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Conformational Analysis of a Tyrosinase Inhibitory Cyclic Pentapeptide, Pseudostellarin A, from *Pseudostellaria heterophylla*[1)]

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Abstract: Conformational studies of pseudostellarin A were performed by a combination of high field NMR and computational chemical evidences to elucidate a relationship between the structures and their tyrosinase inhibitory activities in a series of cyclic peptides, pseudostellarins, and to establish a pharmacophore model. The three-dimensional structure of pseudostellarin A was characterized by γ- and β-turn structures, fixed by trans-annular hydrogen bonds between Gly and Leu.

Introduction

Recently, a number of naturally occurring cyclic peptides with unique structures and biological activities have been isolated. In our investigation of bioactive cyclic peptides from higher plants,[2)] we have isolated several cyclic peptides, called pseudostellarins, from the roots of *Pseudostellaria heterophylla* (Caryophyllaceae), showing potent tyrosinase and melanin-production inhibitory activities.[3)] Though these pseudostellarins have different number of different amino acids in different arrangement from each other, they showed the same enzyme inhibitory activity on tyrosinase.

Cyclic oligopeptides are often experimental models for the studies on the structure-biological activity relationships, because their cyclic structures limit the conformational flexibility of the peptide backbones.[4)] In order to elucidate the mechanisms involved in the action of these cyclic peptides, detailed knowledge of their conformational characteristics is required. Therefore, first, we undertook conformational analysis of these cyclic peptides by the spectroscopic examinations. Pseudostellarins are neutral, cyclic peptides consisting of five to nine lipophilic amino acids containing proline and

aromatic residues.[3] On the basis of detailed and exact knowledge of the structures of pseudostellarins in solution under different environmental conditions, the structure-activity relationships may be assuredly discussed.

Of these pseudostellarins, pseudostellarin A, cyclo[Gly-Pro-Tyr-Leu-Ala], is a cyclic pentapeptide, shown in Fig. 1, with reduced molecular flexibility and the number of constituted amino acids is less than those in the other pseudostellarins. Therefore, conformational analysis of pseudostellarin A in solution conducted by the 2D-NMR techniques, temperature effects on the NH protons, NOE experiments and the refinements of the restrained molecular dynamics calculations is most important step for the precise understanding of the structure - activity relationship of tyrosinase inhibitory cyclic peptides. We are also interested in predicting the conformation of the cyclic peptide in a binding site. In this paper, we describe the conformational analysis of pseudostellarin A (1) by a combination of high field NMR and computational chemical methods with the aim of developing a pharmacophore model of tyrosinase inhibitory cyclic peptides.

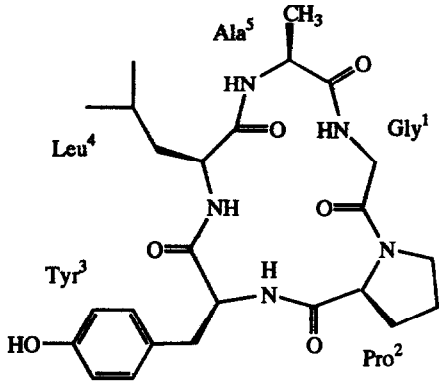

Fig. 1 Structure of Pseudostellarin A (1), Gly was provisionally numbered as the first amino acid.

Results and discussion

Complete assignments of ^1H and ^{13}C NMR signals in DMSO-d_6

Precise knowledge of the conformation of 1 in a polar solvent such as DMSO-d_6 is essential for the studies of the structure-activity relationships and for the design of new derivatives with higher activity. The complete assignments of the signals in various NMR measurements may provide reliable information about the dynamic structures in solution. The assignments of ^1H and ^{13}C-NMR signals of 1, shown in Table 1, were made by the combination of ^1H-^1H COSY, HMQC[5] and HMBC[6] spectra. In spite of the presence of a proline residue, the presence of a single stable conformation in DMSO-d_6 was suggested by the occurrence of well resolved sharp signals and the ^{13}C chemical shifts (δ 29.05 and 24.16) of β and γ positions in Pro residue suggested that the geometry of the proline amide bond was fixed to be *trans*.[7]

Hydrogen bonding

It has been well documented in the literature that effects in temperature have little effect on the chemical shifts of the protons involved in intramolecular hydrogen bonding or which are otherwise shielded from the medium.[8] The temperature effects on the chemical shift of amide hydrogen signals was recorded in ten intervals over the range 300 - 330K in DMSO-d_6, as summarized in Table 2. This result indicated that the NH protons in Gly^1 and Leu^4 were strongly shielded from the solvent, whereas those in Tyr^3 and Ala^5 were exposed to the solvent. Particularly, the NH proton in Gly^1 was involved in a strong intramolecular hydrogen bonding, and the presence of trans-annular hydrogen bonds between Gly^1 and Leu^4 residues was implied. This suggests the presence of a γ-turn which features a seven-membered ring formed *via* a hydrogen bond between the above two residues. The coefficients were almost identical with those which are characteristic of the γ-turn given in the literature.[9]

Table 1. ^1H and ^{13}C NMR Signal Assignments of Pseudostellarin A (1) in DMSO-d_6.

	^1H NMR	^{13}C NMR			
assignment	δ_H (int. mult, J(Hz))	δ_C		δ_H	δ_C
Gly^1			Leu^4		
α	3.47 (1H, dd, 4.2, 15.2)	41.89	α	3.90 (1H, dt, 7.5, 15.0)	53.92
	4.01 (1H, dd, 4.8, 15.2)		β	1.43 (1H, m)	39.81
NH	7.83 (1H, t, 4.9)			1.62 (1H, m)	
C=O		167.62	γ	1.31 (1H, m)	24.38
Pro^2			δ	0.77 (3H, d, 6.5)	22.01
α	4.04 (1H, m)	61.17		0.85 (3H, d, 6.6)	22.43
β	1.51 (1H, m)	29.05	NH	7.91 (1H, d, 7.2)	
	2.03 (1H, m)		C=O		171.50
γ	1.83 (2H, m)	24.16			
δ	3.52 (1H, m)	46.40			
	3.78 (1H, m)				
C=O		170.39[a]			
Tyr^3			Ala^5		
α	4.40 (1H, ddd,7.0,8.9,9.2)	55.23	α	4.07 (1H, m)	48.60
β	2.71 (1H, dd, 8.9, 13.5)	36.74	β	1.25 (3H, d, 7.0)	16.51
	2.81 (1H, dd, 7.0, 13.5)		NH	8.52 (1H, d, 7.6)	
γ		127.26	C=O		172.58
δ	6.93 (2H, d, 8.4)	129.80			
ε	6.63 (2H, d, 8.4)	114.77			
ζ		155.76			
NH	7.22 (1H, d, 9.2)				
C=O		170.32[a]			

a) Assignment may be interchanged.

Table 2 Temperature coefficients ($-d\delta/dT \times 10^3$ ppm/K) of the amide protons in pseudostellarin A

	Gly^1	Tyr^3	Leu^4	Ala^5
Pseudostellarin A	-1.46	3.69	1.17	6.85

Vicinal coupling

Three-bond couplings gave very useful information for the determination of the backbone conformation, because they can directly be converted into dihedral angles *via* Karplus-type equations. The dihedral angles, ϕ in **1**, estimated from the vicinal NH-CαH coupling constants *via* Karplus type equation proposed by Donzel,[10] are shown in Table 4. These dihedral angles resemble those calculated by energy minimizations (See MD and energy minimization section).

Furthermore, the populations of the side chain rotamers were quantitatively assayed by means of homonuclear coupling constants. Though the assignment of each Hβ proton in the side chain of the Tyr^3 was ambiguous, the vicinal coupling constants of 8.9 and 7.0 Hz were observed (Table 1). Using the treatment of Pachler,[11] we calculated the relative populations of the three side-chain rotamers (Fig. 2), the results being shown in Table 3. Thus, the populations of rotamers I and II are almost the same in preference to rotamer III. A different explanation of these predominant rotamers is provided by the T1 relaxation times of the carbon resonances in Tyr^3 (See molecular mobility section) and also supported by ROE enhancements as shown below.

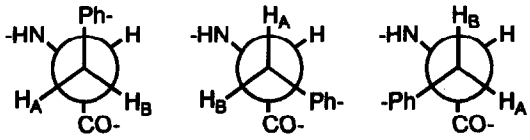

Fig. 2 Rotamers about the Cα - Cβ bond axis for Tyr^3

Table 3. ^{1}H-NMR parameters for Tyr^3 side chain of **1** and the % of rotamers in DMSO-d_6.

Coupling constants (Hz)			Rotamer populations (%)		
J_{AB}	J_{AX}	J_{BX}	I	II	III
13.5	8.9[a)]	7.0[a)]	57.5[b)]	40.1[b)]	2.4

a,b) Each value may be interchanged.

ROE enhancements

The relationship of ROE enhancements in **1** observed by ROESY spectrum[12] is indicated by arrows in Fig. 3. Two important correlations were the ROEs between Tyr^3-Hα and Tyr^3-NH, and between Tyr^3-NH and Pro^2-Hα. These interactions are possible only when the peptide bond between Pro^2 and Tyr^3 takes type II β-turn conformation. Other important correlations observed in the ROESY spectrum were those between Ala^5-Hα and Gly^1-NH, and between Leu^4-Hα and Ala^5-NH, implying the presence of a γ-turn conformation formed by the three residues, Leu^4, Ala^5 and Gly^1. These conformations may be stabilized by the formation of two intramolecular hydrogen bonds between Gly^1 and Leu^4, as mentioned above. Other ROE correlations observed between Gly^1 and Pro^2 residues, and between Tyr^3 and Leu^4 residues also supported the above proposed conformation.

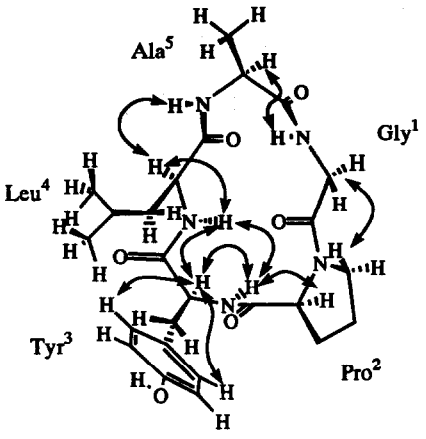

Fig. 3 Some important ROE relationships of pseudostellarin A (1).

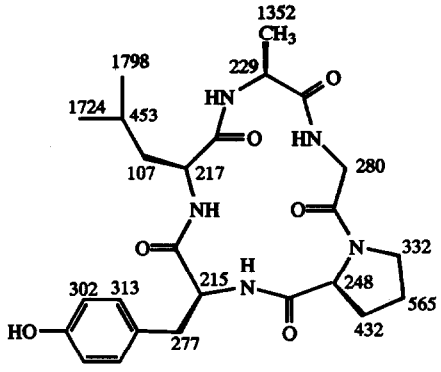

Fig. 4 NT1 values in milliseconds of the carbon atoms of 1 (N=number of attached protons, T1=longitudinal relaxation time).

Molecular mobility

Information about the structural flexibility of this compound can be experimentally obtained from the T1 relaxation times of the carbon resonances. The NT1 values (N=number of attached protons, T1=longitudinal relaxation time) correlate directly with the molecular mobility. This is due to the fact that the ^{13}C relaxation of these carbons is mainly dominated by the single relaxation mechanism, that is, ^{13}C-^{1}H dipolar interaction with directly bonded hydrogens.[13]

The experimental data are given in Fig. 4. These data provide us with information about the rotational motion of the backbone of **1** and about the internal rotations of the side chains. All the α carbons gave very similar NT1 values, indicating the rigidness of the backbone. Interestingly, the NT1 value of the β carbon of Tyr^3 was similar to that of the α-carbon, and was smaller than that of methylene carbon of Pro^2. However, the ortho and meta carbon atoms, which are influenced by the tyrosyl ring rotation, gave larger value than those in fixed tyrosyl rings.[4] These facts about the tyrosyl ring rotation were also supported by the strong ROE correlations between Hδ and Hα of the Tyr^3 residue.

Molecular dynamics and energy minimization

For the purpose of predicting and analyzing complicated conformational features of these cyclic peptides, it is necessary to use a computational method which can give us a result being independent on starting structure. We have already reported that it might be possible to use molecular dynamics techniques as a tool to test simulated annealing.[1,14] The method, applied to a broad class of problems, was also useful for the studies of conformational problems.[15] The starting geometries of

pseudostellarin A for the simulation were modeled by molecular modeling software SYBYL.[16] In order to discriminate between the conformational energy of the pentapeptide ring and that of the Tyr^3 side chain, the following calculation was made with a hypothetical compound in which Tyr^3 was replaced by Ala. Conformation of the Tyr^3 side chain was simulated separately as described below. The distance constraints derived from the ROE experiments were used to show that its solution structure is consistent with the experimental data. Each system was equilibrated for 5400 fs with a thermal bath at 900K and thereafter successively for 900 fs with a thermal bath 10K lower in temperature until a final temperature of 50K was obtained. This temperature was chosen after several trial- and error tests, judging from the arrival at equilibrium between possible conformers. Twenty cycles are performed, and each freezed conformation was sampled from the minimum temperature at 50K. Each low energy conformation was finally minimized by the use of molecular mechanics calculation of AMBER force field.[17] A snapshot with the lowest energy was selected as an relevant conformation. Ten lowest energy conformations have averaged backbone RMSD (STD), 0.03 Å (0.01), relative to the lowest energy conformation. After minimization of the backbone structure, the stable conformer of the side chain of Tyr^3 was obtained by the energy maps calculated by the rotation of $\chi 1$ and $\chi 2$ per 2 degrees. It is clear from the calculated dihedrals for the ten lowest energy conformations shown in Table 4 and the Ramachandran plot shown in Fig. 5 that the conformation of the type II β-turn and γ-turn structures is fulfilled for that in **1**.

Table 4. The calculated backbone dihedrals in **1** by vicinal NH-CαH coupling constants (a) and energy calculations (b).

Residue	Dihedral angle	pseudostellarin A(1)		
		NH-CαH Coupling constant (Hz)	NMR[a]	Calc.[b]
Gly^1	φ	4.9	103.3	132.9(1.6)
	ψ			-154.7(2.9)
Pro^2	φ			-56.4(7.7)
	ψ			102.8(0.9)
Tyr^3	φ	9.2	54.1	56.1(2.3)
	ψ			32.0(4.1)
	χ^1			-46.0
	χ^2			-114.0
Leu^4	φ	7.2	-152.9	-155.0(6.7)
	ψ			-164.9(1.7)
Ala^5	φ	7.6	-89.7	-60.0(0.5)
	ψ			63.0(0.4)

b) Averaged dihedral angles (STD) for ten lowest energy conformations

It is obvious from the stereoscopic view of the lowest energy conformation in Fig. 6 and 7 that the conformation is satisfied with the characteristic ROE relationship and is fulfilled for solution conformer (Table 5 and Fig. 3). Then, the distances between Gly^1-NH and Leu^4-CO, and Leu^4-NH and Gly^1-CO, involving two intramolecular hydrogen bondings were 1.883 and 1.934 Å, respectively, which corresponded to the temperature effects on NH chemical shift as described above.

Table 5. Averaged interatomic distances (Å) involving in the important ROEs and intramolecular hydrogen bondings estimated from the ten lowest energy conformations of 1

Protons		Distances (STD)
Gly^1-NH	Ala^5-Hα	2.481(0.004)
Tyr^3-NH	Pro^2-Hα	2.213(0.017)
Tyr^3-NH	Tyr^3-Hα	2.256(0.004)
Ala^5-NH	Leu^4-Hα	2.520(0.002)
Gly^1-NH	Leu^4-CO	1.883(0.003)
Leu^4-NH	Gly^1-CO	1.934(0.036)

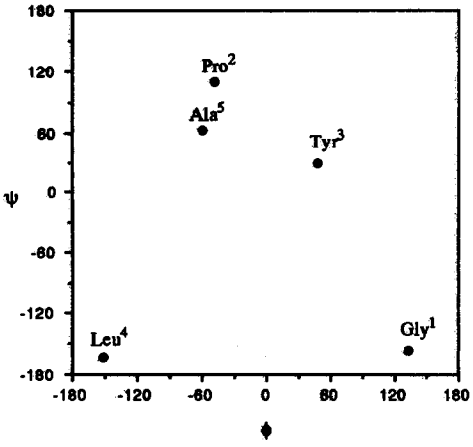

Fig. 5 Ramachandran plot calculated by energy minimization

These findings about the conformation of 1 may be related closely to the mode of action of pseudostellarins which probably interact with specific receptor sites. Studies on the conformation and the biological activity relationship of derived pseudostellarin A and on the precise backbone conformation of the other pseudostellarins are now under investigation.

Experimental

Proton and carbon NMR spectra were recorded on Bruker spectrometers (AM400 and AM500) and processed on a Bruker data station with an Aspect 3000 computer. 15 mg sample of pseudostellarin A in a 5mm tube (0.5ml DMSO-d_6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. ROESY experiments were acquired with mixing times of 90 msec, since no secondary effects were observed at this mixing time. 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.

Materials

The extraction and isolation procedures of 1 from the roots of *Pseudostellaria heterophylla* were performed as described in our previous paper.[3)]

T1 relaxation times (100MHz)

All spectra were recorded on a Brucker AM400 spectrometer at 100.6MHz using proton broad-band decoupling at 303K. The spectra contained 32K data points over a 24KHz frequency range. Relaxation data were obtained by using the inversion-recovery 180-τ-90° pulse sequence. Repetition time between two acquisitions was 30 s for pseudostellarin A (1) in DMSO-d_6. The spin-lattice relaxation times were determined from the relaxation data by using a regression analysis incorporated in the T1 routine of the Bruker acquisition and processing program and given by the expression

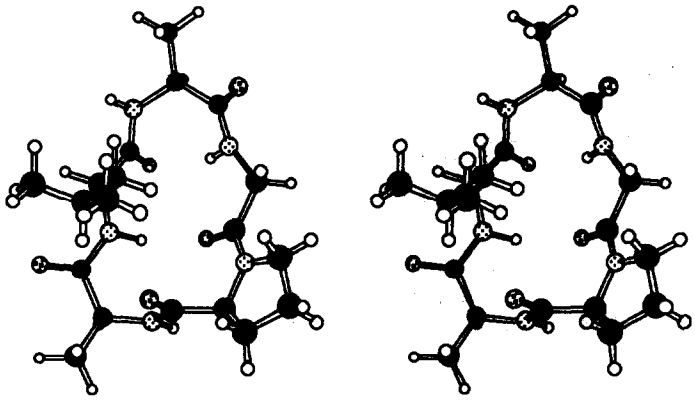

Fig. 6 Stereoscopic view of a stable backbone structure (53.382 kcals/mol) of the hypothetical compound in which Tyr^3 of 1 is replaced by Ala given by MD and energy minimizations

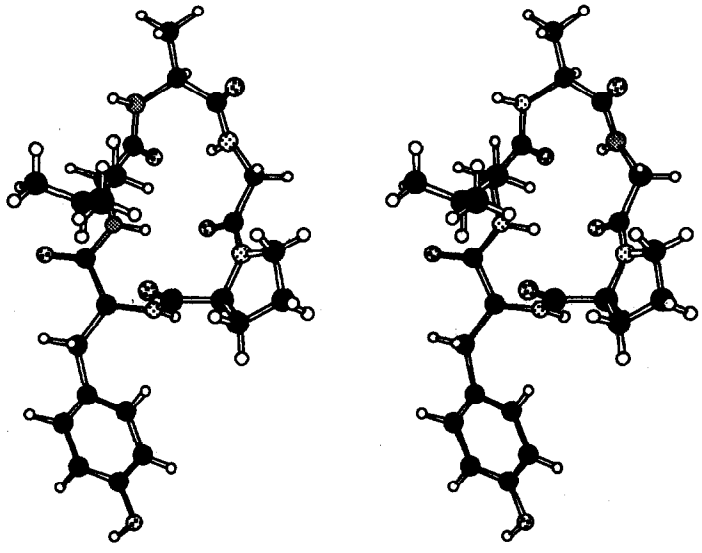

Fig. 7 Stereoscopic view of pseudostellarin A (1) of the lowest energy conformer given by energy map

Y=A3 + A2*exp(-t/T1) in which A3 and A2 are the constants representing the delay times between the 180° and 90° pulses. For the calculation of T1, we used the relative intensities of the ^{13}C signals at 12 different values in an appropriate range. Standard deviations were in the range of 0.008 to 0.030s.

Simulated annealing calculation.

Computer modeling and all calculations were performed by 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 the coordinates modeled by NOE restraint energy minimization using AMBER force field. Molecular mechanics and dynamics calculations were performed with the AMBER force field.[17] The dielectric constant (ε) was assumed to be proportional to the interatomic distances (r) as ε=r. Solvent molecules were not included in the calculations. The NOE relationships involved in the β-turn structure between Tyr^3-Hα and Tyr^3-NH and between Pro^2-Hα and Tyr^3-NH, and those involved in the γ-turn structure between Ala^5-Hα and Gly^1-NH and between Leu^4-Hα and Ala^5-NH, shown in Fig. 3, and two intramolecular hydrogen bondings of Gly^1-NH - Leu^4-CO and Leu^4-NH - Gly^1-CO were taken into account in the calculations of the constraint minimizations and dynamics 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 (K=500). In order to discriminate between conformational energy of the pentapeptide ring and that of the Tyr^3 side chain, calculation was made with a hypothetical compound in which Tyr^3 was replaced by Ala. A simulation was performed by 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 900K, 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 were 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. After the above calculation, the stable conformer involved in the side chain of Tyr^3 was obtained by the energy maps calculated by the rotation of χ1 and χ2 per 2 degrees using a systematic search in SYBYL. Each energy minimization was carried out until the derivatives became less than 0.01 $kcal \cdot mol^{-1} \cdot Å^{-1}$.

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