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Conformational Analysis of an Antitumour Cyclic Pentapeptide, Astin B, from *Aster tataricus* [1)]

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Abstract: Conformational analysis of an antitumour cyclic pentapeptide, astin B, isolated from *Aster tataricus*, was conducted by 2D-NMR techniques, temperature effects on NH protons, rate of hydrogen-deuterium exchange, vicinal NH-CαH coupling constants, and NOE experiments. The methods of molecular mechanics and restrained molecular dynamics calculations were applied to understand the energetic preferences of various conformations of astin B. Distances involving in three intramolecular hydrogen bonds and the NOE correlations were used for the refinements using AMBER program. These results indicated that the conformation in the solution state was, on the whole, homologous to that observed in the solid state. Conformational difference between astin B, showing a *cis* configuration in a proline amide bond, and cyclochlorotine from *Penicillium islandicum*, showing all *trans* amide configurations, was also discussed.

Introduction

A series of antitumour cyclic pentapeptides, astins (astins A - H),[1-3)] from *Aster tataricus* (Compositae), have been characterized structurally by NMR spectroscopic or X-ray diffraction studies. The cyclic pentapeptides of astins contain 16 membered-ring system with unique mono or di-chlorinated proline and/or *allo*threonine residues. As shown in Figure 1, the main active principle, astin B (1) contains a β,γ-dichlorinated proline, an *allo*threonine, a serine, a β-phenylalanine and an α-aminobutyric acid. In the previous paper,[2)] the conformation of astin B in solid state was shown to be different from that of cyclochlorotine[4)] which have been isolated from *Penicillium islandicum*

Sopp as one of the toxic principles, and possessed a *trans* proline amide bond and a typical type I β-turn between Pro[1] and Abu[2], by the comparison of X-ray diffraction study.

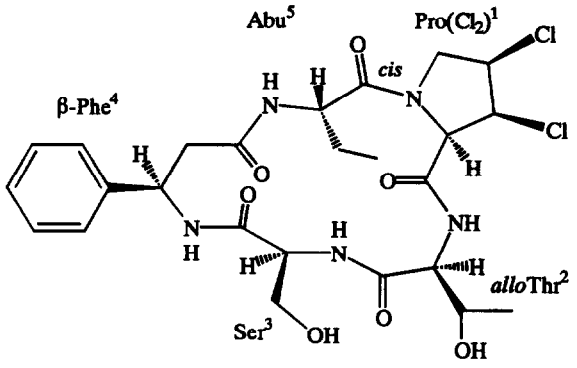

Fig. 1. Structures of astin B (1); Pro in 1 was provisionally numbered as a first amino acid. The structure of cyclochlorotine is cyclo(Pro(Cl_2)-Abu-Ser-β-Phe-Ser).

In order to understand the mechanisms involved in the action of cyclic peptides, it is necessary to have knowledge of their conformational characteristics. As part of our ongoing investigation of bioactive cyclic peptides from higher plants,[5] and to study the structure-activity relationship of astins, to determine the precise backbone conformation of astin B in solution is considered to be of important significance. We are also interesting in the correlation between the solution conformation of astin B and that of cyclochlorotine, both of which possess the similar peptide sequencing. The combination of 2D-NMR analysis with molecular dynamics and mechanics calculations led us to determine the energetically favorable conformation of astin B in solution. Here we report on the conformational analysis of astin B in the solution state by spectroscopic and computational chemical methods including molecular dynamics and molecular mechanics calculations.

Results and Discussion

Conformation in solution

A detailed knowledge of the conformation of 1 under a polar solvent such as [$^2H_6$]DMSO is considered to be the basis for structure-activity relationships allowing the design of new derivatives with higher activity. According to the NMR spectrum of astin B (1), 1 existed in a single stable conformational state in polar solvents such as [$^2H_5$]pyridine, [$^2H_3$]MeOD and [$^2H_6$]DMSO. The

complete assignments of the signals in various NMR measurements may provide more reliable information about the dynamic structures in solution. The assignments of ^{1}H and ^{13}C-NMR signals of **1** (Table 1) were made by the combination of ^{1}H-^{1}H COSY, HMQC[6] and HMBC[7] spectra, and reported in the previous paper.[3] The HMBC, which provides ^{1}H-^{13}C long-range couplings, was proved to be extremely valuable for the assignments. The conformational determination of **1** in solution was made on the basis of the results of the following experiments.

Table 1. ^{1}H and ^{13}C-NMR chemical shifts of **1**.

	proton	carbon
Pro(Cl$_2$)^{1}		
α	4.88 (d, 5.3)	64.45
β	5.13 (dd, 4.5, 5.3)	65.23
γ	4.77 (ddd, 4.5, 6.7, 9.6)	54.79
δ	3.40 (dd, 9.6, 11.3) 4.35 (dd, 6.7, 11.3)	51.02
C=O		166.33
allo Thr^{2}		
α	4.24 (t, 9.4)	56.84
β	4.20 (ddd, 5.8, 5.9, 9.4) 5.79 (d, 5.9; OH)	65.91
γ	1.22 (d, 5.8)	21.96
NH	8.39 (d, 9.4)	
C=O		169.78
Ser^{3}		
α	3.80 (m)	58.27
β	3.66 (br s)	60.21
NH	8.84 (d, 4.2)	
C=O		169.14
β-Phe^{4}		
α	2.10 (t, 12.7) 2.82 (dd, 4.7, 12.7)	42.84
β	4.91(ddd, 4.7, 6.8, 12.7)	51.33
γ		142.80
δ		125.58
ε	7.21 - 7.30 (m)	128.16
ζ		126.60
NH	7.38 (d, 6.8)	
C=O		171.07
Abu^{5}		
α	4.32 (td, 3.7, 9.3)	53.32
β	1.50 (m) 1.75 (m)	22.65
γ	0.97 (t, 7.4)	10.43
NH	8.58 (d, 3.7)	
C=O		172.12

Measurements were peformed in [$^{2}H_6$]DMSO at 500 MHz (^{1}H) and 125 MHz (^{13}C).

Hydrogen bonding

The first step in the procedures of the determination of the secondary structure of peptides in solution by NMR is to distinguish the NH protons exposed to the solvent or shielded from the solvent either sterically or through hydrogen bonding. The common procedure for that purpose is to determine the temperature effects on the NH protons,[8] which the NH protons exposed to solvents will show a higher temperature dependence, and rate of hydrogen - deuterium exchange.[9] The temperature coefficients ($d\delta/dT$) of **1** given in Table 2 clearly show that Ser^{3}-NH, β-Phe^{4}-NH and *allo*Thr^{2}-NH are shielded from the solvent, whereas Abu^{5}-NH is exposed to the solvent as shown by the temperature effects. A hydrogen-deuterium (H-D) exchange experiment in [$^{2}H_6$]DMSO-D_2O mixtures showed that the exchange half-life times ($t_{1/2}$) for Abu^{5}-NH, β-Phe^{4}-NH, *allo*Thr^{2}-NH, and Ser^{3}-NH were ≈ 0.5, ≈ 2.8, ≈ 4.0 and ≈ 6.0 days, respectively. These results gave the similar propensity to the temperature effects as indicated above.

The qualitative closeness between the above results about hydrogen bondings and the crystallographic data, which have already been reported in the previous paper,[2] are indicated as below. The Ser^3-NH, which involved in an intramolecular hydrogen bond to an hydroxyl oxygen in the side chain of *allo*Thr^2 in the crystal state, was strongly shielded from the solvent, which is characteristic of a proton involving in a strong hydrogen bond. The *allo*Thr^2-NH and β-Phe^4-NH, corresponding to weak intramolecular hydrogen bonds between *allo*Thr^2-NH and β-Phe^4-CO, and between β-Phe^4-NH and β-Phe^4-CO in the crystal state, involved in weaker hydrogen bonds. On the other hand, Abu^5-NH showed the values characteristic of solvent-exposed NH groups, also corresponding to the crystal data.

Table 2 Effect of temperature on the NH chemical shifts ($-d\delta/dT \times 10^3$ ppm/K) and rate of hydrogen - deuterium exchange (day) on addition of trace amounts of D_2O of astin B (1)

	solvent	*allo*Thr^2	Ser^3	β-Phe^4	Abu^5
A	[$^2H_6$]DMSO	3.0	2.2	2.8	4.5
B	[$^2H_6$]DMSO + D_2O	4.0	6.0	2.8	0.5

A: temperature effect B: H-D exchange rate

Vicinal NH-CαH coupling

Three-bond couplings gave very useful information to determine the backbone conformation because they can directly be converted into dihedral angles *via* Karplus-type equations. The dihedral angles, φ in **1**, estimated from vicinal NH-CαH (NH-CβH for β-Phe^4) coupling constants *via* Karplus-type equation proposed by Bystrov et al.,[10] were shown in Table 3. As can be seen from Table 3, the φ angles of *allo*Thr^2 and Abu^5 in Pro-neighboring residues showed a slight difference from those in the crystal state. However, both φ angles of Ser^3 and β-Phe^4 from coupling constants and those from X-ray analysis were almost identical.

NOE enhancements

The relationship of NOE enhancements in astin B observed by phase sensitive NOESY spectrum[11] is shown in Figure 2. The NOE observed between Pro^1-Hα and Abu^5-Hα provides an evidence in favor of *cis* amide bond between Pro^1 and Abu^5. In the Pro^1 residue, the presence of cis β,γ-dichloro atoms was suggested by the NOEs among Hα, Hβ and Hγ in Pro^1. Two characteristic NOEs, which imply the weak intramolecular hydrogen bonds between *allo*Thr^2-NH and β-Phe^4-CO, and between β-Phe^4-NH and β-Phe^4-CO in the crystal state, were observed between *allo*Thr^2-NH and Abu^5-Hα, and between β-Phe^4-NH and β-Phe^4-Hα1. Especially, it seems likely that the *allo*Thr^2-NH is directed inside the backbone ring more than in the case of the crystal state (*allo*Thr^2-NH ...Abu^5-Hα 2.743 Å).

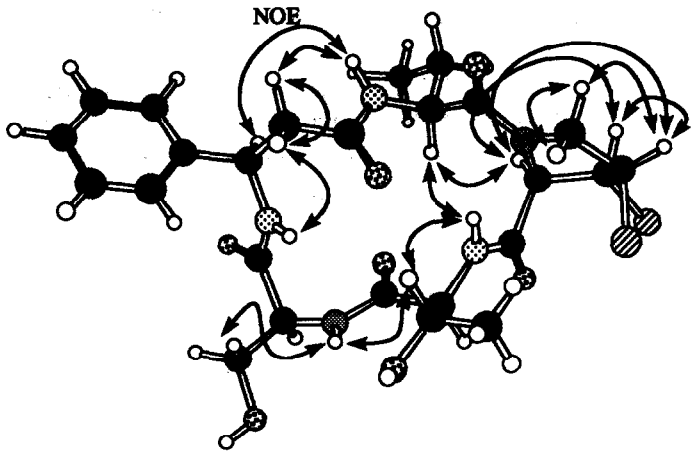

Fig. 2 NOE enhancements of astin B (1); The arrows show the NOE relationships confirmed by NOESYPH experiments in [$^2H_6$]DMSO at 303K.

Quenched molecular dynamics

We applied computational procedures using the NMR data to the elucidation of the solution conformation of astin B and further to the disclosure of the difference between the conformations in the solid and solution states. We have already reported that the possibility of using MD techniques as a tool for simulated annealing is tested in the case of the molecules of tropoloisoquinoline alkaloids,[12] surfactins[13] and trichorabdal diterpenes.[14] The method, applied to a broad class of problems, has also shown its practical utility in the case of conformational problems.[15] We performed the NOE constraint molecular dynamics calculation as a tool for simulated annealing, starting with the X-ray structure. Three distance constraints involved in the hydrogen bondings, as also found in the crystal state, and four distance constraints derived from the NOE experiments were used to show that this solution structure of astin B is consistent with the experimental data.[16] The program used for the MD calculations and the refinements of astin B were obtained from the AMBER 3.0 A program package.[17] All calculations were performed on IRIS 4-D work station. A simulation was performed using a time step of 1 fs, and the structures were sampled every 90 fs. Each system was equilibrated for 5400 fs with a thermal bath at 500K, and thereafter, successively, for 900 fs with a thermal bath 10 K lower in temperature, until a final temperature of 50 K was obtained. Twenty cycles are performed, giving a total simulation time of 126 ps, and each freezed

Table 3 The calculated backbone dihedrals in astin B (1) by vicinal NH-CαH coupling constants, energy calculations and X-ray analysis, and those of cyclochlorotine by X-ray analysis

Residue	Dihedral angle	Astin B(1)				Cyclochlorotine
		NH-CαH Couling constant (Hz)	NMR[a]	Calc.[b]	X-ray[c]	X-ray[c]
Pro(Cl$_2$)^1	φ			-79.6	-96.2	-60.4
	ψ			-1.3	7.4	-22.8
	ω			170.8	169.2	176.8
*allo*Thr^2	φ	9.4	-96.9	-82.8	-83.3	-105.4
(Abu^2)	ψ			-156.6	-149.5	5.4
	ω			173.4	-171.4	-178.8
Ser^3	φ	4.2	-65.5	-54.0	-69.0	-144.6
	ψ			-27.4	-26.5	-110.3
	ω			179.4	176.9	177.6
β-Phe^4	φ*	6.8	-160.0	-160.9	-159.1	-141.6
	ψ$_1$**			49.7	56.6	63.5
	ψ$_2$**			77.6	87.9	-93.5
	ω			-179.9	-168.2	170.8
Abu^5	φ	3.7	-62.7	-55.0	-71.4	-69.2
(Ser^5)	ψ			137.4	140.7	175.7
	ω			8.1	15.4	-163.3

The amino acids in parenthesis indicate those in cyclochlorotine.

*φ in β-Phe^4=C$_{C=O}$-N-Cβ-Cα

**ψ$_1$ in β-Phe^4=N-Cβ-Cα-C$_{C=O}$, ψ$_2$ in β-Phe^4=Cβ-Cα-C$_{C=O}$-N

a - c) dihedral angles calculated from vicinal NH-CαH coupling constants, energy calculations and X-ray analysis, respectively

Table 4 Distances (Å) between some protons and intramolecular hydrogen bondings estimated from the calculated stable conformer and crystal structure of astin B (1)

Protons		Calcd. conformer	Crystal structure
Pro^1-Hα	Abu^5-Hα	2.387	2.118
*allo*Thr^2-NH	Abu^5-Hα	2.668	2.743
β-Phe^4-NH	β-Phe^4-Hα1	2.466	2.464
β-Phe^4-Hβ	Abu^5-NH	2.506	2.892
Ser^3-NH	*allo*Thr^2-OH	1.843	1.892
*allo*Thr^2-NH	β-Phe^4-CO	2.082	2.409
β-Phe^4-NH	β-Phe^4-CO	2.510	2.473

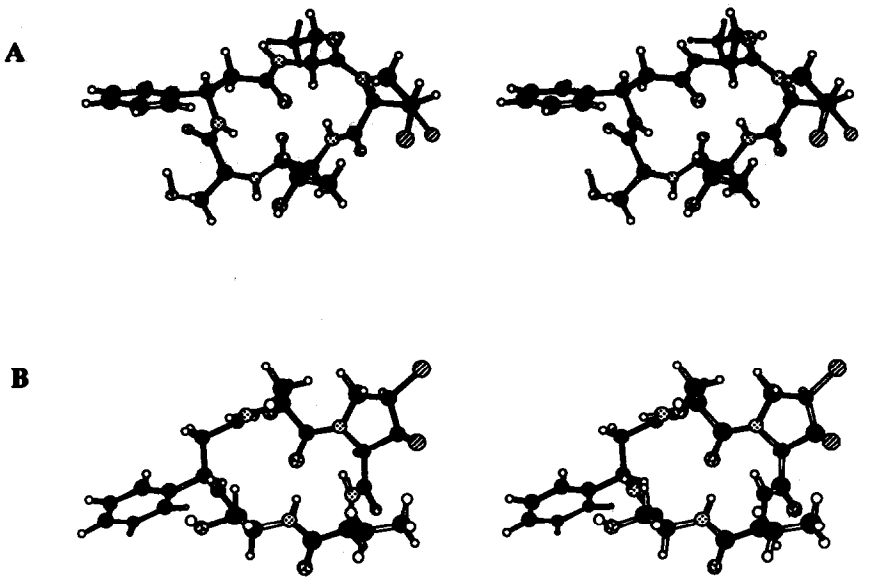

Fig. 3 Stereoscopic view of astin B of the lowest energy conformer (46.231 kcals/mol) given by MD and energy minimizations, and that of cyclochlorotine by X-ray crystallographic analysis[4]; A: the lowest energy conformer of astin B, B: the X-ray structure of cyclochlorotine

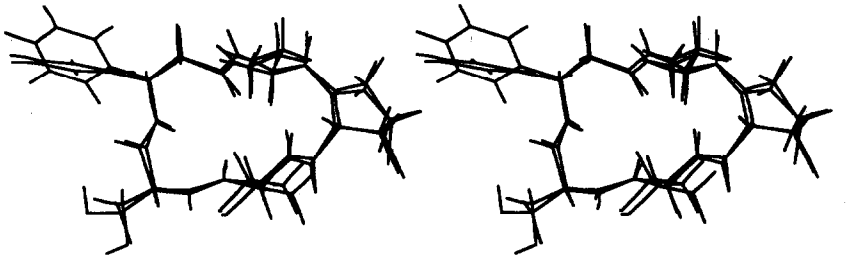

Fig. 4 A stereoview of a superposition of the energy minimized and crystal conformers of astin B (RMSD=0.1700 Å for the best fit of each Cα carbons)

conformation was sampled from the minimum temperature at 50 K and then energy minimized. The resulting structures were characterized in terms of relative energies and conformational properties. A snapshot with the lowest energy (46.231 kcals/mol) was selected as an relevant conformation.

It is obvious from the stereoscopic view of the lowest energy conformation in Fig. 3 and the calculated dihedrals in Table 3 that the conformation is fulfilled for solution conformer, the structure of which involves the turns formed by the residues 5 → 1 and 3 → 4 by stabilization of three intramolecular hydrogen bonds. Furthermore, this conformation is satisfied with the characteristic NOE relationship observed in solution in the following way (See Table 4). The distances between Pro^1-Hα and Abu^5-Hα (2.387 Å *versus* 2.118 Å in the crystal), between *allo*Thr^2-NH and Abu^5-Hα (2.668 Å *versus* 2.743 Å in the crystal), and between β-Phe^4-NH and β-Phe^4-Hα1 (2.466 Å *versus* 2.464 Å in the crystal) were almost identical with those calculated from X-ray data. As can be seen from Table 3, the φ angles in this lowest energy conformer showed a similar propensity to the solution dihedral calculated from vicinal NH-CαH coupling. Furthermore, a side chain dihedral angle (150.0°)[18] of *allo*Thr^2 calculated from vicinal CαH-CβH coupling (9.4 Hz) in ^{1}H-NMR spectrum was almost identical with the energy minimized conformer (-174.3° in the calcd. conformer *versus* 178.1° in the crystal). Despite the fact that conformational freedom of the β-amino acid residue is larger than that of the corresponding α-amino acid, it seemed that the allowed degrees of β-Phe were surprisingly restricted.[19]

The above comparisons indicate that our calculations find crystal conformations to lie within a reasonable energy range relative to the global minima of astin B, as shown in Fig. 4 which exhibit a superposition of the energy minimized and crystal conformers. The qualitative closeness between the crystallographic data and the results of our calculations are suggested. However, the intramolecular hydrogen bondings, especially the one between *allo*Thr^2-NH and β-Phe^4-CO, being present in astin B in solution may be appreciably stronger than those in solid state, judging from the above calculation, slow hydrogen-deuterium exchange rate and NOE correlation described above (see Table 4).

On the other hand, the analogous cyclic pentapeptide, cyclochlorotine[4] adopted a stable type I β-turn structure between $Pro(Cl_2)$ and Abu with a trans proline amide bond and a transannular hydrogen bond as shown in Fig. 3. Table 3 show the backbone dihedral angles in cyclochlorotine, taking a quite different conformation from that of astin B in solid states. S. Lee et al. have reported that a synthetic cyclochlorotine analog took a similar solution conformation to cyclochlorotine in the solid state.[20] In this study, direct comparison of astin B to cyclochlorotine could not be conducted. It is interesting to analyze the conformational homogeneity between astin B and cyclochlorotine, and the antitumour activity of cyclochlorotine.

Experimental

Proton and carbon NMR spectra were recorded on a Bruker spectrometer (AM500) at 303K and processed on a Bruker data station with an Aspect 3000 computer. The 10 mg sample of astin B in a 5 mm tube (0.5 ml [$^2H_6$]DMSO, degassed) was used for the homonuclear and heteronuclear measurements. NOESYPH experiments were made with a mixing time of 0.6s. The NMR coupling constants (*J*) are given in Hz. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec. The temperature effect of the amide hydrogen chemical shift was recorded in five intervals over the range 300 - 330 K in [$^2H_6$]DMSO.

Materials

Roots of *Aster tataricus*, used in this experiment, were purchased from Uchida Wakanyaku Co. in Japan and astin B was isolated according to the previous procedure.[2)]

Simulated annealing calculation

Computer modeling and all calculations were performed using the molecular-modeling software SYBYL ver. 6.03 (Tripos Associates, St. Louis, MO) on an IRIS 4-D work station. Initial calculations started with coordinates for the X-ray structure of astin B. Molecular mechanics and dynamics calculations were performed with the AMBER force field.[17)] The dielectric constant (ε) was assumed to be proportional to interatomic distances (r) as ε=r. Solvent molecules were not included in the calculations. Considering the NOE between Pro^1-Hα and Abu^5-Hα, between alloThr^2-NH and Abu^5-Hα, between β-Phe^4-NH and β-Phe^4-Hα1 and between β-Phe^4-Hβ and Abu^5-NH, and three intramolecular hydrogen bondings of Ser^3-NH - *allo*Thr^2-OH, *allo*Thr^2-NH - β-Phe^4-CO and β-Phe^4-NH - β-Phe^4-CO, constraint minimizations and dynamics were calculated with an extra harmonic term of the form $E= \Sigma K (r-r_{max})^2$ for $r > r_{max}$ and E=0.0 for $r < r_{max}$ added to the force field. A simulation was performed using a time step of 1 fs, and the structures were sampled every 90 fs. Each system was equilibrated for 5400 fs with a thermal bath at 500K, and thereafter, successively, for 900 fs with a thermal bath 10 K lower in temperature, until a final temperature of 50 K was obtained. Twenty cycles are performed, giving a total simulation time of 126 ps, and each freezed conformation was sampled from the minimum temperature at 50 K. The snapshots from the minimum temperature at 50K were then energy minimized with the AMBER force field. A snapshot with the lowest energy was selected as an relevant conformation.

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