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Conformational studies of (Abu^{3,11})-SFTI-1, an analogue of the trypsin inhibitor isolated from sunflower seeds

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Abstract: With only 14 amino acid residues, the trypsin inhibitor SFTI-1 is the smallest naturally occurring serine proteinase inhibitor. It consists of two cyclic fragments (with head-to-tail cyclization and a disulfide bridge). In our previous paper, we showed that the removal of the disulfide bridge produced 2.4-fold lower activity. Here, we present the total conformational analysis of the [Abu^{3,11}]-SFTI-1 analog by means of 2D NMR spectroscopy in conjunction with theoretical methods. The peptide was synthesized by Fmoc SPPS. It was cyclized with PyBop and DIPEA in DMF. The NMR studies were performed in DMSO-d_6 at 303 K. Conformations of the peptide studied were calculated by the following three approaches: distance geometry (DG), molecular dynamics (MD) and determination of the statistical weights of conformations. The first two algorithms use a CHARMM force field, whereas the last uses an ECEPP/3 force field. Our calculations resulted in three sets of conformers with 7, 9 and 6 representatives, respectively. All our results were compared with published ones. It was found that the peptide has an ill-defined structure. Despite its conformational flexibility, the binding loop (3–11 fragment) displayed geometry similar to the corresponding fragments of the other SFTI-1 analogs and to the inhibitor itself. Furthermore, the peptide bond between the Ile7 and Pro8 residues adopts *cis* geometry, which is essential for inhibitory activity. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: SFTI-1 analog; NMR; structure; DG; MD; EDMC; trypsin inhibitor

INTRODUCTION

SFTI-1 is the smallest and most potent from Bowman-Birk inhibitors (BBIs) [1,2]. Its native structure in complex with bovine β-trypsin was established by Luckett *et al.* in 1999 using X-ray crystallography [3]. Over recent times, a lot of attention has been focused on this small peptide. SFTI-1 is a 14-amino acid residue homodetic cyclic peptide, which structure is additionally stabilized by the disulfide bridge. The next important structural element is *cis* Lys7-Pro8 peptide bond, which is, among others, responsible for binding to trypsin [1,2]. The dominant secondary structural element in the binding loop is β-hairpin [3]. Because of this, native SFTI-1 is rather stiff and has restricted conformational freedom; its structure determined by means of NMR spectroscopy in solution does not vary a lot in comparison to that from crystallography [4]. Extensive studies of SFTI-1 analogs revealed the importance of each of the structural features. In one of our previous paper [5], we described kinetic studies of native SFTI-1 and its two analogs: the acyclic and the one without a disulfide bond. It was found that head-to-tail cyclization does not influence inhibitory activity, whereas analogs with isosteric substitution of Cys by α-aminobutyric acid (Abu) yielded the analog [Abu^{3,11}]-SFTI-1, which was 2.4 times less active than the native SFTI-1 [5]. Recently, Korsinczky *et al.* [6] have shown that this analog displayed a solution structure similar to SFTI-1, but with a reduction in rigidity. They used distance and dihedral angle constraints derived from 1D and 2D ^{1}H NMR recorded in aqueous solution (pH 4.5) at 290 K to calculate the [Abu^{3,11}] SFTI-1 conformation, applying the *Simulated Annealing* (SA) protocol followed by molecular dynamics (MD) and energy minimization.

We decided to extend these investigations and perform a total conformational analysis of this SFTI-1 analog by combined 2D NMR spectroscopy and three approaches to calculate 3D structures.

The *Distance Geometry* (DG) and *Restrained* MD protocols implemented in the XPLOR program were used.

In these two approaches, the 3D structure of the peptide studied was computed using interproton distances calculated from the NOE intensities with torsion angles calculated from vicinal coupling constants as constraints.

In the third approach, a global conformational search of [Abu^{3,11}]-SFTI-1 was performed using the electrostatically driven Monte-Carlo (EDMC) method with an ECEPP/3 force field. The NOE effects and the $^3J_{NH-H\alpha}$ coupling constants were computed for the conformations obtained, and then the statistical

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weights of these conformations were calculated by fitting the theoretical NOE effects and $^3J_{NH-H\alpha}$ coupling constants values to the experimental NMR data.

MATERIALS AND METHODS

Peptide Synthesis

The peptide was synthesized manually by the solid-phase method using Fmoc on 2-chlorotrityl chloride resin (Calbiochem-Novabiochem AG, Switzerland; substitution of Cl 1.46 meq/g). After completing the synthesis, the peptide was cleaved from the resin using a mixture of AcOH/TFE/DCM (2 : 2 : 6, v/v/v) for 1.5 h at room temperature. Under these conditions, the cleaved peptide was protected in their side chain functions. In the next step, the cyclization was performed. A solution of 148 mg (0.1 mmol) of peptide in 5 ml of DMF was added dropwise during 15 min to a solution of 90 ml of PyBop/DIPEA (molar ratio 1 : 2) in DMF. 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). Purification was performed using an HPLC method and a semipreparative PR C_8 column. All additional features of this synthesis are described in our previous work [7].

NMR Measurements

For the peptide studied, the following spectra were obtained: TOCSY [8], NOESY [9], ROESY [10], double quantum filtered COSY (DQF-COSY) [11,12]. In all cases, a temperature of 303 K was applied. Additionally, mixing times were 80 ms for TOCSY, 300 ms for NOESY and 200 ms for ROESY. All spectra excluding ROESY were recorded on a Varian Mercury Spectrometer, operating at a 400 MHz resonance frequency. ROESY spectrum was recorded on a Varian Unity 500 Plus Spectrometer. The peptide concentration was 4 mM in DMSO–d_6. Assignment of the proton chemical shifts was accomplished by means of all the 2D spectra mentioned above. The vicinal coupling constants $^3J_{NH-H\alpha}$ were extracted from the 1D NMR spectrum. NOE effects were picked up from the NOESY spectrum. All data was processed using the program XEASY [13]. The interproton distances and the torsion angles were generated using the CALIBA and HABAS algorithms of the DYANA package, respectively [14].

Structure Calculations

The following approaches were used when defining the structure of this peptide: DG and MD implemented in the XPLOR 3.1 package [15] and determination of the statistical weights of the obtained ensemble of conformations. Two force fields were used, CHARMM [16] and ECEPP/3, respectively [17]. The CHARMM force field is an integral part of the XPLOR 3.1 package. It was used for both DG and MD simulations. Total energy (E_{tot}) is a function of the position of the atoms (R) in a particular system. E_{tot} is treated as the sum of internal energy and called bond energy (E_b), while external energy that is connected with nonbonded interactions is called (E_{nb}). Bond energy consists of three additional elements: bond stretch energy (E_s), bend energy (E_{be}) and rotation energy (E_{rot}). The ECEPP/3 force field assumes rigid valence geometry. The total energy (E_{tot}) is a sum of the electrostatic energy (E_{es}), nonbonded energy (E_{nb}), and torsional energy (E_{tor}). Solvation free energy (E_{solv}) is also included. It was calculated using the surface-solvation model parameterized to describe the energy of solvation in DMSO [18]. According to the NMR data, the geometry of all peptide bonds except for Ile7-Pro8 was fixed to *trans*, and the chirality of all C_α (except for Gly residue) was fixed to L.

Distance geometry calculations. The structures of each peptide were created using DG and SA calculations applied by the XPLOR program [15]. Initial structures were generated by metric matrix DG embedding using all atoms which were subjected to restrained SA, followed by SA refinement. Molecules were heated and annealed at 2000 K for 3 ps (1000 steps), next cooled to 100 K for 5 ps (1000 steps), and finally for 200 steps the energy was minimized using the Powell algorithm [19]. During the refinement, the molecule was slowly cooled from 1000 to 100 K for 5 ps (2000 steps).

Molecular dynamics calculations. The calculations were starting from a random conformation and using standard modules of the XPLOR program's SA algorithm. Additionally, NMR-derived constraints for interproton distances of dihedral angles were added to the target function with force constants $f = 50$ kcal/mol Å^2 and $f = 50$ kcal/mol rad^2, respectively. Furthermore, constraints for the ω angles of the peptide groups were added to keep them in a *trans* configuration (except Pro8) $-f = 500$ kcal/mol rad [2]. The chirality of C^α atoms (except for Gly) was held by imposing a 3-fold potential on the improper N–CO–C^α–C^β torsion angles $-f = 500$ kcal/mol rad [2].

Determination of the statistical weights of the conformations. This approach consists of two steps: (i) generation of the ensemble of 3000 conformations, and (ii) determination of the statistical weights in order to characterize the conformational equilibrium. The first step calculations were carried out using an EDMC in an ECEPP/3 force field [20] as described above. All the peptide bonds except for Ile7-Pro8 were kept in *trans* geometry. The chirality of C^α atoms and geometry of the peptide bonds were the only restraints while generating the ensemble. After this, obtained conformations were clustered using the minimum variance algorithm [21]. The root-mean-square deviation (RMSD) between the heavy atoms at an optimum superposition was taken as a measure of the distance between conformations, and a cut-off value of 0.05 Å was used to separate the families of the peptide. In this particular case, we obtained 774 families for the analog studied. The statistical weights of selected conformations were calculated by fitting the theoretical NOE spectra and $^3J_{NH-H\alpha}$ coupling constants to the experimental spectra using the ANALYZE algorithm [22]. The intensities of the NOE effects were computed by solving the Bloch differential equations system [23] applied in the MORASS program [24,25]. The $^3J_{NH-H\alpha}$ coupling constants were calculated using the empirical Bystrov-Karpulus equation [26].

RESULTS AND DISCUSSION

The assignment of the chemical shifts of protons was started with the help of the DQF-COSY spectrum. We

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Table 1 The chemical shifts (ppm) of [Abu^{3,11}]-SFTI-1 in DMSO-d_6 at 303 K

Amino acid	Chemical shifts (ppm)					
	NH	α-CH	β-CH	γ-CH	δ-CH	Others
Gly1	8.18	3.74				
Arg2	7.44	4.23	1.96	1.65	3.06	ε-NH 7.35
			1.72			HH1 7.26
Abu3	8.01	4.26	1.71	0.85		
Thr4	7.74	4.21	3.98	1.03		
Lys5	8.38	4.30	1.81	1.31	1.53	ε-NH$_3$ 2.76
Ser6	7.25	4.31	3.74			
			3.50			
Ile7	8.18	4.23	1.72	1.17	0.79	
Pro8	—	4.52	2.74	2.56	4.28	
Pro9	—	4.23	2.34	1.96	3.73	
					3.58	
Ile10	8.42	4.60	1.59	1.33	0.83	
Abu11	8.34	5.32	1.65	0.83		
Phe12	7.56	4.31	3.02			3,5H 7.17
			2.72			4H 7.26
Pro13	—	4.19	2.24	1.88	3.95	
			2.09		3.78	
Asp14	8.41	4.57	2.92			
			2.88			

found 13 signals in the amide region which were the couplings between alpha and amide protons of amino acids. For each shift of H$^{\alpha}$, we determined the resonance lines of particular spin systems in TOCSY spectrum, and collected shifts of rest of the protons. The Pro residues were identified by the couplings between H$^{\alpha}$ and H$^{\delta}$. Additional sets of minor signals were found in the spectra but they were excluded from further studies. The Arg residue protons were assigned on the basis of the characteristic spin system. The Ser residue was found by the high values of the H$^{\beta}$ shifts. The chemical shifts of the Abu and Asp protons were identified with the help of the couplings between H$^{\alpha}$ and methylene protons of these residues. The correctness of the assignment in NOESY was confirmed by finding the sequential couplings. Analysis of the H$^{\alpha}$–H$^{\alpha}$ region of the NOESY spectrum revealed the existence of the *cis* peptide bond between Lys7 and Pro8 residues. All chemical shifts are listed in Table 1. The NOE effect pattern obtained and the values of vicinal coupling constants are shown in Figure 1. A low value of the vicinal coupling constant of Ser6 suggests the existence of β turn in this region. Other values are high and there is a lack of coupling between H$^{\alpha}$ protons. This suggests that peptide adopts an extended or ill-defined conformation. Additionally, the low amount of the observed interresidue NOE effects confirms the flexible nature of this peptide. Because of the fact that there is not only one stiff conformation in solution, but also they exist in conformational equilibrium, we decided to present our results by using three approaches in structural calculation. As mentioned earlier, the three approaches we applied are: DG, MD and determination of the statistical weights of the conformations obtained. As a result, we chose seven, nine and six conformations, respectively. In the first two cases, the lowest energies were the criterion, and in the other one the statistical weights higher than 8.5%. We put the chosen conformers, their total energies and statistical weights, and types and positions of β-turns in Table 2. The superposition of C$^{\alpha}$ atoms of whole molecules gave RMSDs of 2.13, 2.01 and 0.65 Å for DG, MD and EDMC, respectively. The binding loops of the obtained structures are more similar (not shown). The superposition of C$^{\alpha}$ atoms of 3–11 fragments of selected conformations gave RMSD values of 1.64, 1.45 and 0.42 Å for DG, MD and EDMC, respectively (Figure 2). The differences among these conformers are caused because of applying different methods of calculations. Nevertheless, the superposition shows the flexibility of this peptide (except for those from EDMC structures). Methods which involve the SA protocols (DG and MD) give a much more varied ensemble of conformations than the EMDC method. In Figure 3, we show the superposition of the representative structures

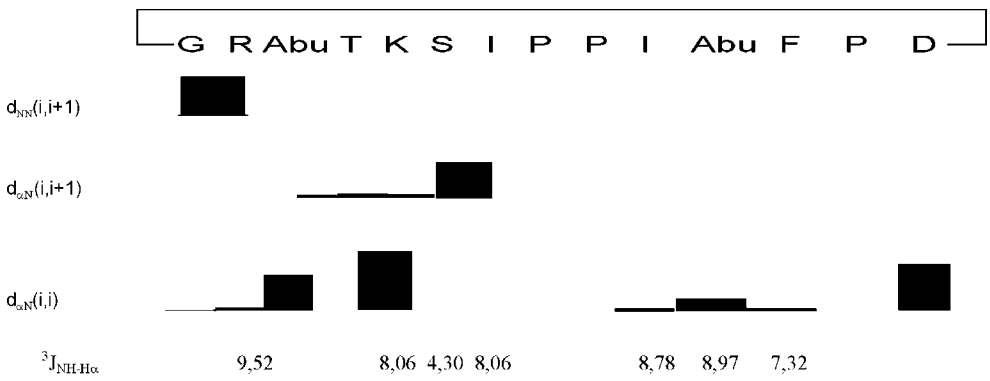

Figure 1 The internal intensities of off-diagonal signals in the NOESY spectrum of [Abu^{3,11}]-SFTI-1 and vicinal coupling constants.

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Table 2 Total energies, statistical weights and position and types of β-turns of obtained conformations

Distance geometry		
No.	Energies [kcal/mol]	Positions ($i+1$ and $i+2$) and types of β-turns
1	26.098	Ser6-Ile7 type VI Ile7-Pro8 type IV Phe12-Pro13 type II′
2	27.834	Lys5-Ser6 type IV Ser6-Ile7 type VI Ile7-Pro8 type IV Ile10-Abu11 type II′
3	27.848	Ser6-Ile7 type VI Ile7-Pro8 type IV Phe12-Pro13 type II′
4	27.967	Ser6-Ile7 type VI Ile7-Pro8 type IV Phe12-Pro13 type I
5	28.358	Thr4-Lys5 type IV Ile7-Pro8 type IV Phe12-Pro13 type I
6	28.717	Ile7-Pro8 type IV Ile10-Abu11 type I
7	29.602	Lys5-Ser6 type IV Ser6-Ile7 type VI Ile7-Pro8 type IV Pro9-Ile10 type I Phe12-Pro13 type IV
Molecular Dynamics		
1	26.713	Arg2-Abu3 type IV Ile7-Pro8 type IV Ile10-Abu11 type IV
2	26.942	Ile7-Pro8 type IV Phe12-Pro13 type I
3	27.420	Abu3-Thr4 type II Ser6-Ile7 type VI Ile7-Pro8 type IV Abu11-Phe12 type VII Phe12-Pro13 type IV
4	28.086	Arg2-Abu3 type I Ser6-Ile7 type VI Ile7-Pro8 type IV Ile10-Abu11 type IV
5	28.549	Arg2-Abu3 type I Thr4-Lys5 type VII Ile7-Pro8 type IV Ile10-Abu11 type VII
6	28.747	Ser6-Ile7 type VI Ile7-Pro8 type IV
7	28.793	Ser6-Ile7 type VI Ile7-Pro8 type IV
8	29.308	Ile7-Pro8 type IV Abu11-Phe12 type IV
9	29.339	Ile7-Pro8 type IV

Table 2 (*Continued*)

EDMC		
No.	Statistical Weights (%)	Positions ($i+1$ and $i+2$) and types of β-turns
1	13.16	Thr4-Lys5 type IV Lys5-Ser6 type III′ Ile7-Pro8 type IV Pro8-Pro9 type IV
2	10.59	Abu3-Thr4 type I′ Thr4-Lys5 type IV Lys5-Ser6 type III′ Ile7-Pro8 type IV
3	9.03	Abu3-Thr4 type I′ Thr4-Lys5 type IV Lys5-Ser6 type III′ Ile7-Pro8 type IV
4	8.95	Abu3-Thr4 type I′ Thr4-Lys5 type IV Lys5-Ser6 type III′ Ile7-Pro8 type IV
5	8.91	Abu3-Thr4 type I′ Thr4-Lys5 type IV Lys5-Ser6 type III′ Ile7-Pro8 type IV Abu11-Phe12 type I
6	8.87	Abu3-Thr4 type I′ Thr4-Lys5 type IV Lys5-Ser6 type III′ Ile7-Pro8 type IV Abu11-Phe12 type I

Table 3 The RMSD values of superposition of the binding loop regions of obtained conformations and reference ones

	DG (Å)	MD (Å)	EDMC (Å)
Acyclic permutant SFTI-1 NMR structure [27]	2.84	3.18	3.45
[Abu^{3,11}]-SFTI-1 NMR structure [7]	2.63	2.97	3.34
Native SFTI-1 NMR structure [27]	2.81	3.16	3.46
Native SFTI-1 cryst. structure [26]	2.73	3.17	3.51

calculated using the methods mentioned. Analyzing β-turns, we can say that there are the following amino acids in positions $i+1$ and $i+2$ Thr4–Lys5 – type IV; Lys5–Ser6–type III′; Ile7–Pro8 – type IV in each EDMC conformation whereas in all SA conformations there is a β-turn of type IV involving Ser6–Pro9 fragments. The existence of a β turn in this region is established by

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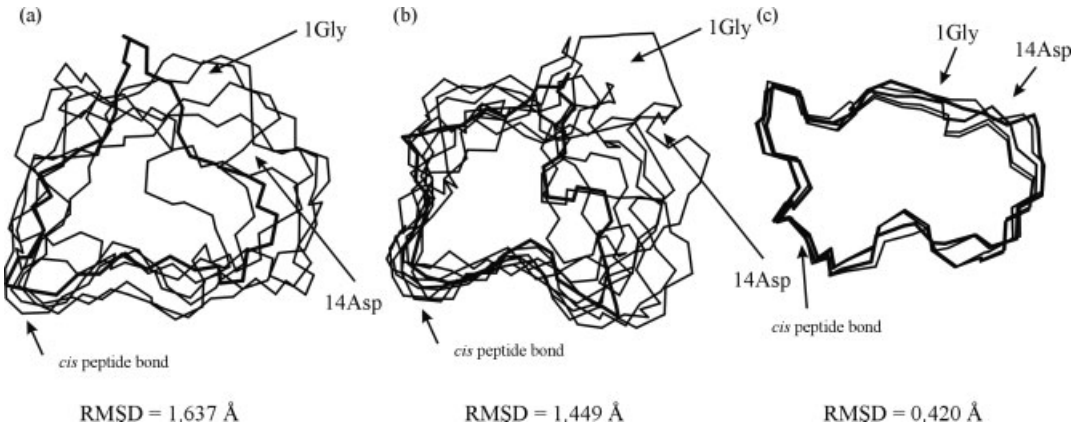

Figure 2 The superposition of C$^{\alpha}$ atoms of 3–11 fragments of the obtained sets of conformations. The superposition of DG (a), MD (b) and EDMC (c) conformations.

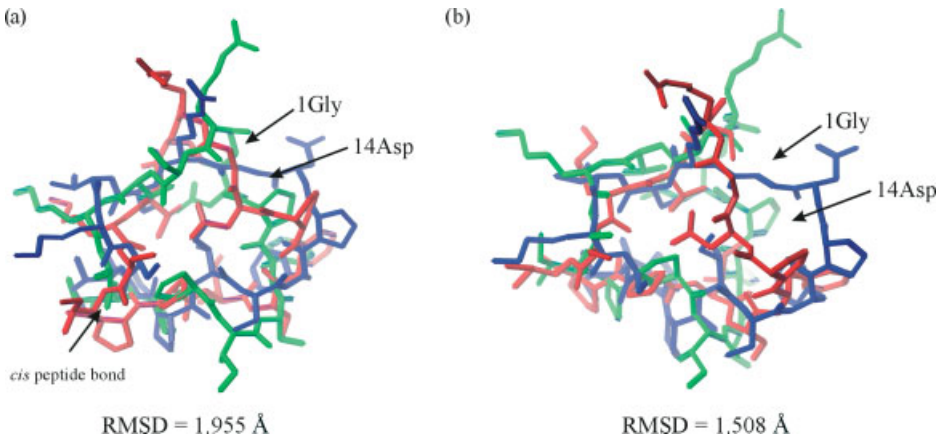

Figure 3 The superposition of the lowest energy DG conformation (red line), the lowest MD conformation (green line) and the conformation of the highest statistical weight (blue line). (a) The superposition of all C$^{\alpha}$ atoms, (b) The superposition of C$^{\alpha}$ atoms of 3–11 fragments of representative conformations.

a *cis* peptide bond and corresponds with the values of coupling constants.

CONCLUSIONS

The conformations observed correspond to solution and X-Ray structures of native SFTI-1 [27,28], acyclic SFTI-1 [28] and [Abu^{3,11}]-SFTI-1 determined by Korsinczky *et al.* [6] Despite the conformational flexibility of the peptide studied under the conditions applied in the NMR experiment, there are certain similarities between conformations generated by us and those deposited in the PDB database, especially in the binding loop region. In addition to the similar geometry of the binding loop of native and monocyclic SFTI-1 inhibitors, a common feature in all the cases is the *cis* geometry of the Ile7-Pro8 peptide bond. We have presented the calculated RMSD values for binding loop regions of the reference material and our structures in Table 3. The observed structural results are in good agreement with kinetic studies [5]. All three peptides inhibit bovine β-trypsin. A 2.4-fold lower inhibitory activity of the peptide studied and its lower proteolytic resistance, as compared with SFTI-1, is connected with its higher conformational flexibility. It is worth noticing that the solution structure of [Abu^{3,11}]-SFTI-1 described by Korsinczky *et al.* [6] is much better defined. This is probably due to the different conditions (a low temperature of 290 K) applied in the NMR experiment, whereas we applied 303 K, which is closer to physiological conditions.

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REFERENCES

1. Craik DJ, Simonsen S, Daly NL. The cyclocides: Novel macrocyclic peptides as scaffolds in drug design. *Curr. Opin. Drug Discov. Devel.* 2002; **5**: 251–260.

2. Jaulent AM, McBride JD, Leatherbarrow RJ. In *Proceedings of the Second International and Seventeenth American Peptide Symposium*, Lebl M, Houghten RA (eds). American Peptide Society: San Diego, CA, 2001; 547.
3. Luckett S, Santiago Garcia R, Barker JJ, Konarev Al V, Shevry PR, Clarke AR, Brady RL. High resolution structure of a potent, cyclic proteinase inhibitor from sunflower seeds. *J. Mol. Biol.* 1999; **290**: 525–533.
4. Korsinczky ML, Shirra HJ, Rosengren KJ, West J, Condie BA, Otvos L, Anderson MA. Solution structures by 1H NMR of the novel cyclic trypsin inhibitor SFTI-1 from sunflower seeds and an acyclic permutant. *J. Mol. Biol.* 2001; **311**: 579–591.
5. Zabłotna E, Kaźmierczak K, Jaśkiewicz A, Stawikowski M, Kupryszewski G, Rolka K. Chemical synthesis and kinetic study of the smallest naturally occuring trypsin inhibitor SFTI-1 isolated from sunflower seeds and its analogues. *Biochem. Biophys. Res. Commun.* 2002; **292**: 855–859.
6. Korsinczky ML, Clark RJ, Craik DJ. Disulfide bond mutagenesis and the structure and function of the head-to-tail macrocyclic trypsin inhibitor SFTI-1. *Biochemistry* 2005; **44**: 1145–1152.
7. Zabłotna E, Jaśkiewicz A, Łęgowska A, Lesner A, Rolka K. Designing of proteinase inhibitors by combinatorial chemistry using trypsin inhibitor SFTI-1 as a starting structure. *J. Pept. Sci.* 2007; **13**: 749–755.
8. Bax A, Davis DG. Assignment of complex H-1 NMR spectra via two-dimensional homonuclear Hartmann-Hahn spectroscopy. *J. Am. Chem. Soc.* 1985; **107**: 2820–2821.
9. Jeener J, Meier BH, Bachmann P, Ernst RR. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Chem. Phys.* 1979; **71**: 4546–4553.
10. Bax A, Davis DG. Practical aspects of two-dimensional transverse NOE spectroscopy. *J. Magn. Reson.* 1985; **63**: 207–213.
11. Bax A, Davis DG. MLEV-17 based two-dimensional homonuclear megnetization transfer spectroscopy. *J. Magn. Reson.* 1985; **65**: 355–360.
12. Piantini U, Sørensen OW, Ernst RR. Multiple quantum filters for elucidating NMR coupling network. *J. Am. Chem. Soc.* 1982; **104**: 6800–6801.
13. Bartels CT, Xia TH, Billeter M, Güntert P, Wüthrich K. The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J. Mol. Biol.* 1995; **6**: 1–10.
14. Güntert P, Braun W, Wüthrich K. Efficient computation of three-dimensional protein structures in solution from nuclear magnetic resonance data using the program DIANA and the supporting programs CALIBA, HABAS and GLOMSA. *J. Mol. Biol.* 1991; **217**: 517–530.
15. Brünger AT. *The X-PLOR Software Manual, Version 3.1.* Yale University Press: New Heaven, CT, 1992.
16. Brooks B, Bruccoleri R, Olafson BO, States DJ, Swaminathan S, Karplus M. CHARMM: a program for macromolecular energy minimization, and molecular dynamics calculations. *J. Comput. Chem.* 1983; **4**: 187–217.
17. Némethy G, Gibson KD, Palmer KA, Yoon CN, Paterlini G, Zagari A, Rumsey S, Scheraga HA. Improved geometrical parameters and nonbonded interactions for use in the ECEPP/3 algorithm with application to Proline-containing peptides. *J. Phys. Chem.* 1992; **96**: 6472–6484.
18. Groth M, Malicka J, Rodziewicz-Motowidło S, Czaplewski C, Klaudel L, Wiczk W, Liwo A. Determination of conformational equilibrium of peptides in solution by NMR spectroscopy and theoretical conformational analysis: application to the calibration of mean-field solvation models. *Biopolymers* 2001; **60**: 79–95.
19. Powell MJD. Restart procedures for the conjugate gradient method. *Math. Prog.* 1977; **12**: 241–254.
20. Ripoll DR, Scheraga HA. On the multiple-minima problem in the conformational analysis of polypeptides. II. An electrostatically driven Monte Carlo method. Tests on poly(L-Alanine). *Biopolymers* 1988; **27**: 1283–1303.
21. Späth HJ. Cluster analysis algorithms. *J. Comput. Chem.* 1980; **10**: 209.
22. Groth M, Malicka J, Czaplewski C, Ołdziej S, Liwo A, Łankiewicz L, Wiczk W. Maximum entropy approach to the determination of solution conformation of flexible polypeptides by global analysis and NMR spectroscopy–application to DNS1-c-[D-A(2)bu(2), Trp(4),Leu(5)]-enkephalin and DNS1-c-[D-A(2)bu(2), Trp(4), D-Leu(5)]enkephalin. *J. Biomol. NMR* 1999; **15**: 315.
23. Massewski WJ, Bolton PH. Quantitive analysis of nuclear overhauser effects. *J. Magn. Reson.* 1985; **65**: 526–530.
24. Meadows RP, Post CB, Luxon BA, Gorenstein DG. MORASS 2.1, Purdue University, W. Lafayette, 1994.
25. Post CB, Meadows RP, Gorenstein DG. On the evaluation of interproton distances for three-dimensional structure determination by NMR using a relaxation rate matrix analysis. *J. Am. Chem. Soc.* 1990; **112**: 6796–6803.
26. Bystrov VF. Spin-spin coupling and the conformational states of peptide systems. *Prog. NMR Spectrosc.* 1976; **10**: 41–81.
27. Luckett S, Garcia RS, Barker JJ, Konarev AV, Shewry P, Clerke AR, Brady RL. High resolution structure of a potent, cyclic protease inhibitor from sunflower seeds. *J. Mol. Biol.* 1999; **290**: 525–533.
28. Korsinczky MLJ, Schirra HJS, Rosengren KJ, West J, Condie BA, Otvos L, Anderson MA, Craik DJ. Solution structures by 1H NMR of the novel cyclic trypsin inhibitor SFTI-1 from sunflower seeds and an acyclic permutant. *J. Mol. Biol.* 2001; **311**: 579–591.