypsin inhibitory activity of the linear analogue of SFTI-1 indicates that peptoid monomers can still be considered as attractive derivatives in designing biologically active peptoids with a simplified structure.

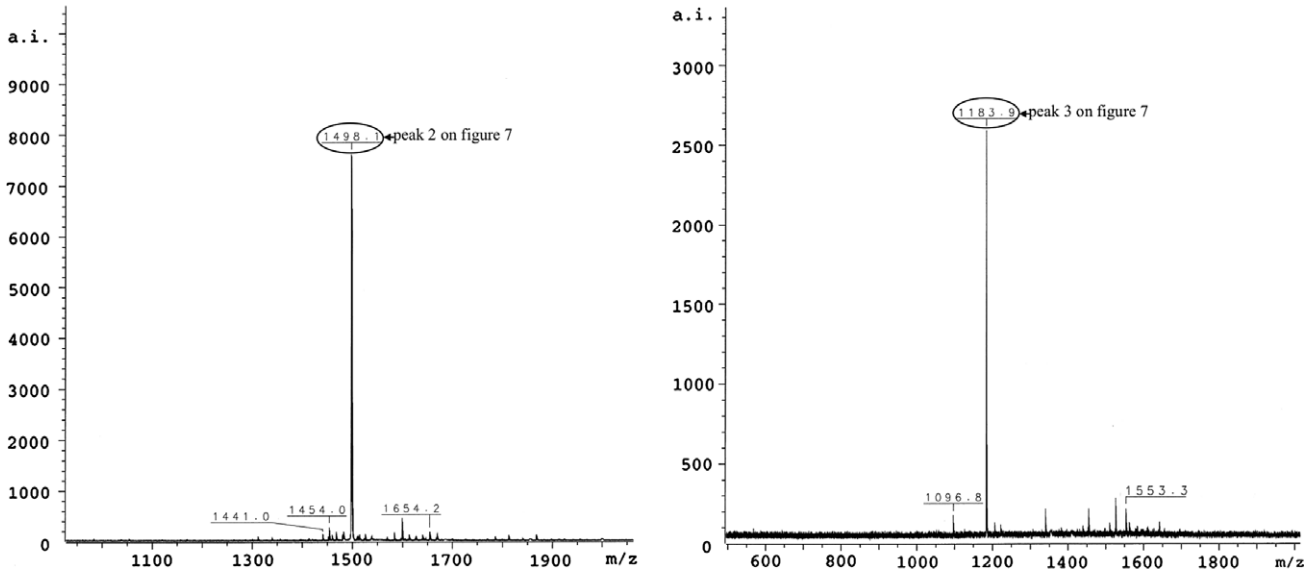


Figure 8. MS spectrum of peptide's resulted from incubation of analogue **5** with β-trypsin, pH 8.3, incubation time 24 h.

4. Materials and methods

4.1. 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). The C-terminal amino acid residue (Fmoc-Asp(O*t*-Bu)) was attached to 2-chlorotrityl chloride resin (substitution of Cl 1.46 meq/g) (Calbiochem-Novabiochem AG, Switzerland) in the presence of an equimolar amount of DIPEA in relation to the amino acid in anhydrous condition in DCM solutions. Peptide chains were elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with 20% piperidine in the mixture of DMF/NMP (1:1, v/v) with the addition of 1% Triton X-100, whereas the chain elongation was achieved with standard DIC/HOBt chemistry; 3 equiv of protected amino acid derivatives was used. N-substituted glycine derivatives (Nphe and Nlys(Boc)) were introduced into the peptide chain by the submonomeric approach.[13] In the first step, bromoacetic acid (5 equiv) was attached to the peptidyl-resin using DIC/HOBt method, followed by the nucleophilic replacement of bromine with primary amines: benzylamine (8 equiv) and *N*-1-Boc-1,4-diaminobutane × HCl (4 equiv). 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 (88:5:2.5, v/v/v).[19] In the case of analogue **4**, the disulfide bridge was formed by 0.1 M solution of J_2 in MeOH applying the procedure described elsewhere.[20] In order to obtain analogues **5** and **7**, the peptides were cleaved from the resin using a mixture of AcOH/TFE/DCM (2:2:6, v/v/v) for 1.5 h at room temperature.[21] Under these conditions, the cleaved peptides were protected in their side chain functions. In the next step, the head-to-tail cyclization of **5**, **7** and **9** was performed by the method with PyBop/DIPEA (molar ratio 1:2) in DMF.[22] The progress of the reaction was monitored by HPLC. After cyclization, the protecting groups on the side chains were removed using a mixture of TFA/phenol/triisopropylsilane (88:5:2.5, v/v/v). 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). Isocratic condition or linear gradient was applied, flow rate 3.0 mL/min, monitored at 226 nm. The purity of the peptides synthesized was checked on an RP Kromasil 100, C_8, 5 μm column (4.6× 250 mm) (Knauer, Germany). 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, was used. The mass spectrometry analysis was carried out on a MALDI-MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using α-CCA matrix.

4.2. 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.[23] The standardized bovine β-trypsin solution was used for the titration of 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 standardized bovine β-trypsin or bovine α-chymotrypsin, with N^{α}-benzoyl-DL-arginine-4-nitroanilide (BAPNA) and Suc-Ala-Ala-Pro-Leu-4-nitroanilide as substrates, respectively. The association constants were measured by the method developed in the laboratory of Laskowski, Jr.[24,25] The interaction between bovine β-trypsin or bovine α-chymotrypsin and the inhibitor was determined in 0.1 M Tris–HCl, pH 8.3, buffer con-

taining 20 mM $CaCl_2$ and 0.005% Triton X-100 at room temperature. Increasing amounts of the inhibitor, varying from 0 to $2E_0$ (E_0—the initial enzyme concentration), were added to the constant amount of the enzyme. The residual enzyme activity was measured using a turnover substrate on a Cary 3E spectrophotometer (Varian, Australia) after appropriate incubation time. Time of incubation was 40 min for analogues **4**, **6**, **7** (low K_a values), 60 min for analogue **5**, and 3 h for analogue **9** (high K_a value). The measurements were carried out at initial enzyme concentrations of 5.3 and 8.0 nM for trypsin and chymotrypsin, respectively. The residual enzyme activity was measured with Phe-Val-Pro-Arg-Anb^{5,2}–NH_2[26] and Z-Phe-Ala-Thr-Tyr-Anb^{5,2}–NH_2[27] as chromogenic substrates for trypsin and chymotrypsin inhibitors, respectively. In all the cases the initial substrate concentration was below 0.1 K_M. The experimental points were analyzed by plotting the residual enzyme concentration [E] versus the initial inhibitor concentration [I_0]. The experimental data were fitted to the theoretical values using the GraFit software package.[28]

4.3. Determination of hydrolysis rates

Peptomeric analogues which exhibited inhibitory activity were incubated in 50 mM sodium acetate, 20 mM $CaCl_2$, pH 3.5, using catalytic amounts of the enzymes (1 mol%).[29] The incubation was carried out at room temperature and sample aliquots of the mixture were taken out periodically and submitted to RP-HPLC analysis. The analysis was performed on an HPLC 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). Linear gradient from 25% to 50% B for 20 min, flow rate 3 mL/min, monitored at 226 nm. The collected fractions of the peaks were analyzed by MALDI-MS. The experiments were repeated in 100 mM Tris–HCl, pH 8.3, buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100. The hydrolysis rate (k) of the inhibitors studies was calculated from the equation describing the intact inhibitor concentration [I] as a function of time

$$[I]_t = [I_0]\exp(-kt)$$

The half-time ($t_{1/2}$) of the inhibitor was calculated from the following equation

$$t_{1/2} = 0.6931/k$$

Acknowledgements

This work was supported by the University of Gdańsk under Grants DS/8290-4-0129-7 and BW/8000-5-0399-7.

References and notes

1. Abbenante, G.; Fairlie, D. P. *Med. Chem.* **2005**, *1*, 71.
2. Luckett, S.; Santiago Garcia, R.; Barker, J. J.; Konarev, A. V.; Shewry, P. R.; Clarke, A. R.; Brady, R. L. *J. Mol. Biol.* **1999**, *290*, 525.
3. Bode, W.; Huber, R. *Eur. J. Biochem.* **1992**, *204*, 433.
4. Laskowski, M., Jr.; Qasim, M. A. *BBA* **2000**, *1477*, 324.
5. Lu, W.; Apostol, I.; Qasim, M. A.; Warne, N.; Wynn, R.; Zhang, W. L.; Anderson, S.; Chiang, Y. W.; Ogin, E.; Rotherg, I.; Ryan, R.; Laskowski, M., Jr. *J. Mol. Biol.* **1997**, *266*, 441.
6. Krowarsch, D.; Dadlez, M.; Buczek, O.; Krokoszynska, I.; Smalas, A. O.; Otlewski, J. *J. Mol. Biol.* **1999**, *289*, 175.
7. Korsinczky, M. L.; Schirra, H. J.; Rosengren, K. J.; West, J.; Condie, B. A.; Otvos, L.; Anderson, M. A.; Craik, D. J. *J. Mol. Biol.* **2001**, *311*, 579.
8. McBridge, J. D.; Watson, E. M.; Brauer, A. B. E.; Jaulent, A. M.; Leatherbarrow, R. J. *Biopolymers* **2002**, *66*, 79.
9. Korsinczky, M. L. J.; Schirra, H. J.; Craik, D. J. *Curr. Protein Pept. Sci.* **2004**, *5*, 351.
10. Qi, R.-F.; Chi, C. *Acta Biochim. Biophys. Sin.* **2005**, *37*, 283.
11. Zabłotna, E.; Kaźmierczak, K.; Jaśkiewicz, A.; Stawikowski, M.; Kupryszewski, G.; Rolka, K. *Biochem. Biophys. Res. Commun.* **2002**, *292*, 855.
12. Stawikowski, M.; Stawikowska, R.; Jaśkiewicz, A.; Zabłotna, E.; Rolka, K. *ChemBioChem* **2005**, *6*, 1057.
13. Simon, R. J.; Kania, R. S.; Zuckermann, R. N.; Huebner, V. D.; Jewell, D. A.; Banville, S.; Ng, S.; Wang, L.; Rosenberg, S.; Marlowe, C. K.; Spellmeyer, D. C.; Tan, R.; Frankel, A. D.; Santi, D. V.; Cohen, F. E.; Bartlett, P. A. *PNAS* **1992**, *89*, 9367.
14. Miller, S. M.; Simon, R. J.; Ng, S.; Zuckermann, R. N.; Kerr, J. M.; Moos, W. H. *Drug Dev. Res.* **1995**, *35*, 20.
15. Ardelt, W.; Laskowski, M., Jr. *J. Mol. Biol.* **1991**, *220*, 1041.
16. Otlewski, J.; Zbyryt, T. *Biochemistry* **1994**, *33*, 200.
17. Zabłotna, E.; Jaśkiewicz, A.; Łęgowska, A.; Lesner, A.; Rolka, K. *J. Pept. Sci.* **2007**, *13*, 749.
18. Spengler, J.; Jimenez, J. C.; Burger, K.; Giralt, E.; Albericio, F. *J. Pept. Sci.* **2005**, *65*, 550.
19. Sole, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399.
20. Yang, Y.; Sweeney, W. V.; Schneider, K.; Chait, B. T.; Tam, J. P. *Protein Sci.* **1994**, *3*, 1267.
21. Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513.
22. Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205.
23. Chase, T.; Schaw, E. *Methods Enzymol.* **1970**, *19*, 20.
24. Empie, M. W.; Laskowski, M., Jr. *Biochemistry* **1982**, *21*, 2274.
25. Park, S.J. PhD thesis, Purdue University, IN, USA, **1985**.
26. Zabłotna, E.; Dysasz, H.; Lesner, A.; Jaśkiewicz, A.; Kaźmierczak, K.; Miecznikowska, H.; Rolka, K. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 185.
27. Wysocka, M.; Kwiatkowska, B.; Rzadkiewicz, M.; Lesner, A.; Rolka, K. *Comb. Chem. High Throughput Screen.* **2007**, *10*, 171.
28. Leatherbarrow, R. J. GraFit Version 5. Erithacus Software, Horley, **2001**.
29. Jaulent, A. M.; Leatherbarrow, R. J. *Protein Eng. Des. Select.* **2004**, *17*, 681.