Structural and Conformational Analysis of Hydroxycyclochlorotine and Cyclochlorotine, Chlorinated Cyclic Peptides from *Penicillium islandicum*

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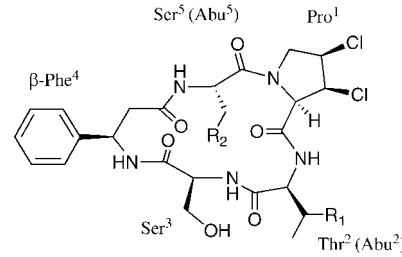
A new chlorinated cyclic pentapeptide, hydroxycyclochlorotine (**1**), has been isolated from *Penicillium islandicum*, and the structure including absolute stereochemistry of **1** and conformational properties of **1** and cyclochlorotine (**2**) in DMSO-d_6 were elucidated by using extensive 2D NMR and chemical means. Hydroxycyclochlorotine (**1**) and astin B (**3**) from *Aster tataricus*, each containing an *allo* threonine at residue 2, have a *cis* proline configuration, whereas cyclochlorotine (**2**) has two conformational states in solution, which may be produced from *cis*−*trans* isomerization of the proline amide bond. The presence of an intramolecular hydrogen bond between Ser^3-NH and a hydroxyl oxygen atom of *allo*Thr^2 may serve to maintain the backbone conformation with a *cis* proline amide bond.

Penicillium islandicum Sopp, a commonly found storage mold occurring on a variety of foodstuffs such as rice and wheat, is known to produce hepatotoxic mycotoxins such as islanditoxin and cyclochlorotine.[1] Cyclochlorotine, whose structure was established by X-ray crystallographic analysis,[2] is a periportal toxic agent causing peripheral damage to the liver lobule[3] and has a strong stabilizing action on actin filaments.[4]

In view of the public health need to establish a practical method for the detection of cyclochlorotine in natural foodstuffs and to protect people against hazards from "yellow rice", we undertook a study on the microbial production of this toxin and isolated a new cyclic peptide, hydroxycyclochlorotine (**1**), together with the known related peptide cyclochlorotine (**2**). Hydroxycyclochlorotine (**1**) presented a single stable conformational state in solution, whereas cyclochlorotine (**2**) exhibited two stable conformational states, one of them similar to that of **1** and the other similar to that of the antitumor cyclic pentapeptide astin B (**3**), isolated from the roots of *Aster tataricus*.[5] This paper describes the isolation and structure elucidation of **1** and conformational analysis of the three related cyclic peptides hydroxycyclochlorotine (**1**), cyclochlorotine (**2**), and astin B (**3**) by using extensive 2D NMR and chemical means.

The filtrate of the broth of *P. islandicum* TSY-1084 was passed through active carbon and then extracted with *n*-BuOH. The *n*-BuOH-soluble materials were subjected to a C_{18} HPLC twice (gradient solvent system: first, 20% → 90% CH_3OH−0.1% formic acid, second, 10% → 50% CH_3CN−0.1% formic acid) to give hydroxycyclochlorotine (**1**, 7.0 mg) as a colorless solid together with cyclochlorotine (**2**, 14.0 mg).

Hydroxycyclochlorotine A (**1**) showed a pseudomolecular ion peak at *m*/*z* 588 $[M + H]^+$, and the molecular formula, $C_{24}H_{32}N_5O_8Cl_2$, was established by HRTOFMS (*m*/*z* 588.1661, $[M + H]^+$, Δ +3.3 mamu). IR absorptions implied the presence of amide NH (3090 cm^{-1}) and amide carbonyl (1660 cm^{-1}) functionalities. Amino acid analysis of the hydrolysates of **1** showed the presence of 1 equiv each of *β*-Phe and Thr and 2 equiv of Ser. Chiral HPLC analysis suggested the presence of Ser, *allo*Thr, and *β*-Phe and showed that all of the amino acids had the L configuration. Analysis of ^1H and ^{13}C NMR data (Table 1) and the HMQC spectrum of **1** revealed the presence of five carbonyl signals (δ_C 166.8, 169.4, 170.1, 170.8, and 172.4) containing a proline-like residue and four amide protons (δ_H 7.46, 8.44, 8.58, and 8.86), indicating the pentapeptide nature of **1**. The last amino acid containing two chlorine atoms was disclosed to be 2,3-dichloroproline by the coupling sequence from the α-proton as shown in Figure 1. This residue can be found in a series of astins isolated from the roots of *Aster tataricus*.[5] The relative orientation of the two chlorines attached to the *β*- and *γ*-carbons in Pro(Cl_2) was established to be *cis* by the similar coupling constants in Pro(Cl_2) of the astins (Figure 1) and the NOE correlation observed between H*β* and H*γ* of Pro(Cl_2) in the NOESY spectrum (Figure 1). In addition, the lack of terminal amino group protons in the ^1H NMR spectrum suggested **1** to be a cyclic pentapeptide.



hydroxycyclochlorotine (**1**: R_1=OH, R_2=OH)
cyclochlorotine (**2**: R_1=H, R_2=OH)
astin B (**3**: R_1=OH, R_2=H)

Analyses of $^1H-^1H$ COSY and HOHAHA spectra led to the assignment of each individual amino acid unit (Table 1). The five peptide bonds in the cyclic backbone and the amino acid sequence were elucidated by correlations found in the carbonyl region of the HMBC spectrum (Figure 2). Thus, the gross structure of hydroxycyclochlorotine (**1**) was elucidated to be a cyclic pentapeptide with a dichlorinated proline residue similar to those of cyclochlorotine[2] and the antitumor cyclic pentapeptides, the astins, isolated from the roots of *Aster tataricus* (Compositae).[5]

To confirm the proposed sequence and elucidate the conformation of hydroxycyclochlorotine (**1**), NOESY correlations were analyzed as shown in Figure 3. The sequence of Pro(Cl_2)^1-Thr^2-Ser^3-*β*-Phe^4-Ser^5 in **1** was supported by NOESY correlations between the amide proton and each adjacent amino acid residue. Furthermore, the NOESY correlation of Pro^1-H*α*/Ser^5-H*α* indicated the cyclic nature and the presence of *cis* geometry of the Pro(Cl_2)^1 amide bond for **1**.

Cyclic peptides are constrained, as they contain turns in the backbones, and these turns are often stabilized by intramolecular hydrogen bonds. NOESY correlations of Thr^2-H*β*/Thr^2-NH and

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Table 1. ^{1}H and ^{13}C NMR Data of Hydroxycyclochlorotine in DMSO-d_6 at 300 K[a]

position	δ_H	δ_C	HMBC
Pro$(Cl_2)^1$			
α	5.24 (1H, d, 4.8)	64.5	
β	5.18 (1H, dd, 4.8, 4.1)	65.1	Pro$(Cl_2)^1$: Hα, H*δ*
γ	4.60 (1H, m)	55.5	Pro$(Cl_2)^1$: Hα, H*δ*
δ	3.39 (1H, dd, 10.8, 10.3)	51.3	Pro$(Cl_2)^1$: H*β*
	4.40 (1H, dd, 10.8, 6.5)		
C═O		166.8	Pro$(Cl_2)^1$: Hα, H*β*; Thr^2: Hα, NH
Thr^2			
α	4.24 (1H, dd, 9.8, 9.6)	57.0	Thr^2: H*β*, H*γ*
β	4.18 (1H, m)	66.3	Thr^2: Hα, H*γ*
γ	1.20 (3H, d, 5.9)	22.2	Thr^2: Hα
C═O		170.1	Thr^2: Hα, H*β*; Ser^3: Hα, NH
NH	8.44 (1H, d, 9.8)		
Ser^3			
α	3.78 (1H, m)	58.7	Ser^3: H*β*
β	3.68 (1H, m)	60.5	Ser^3: Hα
	3.68 (1H, m)		
C═O		169.4	Ser^3: Hα; *β*-Phe^4: NH
NH	8.86 (1H, d, 4.1)		
β-Phe^4			
α	2.12 (1H, dd, 12.6, 12.5)	43.1	*β*-Phe^4: H*β*, NH
	2.73 (1H, dd, 12.6, 4.1)		
β	4.89 (1H, m)	51.4	*β*-Phe^4: Hα, NH
γ		143.1	*β*-Phe^4: H*β*, H*ϵ*
δ	7.29 (2H, m)	125.8	*β*-Phe^4: H*β*, H*ζ*
ϵ	7.29 (2H, m)	128.5	
ζ	7.20 (1H, m)	126.9	*β*-Phe^4: H*δ*
C═O		170.8	*β*-Phe^4: Hα; Ser^5: NH
NH	7.46 (1H, d, 6.9)		
Ser^5			
α	4.46 (1H, m)	54.0	Ser^5: H*β*
β	3.56 (1H, dd, 9.6, 9.6)	61.6	Ser^5: Hα
	3.63 (1H, m)		
C═O		172.4	Pro$(Cl_2)^1$: H*δ*; Ser^5: Hα, H*β*, NH
NH	8.58 (1H, m)		

[a] *δ* in ppm.

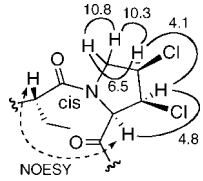

Figure 1. Selected coupling and NOESY correlations in Pro$(Cl_2)^1$ of **1**. Numbers indicate coupling constants in Hz.

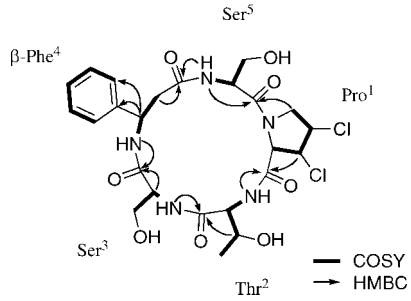

Figure 2. Selected two-dimensional NMR correlations for hydroxycyclochlorotine (**1**).

Ser^3-NH, Thr^2-H*γ*/Thr^2-NH, and Thr^2-Hα/Ser^3-NH indicated that the side chain of Thr^2 does not rotate freely. The conformations of cyclochlorotine (**2**)[2] and astin B (**3**)[5b] from X-ray diffraction studies are shown in Figure 4. In astin B (**3**), which possesses an *allo*Thr residue at the second position, only an intramolecular hydrogen bond between Ser^3-NH and an hydroxyl oxygen atom in the side chain of *allo*Thr^2 was present [N−O of 2.688(17) Å and HN−O of 1.82(16) Å].[5b] On the other hand, cyclochlorotine (**2**) adopted a stable type I *β*-turn structure between Pro$(Cl_2)^1$ and Abu^5 with a

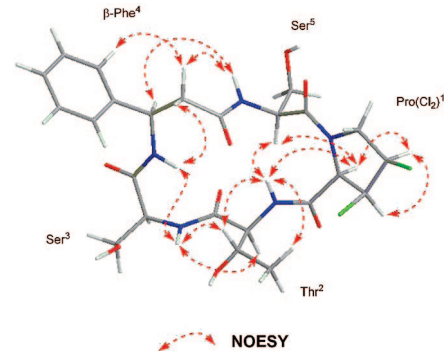

Figure 3. Selected NOESY correlations for hydroxycyclochlorotine (**1**).

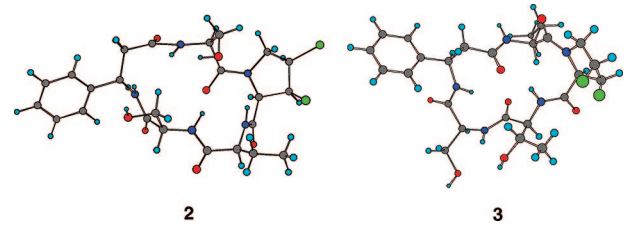

Figure 4. X-ray crystallographic structures of cyclochlorotine (**2**)[2] and astin B (**3**).[3b]

trans proline amide bond and a transannular hydrogen bond, as shown in Figure 5. In this manner, cyclochlorotine (**2**) and astin B (**3**) were found to possess quite different conformations in solid states. In the solution conformation of **1**, the turns were deduced to be formed by the residues 5 → 1 and 3 → 4. The 5 → 1 turn

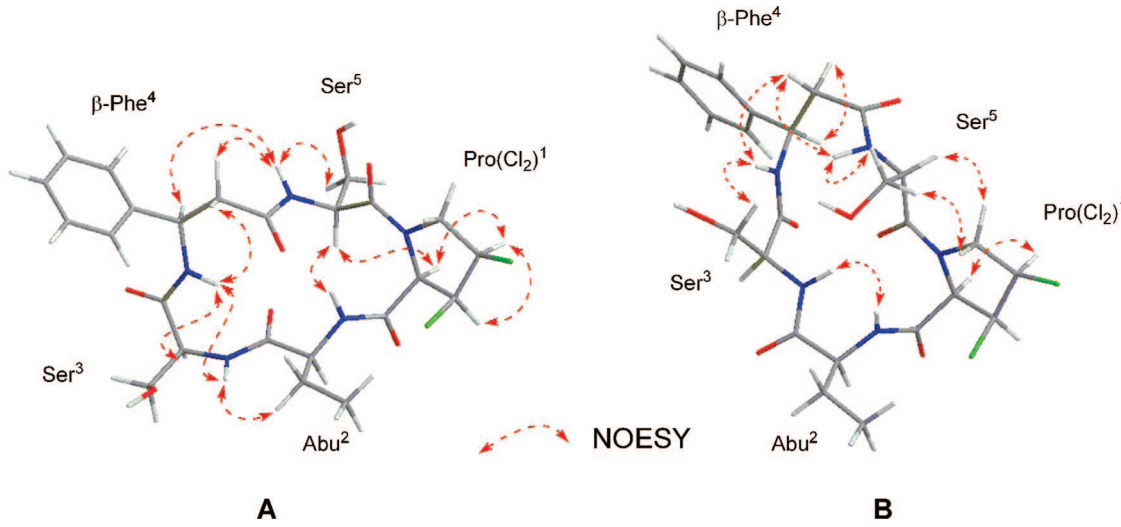

Figure 5. Selected NOESY correlations for two conformers (**A** and **B**) of cyclochlorotine (**2**).

Table 2. ^{1}H and ^{13}C NMR Data of Cyclochlorotine (**2**) in DMSO-d_6 at 300 K[a]

	δ_H		δ_C	
position	major (**A**)	minor (**B**)	major (**A**)	minor (**B**)
Pro(Cl$_2$)^1				
α	5.22 (1H, d, 5.1)	4.74 (1H, d, 4.8)	64.5	66.5
β	5.17 (1H, dd, 5.1, 4.4)	5.05 (1H, m)	63.8	62.1
γ	4.62 (1H, m)	4.95 (1H, m)	55.8	55.3
δ	3.50 (1H, dd, 12.1, 8.9)	3.68 (1H, m)	51.4	50.5
	4.36 (1H, dd, 12.1, 7.3)	4.48 (1H, dd, 10.3, 7.1)		
C═O			166.4	165.9
Abu^2				
α	4.39 (1H, dd, 9.5, 4.8)	4.01 (1H, m)	54.1	55.3
β	1.73 (1H, m)	1.45 (1H, m)	24.4	24.9
	1.94 (1H, m)	1.81 (1H, m)		
γ	0.91 (3H, t, 7.2)	0.86 (1H, t, 7.2)	10.7	11.2
C═O			171.2	171.6
NH	8.02 (1H, d, 9.5)	7.19 (1H, m)		
Ser^3				
α	3.73 (1H, m)	3.99 (1H, m)	59.6	57.8
β	3.68 (1H, m)	3.64 (1H, m)	60.0	59.3
	3.73 (1H, m)	3.72 (1H, m)		
C═O			169.4	167.8
NH	8.06 (1H, d, 5.2)	7.46 (1H, m)		
OH	4.96 (1H, m)	5.05 (1H, m)		
β-Phe^4				
α	2.38 (1H, dd,13.9, 11.7)	2.18 (1H, dd, 12.6, 2.0)	42.1	43.6
	2.66 (1H, dd, 13.9, 4.8)	2.93 (1H, dd, 12.8, 12.6)		
β	4.86 (1H, m)	4.97 (1H, m)	51.0	51.8
γ			142.8	144.0
δ	7.26 (2H, m)	7.34 (2H, d, 7.3)	126.0	126.0
ϵ	7.27 (2H, m)	7.27 (2H, m)	128.4	128.4
ζ	7.19 (1H, t, 7.3)	7.19 (1H, t, 7.3)	126.8	126.8
C═O			170.0	170.7
NH	7.76 (1H, d, 6.5)	8.19 (1H, m)		
Ser^5				
α	4.43 (1H, m)	4.64 (1H, m)	53.2	55.7
β	3.46 (1H, m)	3.75 (1H, m)	62.0	59.9
	3.58 (1H, ddd, 10.3, 5.9, 5.9)	4.14 (1H, m)		
C═O			172.2	170.7
NH	8.20 (1H, d, 4.8)	8.18 (1H, m)		
OH	5.41 (1H, t, 5.9)	5.69 (1H, m)		

[a] *δ* in ppm.

was formed by the *cis*-amide linkage, which was identical with the NOESY data described above. The residue 3 → 4 accommodated the turn by taking on a bent structure similar to type I, which was supported by strong NOESY correlations between *β*-Phe^4-NH/Ser^3-NH and *β*-Phe^4-H*β*. The remaining NOESY correlations observed for **1** also supported the proposed conformation (Figure 3), which was almost identical with the backbone conformation of astin B (**3**) determined by X-ray.[5b]

The NMR data of cyclochlorotine (**2**) have not been reported yet. To elucidate the solution conformation of cyclochlorotine (**2**), complete assignments of the ^{1}H and ^{13}C signals of cyclochlorotine were made by 2D NMR measurements. Surprisingly, two sets of NMR signals appeared in DMSO-d_6 for cyclochlorotine (**2**). The complete assignments of the ^{1}H NMR signals of the major (**A**, 60%) and minor (**B**, 40%) conformers are shown in Table 2. In the major conformer **A**, the chemical shifts and NOESY correlations were

similar to those of hydroxycyclochlorotine (**1**) and astin B (**3**) with a *cis* proline amide bond, whereas in the minor conformer, **B**, NOESY correlations of Ser[5]-Hα/Pro[1]-Hδ and Ser[5]-NH/Pro[1]-Hδ provided evidence of a *trans* proline peptide bond between Ser[5] and Pro(Cl$_2$)[1] (Figure 5). The geometries of the amide bonds including the Pro residue were all defined as *trans* by the NOESY correlations between Hαi and NHi+1 in conformer **B**. It is well known that the ^{13}C chemical shift of the β- and γ-carbons of the Pro residue and the ^{1}H signal of the Pro-Hα possess different values and multiplicity between *cis* and *trans* geometry.[6] However, this trend seems not to apply to both conformers **A** and **B** of cyclochlorotine (**2**), as shown in Table 2. A NOESY correlation between Abu-NH and Ser-NH indicated that conformer **B** took a type I β-turn structure like that of the solid state of cyclochlorotine (**2**).[2]

From the above analysis, hydroxycyclochlorotine (**1**) and astin B (**3**) were found to adopt quite similar backbone conformations with a *cis* amide bond, whereas cyclochlorotine (**2**) exhibited two different conformations in solution, depending on isomerization of the Pro amide bond. The presence of an intramolecular hydrogen bond between Ser[3]-NH and a hydroxyl oxygen atom in the side chain of *allo*Thr[2] may serve to maintain the backbone conformation with a *cis* proline amide bond. Efforts are currently underway to determine the precise backbone conformation and its relationship to biological activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-4 polarimeter. UV spectra were recorded on a Shimadzu UV1600PC spectrophotometer and IR spectra on a Perkin-Elmer 1710 spectrophotometer. ^{1}H and 2D NMR spectra in DMSO-d_6 were recorded on a 600 MHz spectrometer at 300 K, while ^{13}C NMR spectra were measured on a 150 MHz spectrometer. Chemical shifts are reported using residual DMSO-d_5 (δ_H 2.49 and δ_C 39.7) as internal standard. Standard pulse sequences were employed for the 2D NMR experiments. Phase-sensitive NOESY experiments were acquired with mixing times of 750 ms. Mass spectra were recorded on a Waters LCT Premier coupled with UPLC system. Computer modeling was carried out using the molecular-modeling software package SYBYL ver. 7.2.5 (Tripos, Inc., St. Louis, MO).

Fungal Material. *Penicillium islandicum* was identified by one of the authors (Y.S.-K.) and was deposited in the Division of Microbiology, National Institute of Health Sciences.

Extraction and Isolation. The fungus (1×10^5 cells/mL) was grown in Tsunoda broth (6 L) at 25 °C for 40 days. The cultured broth (2.5 L) was filtered. The filtrate of the broth of *P. islandicum* TSY-1084 was passed through active carbon and then extracted by *n*-BuOH. The *n*-BuOH-soluble materials were subjected to a C_{18} HPLC twice (gradient solvent system: first, 20% → 90% CH_3OH−0.1% formic acid, second, 10% → 50% CH_3CN−0.1% formic acid) to give hydroxycyclochlorotine (**1**, 7.0 mg) as a colorless solid together with cyclochlorotine (14.0 mg).

Hydroxycyclochlorotine (1): colorless solid; $[\alpha]^{27}_D$ −63 (*c* 1.0, MeOH); IR (neat) ν_{max} 3090, 1660, 1550, 1410, 1320, and 1030 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); ESIMS *m/z* 588 $[M + H]^+$; HRTOFMS *m/z* 588.1661 (M + H; calcd for $C_{24}H_{32}N_5O_8Cl_2$, 588.1628).

Absolute Configurations of Amino Acids. A solution of **1** (0.1 mg of the peptide) in 6 N HCl was heated at 110 °C for 24 h. After being cooled, the solution was concentrated to dryness. The residue was soluble in water (100 μL), and chiral HPLC analyses were carried out using a Sumichiral OA-5000 column (Sumitomo Chemical Industry, Osaka, Japan; 150 mm; 40 °C, detection at 254 nm). Retention times (min) of authentic amino acids were as follows: L-Ser (11.0), D-Ser (12.0) [eluent: 1 mM $CuSO_4$, flow rate 0.5 mL/min], L-β-Phe (5.5), D-β-Phe (6.0) [eluent: 2 mM $CuSO_4$−2-propanol (90:10), flow rate 1 mL/min], L-Thr (8.0), D-Thr (9.1), L-*allo*Thr (11.1), D-*allo*Thr (12.8) [eluent: 0.5 mM $Cu(CH_3COO)_2$, flow rate 1 mL/min]. Retention times of the hydrolysate of **1** were as follows: L-Ser (11.0), L-β-Phe (5.5), and L-*allo*Thr (11.1).

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