Cyclic Peptides from *Penicillium Islandicum*. A Review and a Reevaluation of the Structure of Islanditoxin

Anil C. Ghosh and Malathi Ramgopal*

SISA Incorporated, and Sheehan Institute for Research, Inc., 763D Concord Ave., Cambridge, Massachusetts 02138

Received March 20, 1980

Dedicated to Professor John C. Sheehan on the occasion of his sixty-fifth birthday.

The structures of cyclochlorotine and islanditoxin, cyclic peptides from *Penicillium islandicum* are reviewed. The peptide A isolated in these laboratories has been identified by gc/ms studies as cyclochlorotine. It is suggested that the structure of islanditoxin [cyclo-(L-dichloroprolyl-D-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyryl-L-seryl-L-serine)] **1** should now be revised to that of cyclochlorotine, [cyclo-(L-dichloroprolyl-D-β-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine)] **2**.

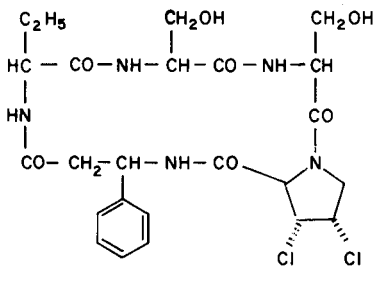
J. Heterocyclic Chem., **17**, 1809 (1980).

Sir:

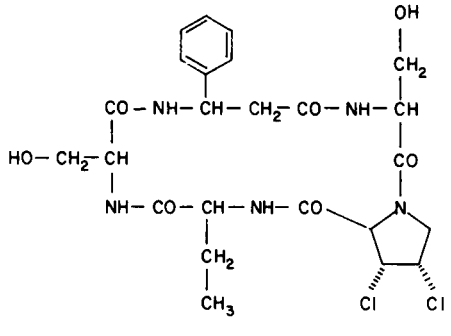
In 1955, Marumo and Sumiki reported the isolation of a toxic cyclic peptide islanditoxin (m.p. 250-251° dec.) from the commonly found storage mold, *Penicillium islandicum* (1-5). After extensive degradative studies, the structure of this unusual peptide was determined as cyclo-(L-dichloroprolyl-D-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyryl-L-seryl-L-serine) **1** (Figure 1). The minimum lethal dose of islanditoxin was found to be 3 mg./kg. in mice (S.C.). Almost simultaneously, Tatsuno and co-workers (6) announced the isolation of another water-soluble mycotoxin, cyclochlorotine (m.p. 251° dec.) from *P. islandicum*. This compound was found to be isomeric with islanditoxin and the structure was established as cyclo-(L-dichloroprolyl-D-β-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine) **2**. The amino acid sequencing of the peptide was confirmed by degradation as well as by x-ray crystallography (7-10). When injected into mice, cyclochlorotine (LD_{50}, mice, 0.475 mg./kg., i.p.) produced violent symptoms such as necrosis, vacuolation of liver cells and development of blood lakes (7,11).

A closer look at the structures and properties of islanditoxin and cyclochlorotine reveals several interesting features. Elemental analysis and physical properties of the two toxins are almost identical. Both structures contain an unusual *cis*-dichloroproline unit. Acid hydrolysis of both preparations led to a mixture of L-serine, α-aminobutyric acid, β-amino-β-phenylpropionic acid (β-phenylalanine), and an unknown substance. The last compound was eventually identified as having been derived from the *cis*-dichloroproline unit. Treatment of islanditoxin with ammonia provided dehydrochlorinated islanditoxinic amide (3) (m.p. 268-273°) (Figure 2). An isomeric dehydrochlorinated cyclochlorotinic amide (4) (m.p. 270-273°) was obtained upon treatment of cyclochlorotine with ammonia. Both of these amides showed uv absorption at 268 nm.

The occurrence of *P. islandicum* on a variety of foodstuffs including rice, wheat, corn, bread, flour, white peppercorns, soybeans, peanut and beans has attracted wide attention to this mold as a potential human health hazard (8,12,13). However, during the past twenty-five years, a major problem confronting research on toxins such as cyclochlorotine and islanditoxin has been the scarcity of these water-soluble peptides. The extremely



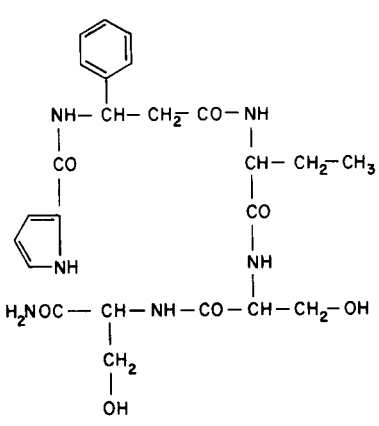
ISLANDITOXIN (1)



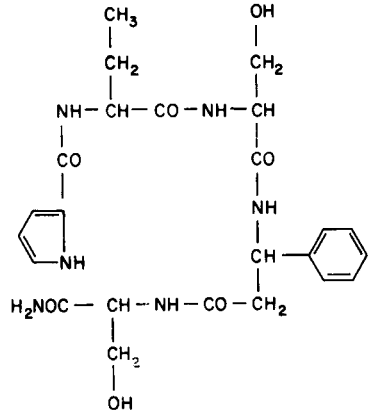
CYCLOCHLOROTINE (2)

Figure 1

0022-152X/80/081809-04$02.25



Dehydrochlorinated Islanditoxic Amide (3)



Dehydrochlorinated Cyclochlorotine Amide (4)

Figure 2

Table I

Compound	Melting Point	UV λ max	IR μ max (cm^{-1})	$(\alpha)_0^{24}$
dehydrochlorinated cyclochlorotinic amide (derived from cyclochlorotine)	270-273° dec.	268 ($\epsilon = 1.42 \times 10^4$)	3430, 3270, 1670, 1645, 1620, 1540	
dehydrochlorinated cyclochlorotinic amide (synthetic)	270-272°	268 ($\epsilon = 1.57 \times 10^4$)	3430, 3270, 1675, 1650, 1620, 1540	+ 2.5° (c = 0.787, DMF)
dehydrochlorinated islanditoxinic amide (derived from islanditoxin)	268-273°	268 ($\epsilon = 1.4 \times 10^4$)		
dehydrochlorinated islanditoxinic amide (synthetic)	254-256°	268 ($\epsilon = 1.69 \times 10^4$)	3460, 3330 1690, 1660, 1635, 1555, 1530	- 5.7° (c = 1.059, DMF)

low yield of the toxins in the culture broth (1.5 mg.-10 mg./litre), their instability, and difficulties involved in their isolation have prevented extensive toxicological research on these peptides (14,15).

A few years ago, in these laboratories, we initiated a systematic study of the isolation, chemistry and biology of metabolites of *P. islandicum* (12,16-19). A large number of quinonoid and non-quinonoid metabolites were isolated, many of which were found to possess interesting antimicrobial and antitumor activities (20). For the production of the toxic peptides, both liquid fermentations as well as solid state fermentations were used (17). More than twenty strains of *P. islandicum* were screened using 10 different grains as substrate. Fermentations were carried out under both static and shaking conditions to achieve the best yield of the chlorine-containing peptides.

Our initial studies on the production of the cyclic peptide by *P. islandicum* were hampered by the lack of any suitable method for detection of these compounds. A previous method for the determination of cyclochlorotine involved tedious fractionation, ammonolysis and ultraviolet photometry (14,15). We have developed a new thin layer chromatographic method for the detection of the peptide from *P. islandicum*. The technique is based upon the color reaction of the peptide with the chlorine-*o*-tolidine reagent (18). Two spots with values of Rf 0.7 (peptide-A), and Rf 0.4 (peptide-B) were detected, indicating that at least two peptides were being produced by *P. islandicum* under our fermentation conditions.

In these studies, the best yield of the purified chlorine-containing peptide was obtained by using *P. islandicum* WF-38-12 and red wheat as the substrate. Whereas peptide-A (Rf = 0.7) melted at 254-255°, the melting points of cyclochlorotine and islanditoxin have been reported to be 250° and 250-251°, respectively. Peptide

B (Rf = 0.4) melted at 215°. Since this compound was clearly different from the known cyclic peptides, it was designated as a new compound and named simatoxin (17). In view of the discrepency in the literature, it was necessary to determine if peptide-A was cyclochlorotine or islanditoxin or a new compound.

Since the purified peptide-A could be isolated only in milligram quantities, it seemed that mass spectrometry would be the best method for the identification of the compound. Application of this powerful tool for the determination of the amino acid sequence of small peptides was first demonstrated by Biemann (21-23). While this method has been utilized in the amino acid sequencing of a large number of small peptides, determination of structures of cyclic peptides presents difficult problems. The interpretation of mass spectra of such peptides is often complicated by the possibility that transannular bond formation either prior to or after ionization, followed by cleavage after electron impact, may take place in such molecules. This can lead to fragments containing two or more amino acids which were not adjacent in the original structure. Also, since neither cyclochlorotine nor islanditoxin has any defined *N*-terminus or *C*-terminus, the sequencing could not be confirmed by conventional sequencing methods such as Edman technique.

The peptide-A was therefore identified by a combination of high resolution mass spectrometry and field desorption mass spectrometry on the intact sample and gas chromatographic mass spectrometry (gc/ms) on partial hydrolyzates thereof (24).

The expected molecular ion corresponding to 571 ($C_{24}H_{31}Cl_2N_5O_7$) for cyclochlorotine or islanditoxin could not be found in the high resolution mass spectra of our chlorine-containing peptide (Rf 0.7). The ion of highest mass was observed at 499.1616, which corresponded to $C_{24}H_{26}ClN_5O_5$ (loss of 2 H_2O + HCl from the molecular ion). A low resolution mass spectrum using field desorption also failed to show any molecular ion although an ion was observed for $(M\text{-}HCl)^+$. Mass spectra of the peptide diacetate, however, showed a protonated molecular ion at m/z 656, indicating that our peptide could be isomeric or identical to either cyclochlorotine or islanditoxin.

While high resolution mass spectra as well as field desorption mass spectra were useful in determining the molecular formula of peptide-A, no conclusive information could be obtained on its amino acid sequence. A combination of nonspecific cleavage by partial acid hydrolysis, derivatization and gc/ms was used to determine the sequence. Hydrolysis of 0.8 mg. of the peptide was carried out using 6*M* hydrochloric acid at 105° for 20 minutes in an evacuated, sealed tube. This was followed by lyophilization, methylation, trifluoroacetylation, reduction and silylation (Fig. 3). The resulting product was analyzed by gc/ms using a Perkin-Elmer Model 990 gas chromatograph equipped with a flame ionization detector, which was coupled via a fitted glass Watson-Biemann separator to a Hitachi-Perkin Elmer RMU-6L mass spectrometer.

A total of five peptide fragments were identified by gc/ms. Reconstruction of the peptide from these fragments established the structure as cyclochlorotine. As shown in Figure 3, the sequence of the L-serine-β-phenylalanine-L-serine was established unequivocally from the mass spectra of the degradation product.

The results from gc/ms also established that peptide-A isolated in our laboratories was not islanditoxin. No fragment corresponding to serine-serine or β-phenylalanine-α-amino-butyric acid could be traced in the mass spectral studies.

These studies also show that gc/ms is a fast and effective method for peptide sequencing. Also, the presence of unusual amino acids such as *cis*-dichloroproline causes no special problem in such sequencing studies.

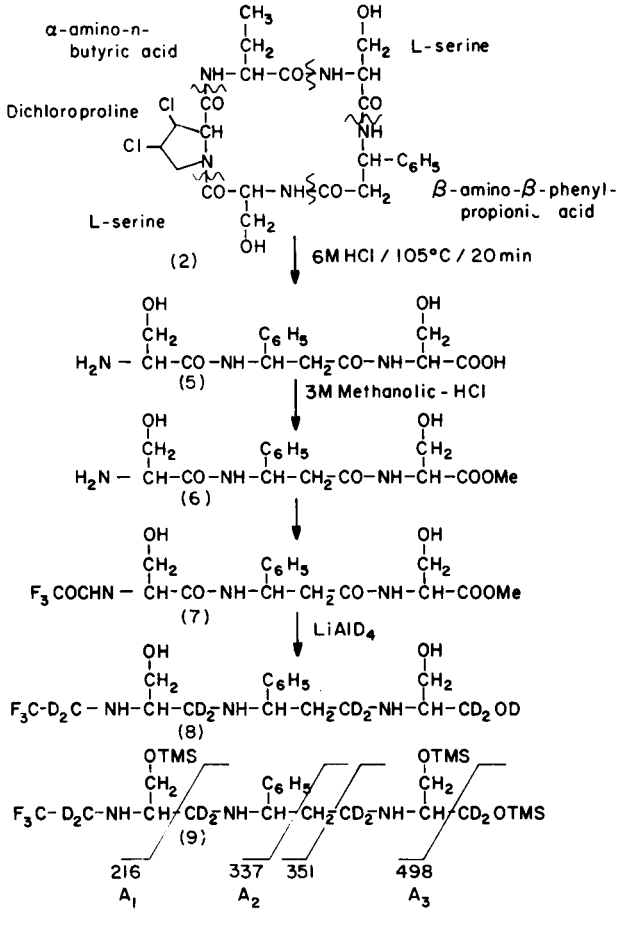


Figure 3

Identification of our chlorine-containing peptide-A as cyclochlorotine now leads to the following question. Is islanditoxin identical with simatoxin or cyclochlorotine? The physical properties of simatoxin indicate that this compound is different from islanditoxin. For example, the melting points of the two compounds are different. While simatoxin shows peak absorption at 262 nm, islanditoxin absorbs at 259 nm. The IR-spectrum of simatoxin (ν max at 3325, 1680, 1660, and 1604 cm^{-1}) is different from that reported for islanditoxin (ν max at 3450, 3270, 1650 cm^{-1}).

In an attempt to isolate islanditoxin, we have carried out a large number of fermentation experiments, using a variety of *P. islandicum* strains. So far, we have been able to detect only two peptides, cyclochlorotine and simatoxin. Tatsuno synthesized both dehydrochlorinated cyclochlorotinic amide and dehydrochlorinated islanditoxinic amide (5,9) and found the two compounds to be different (Table I). This leads one to believe that the structure originally assigned to dehydrochlorinated islanditoxinic amide needs to be reinvestigated. This is further supported by the facts that the physical properties of cyclochlorotine and islanditoxin are similar, and the reported melting points of dehydrochlorinated islanditoxinic amide and dehydrochlorinated cyclochlorotinic amide are almost identical. We therefore suggest the structure of islanditoxin should now be revised to that of cyclochlorotine.

Acknowledgement.

The authors wish to thank their collaborators Professor A. L. Demain and Professor K. Biemann for helpful suggestions. Microbial production and isolation of the peptides were carried out by Mr. J. Townsend, Dr. Manmade and Ms. A. Bousquet. Mass spectral studies were done by Dr. R. Anderegg. This work was supported by the Food and Drug Administration, Washington D.C. (Contract No. 223-74-2209) and the National Science Foundation (Contract No. C-AEN-77-19655 and PRF-7817861).

REFERENCES AND NOTES

(1) S. Marumo and U. Sumiki, *Bull. Agric. Chem. Soc. Japan*, **29**, 305 (1955).

(2) S. Marumo, *ibid.*, **19**, 258 (1955).

(3) S. Marumo, K. Miyao and A. Matsuyama, *ibid.*, **29**, 913 (1955).

(4) S. Marumo, K. Miyao and A. Matsuyama, *ibid.*, **19**, 262 (1955).

(5) S. Marumo, *ibid.*, **23**, 428 (1959).

(6) T. Tatsuno, M. Tsukioka, Y. Sakai, Y. Suzuki and Y. Asami, *Chem. Pharm. Bull.*, **3**, 476 (1955).

(7) K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, O. Yonemitsu, H. Ito, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, *Japan. J. Exp. Med.*, **31**, 19 (1961).

(8) M. Saito, M. Enomoto and T. Tatsuno, in "Microbial Toxins," Vol. VI, A. Ciegler, S. Kadis and S. J. Ajl, Eds., Academic Press, New York, N.Y., 1971, p. 299.

(9) M. Sato and T. Tatsuno, *Chem. Pharm. Bull.*, **16**, 2182 (1968).

(10) H. Yoshioka, N. Nakatsu, M. Sato, and T. Tatsuno, *Chem. Letters*, 1319 (1973).

(11) K. Uraguchi, M. Saito, Y. Noguchi, K. Takahashi, M. Enomoto and T. Tatsuno, *Food Cosmet. Toxicol.*, **10**, 193 (1972).

(12) A. C. Ghosh, A. Manmade and A. L. Demain, in "Mycotoxins in Human and Animal Health," J. V. Rodricks, C. W. Hasseltine and M. A. Mehlman, Eds., Pathotox Publishers, Inc., Park Forest South, Illinois, 1977, p. 625.

(13) S. Shibata, in: Chemistry of Natural Products, vol. 8, p. 109. 8th International Symposium on the Chemistry of Natural Products, New Delhi, India, Butterworth and Co., London 1973.

(14) I. Ishikawa, Y. Ueno, and H. Tsunoda, *J. Biochem.*, **67**, 753 (1970).

(15) K. Ishii and Y. Ueno, *Appl. Microbiol.*, **26**, 359 (1973).

(16) A. C. Ghosh, A. Manmade, B. Kobbe, J. M. Townsend and A. L. Demain, *Appl. Environ. Microbiol.*, **35**, 563 (1978).

(17) A. C. Ghosh, A. Manmade, J. M. Townsend, A. Bousquet, J. F. Howes and A. L. Demain, *ibid.*, **35**, 1074 (1978).

(18) A. C. Ghosh, A. Manmade, A. Bousquet, J. M. Townsend and A. L. Demain, *Experientia*, **34**, 819 (1978).

(19) A. A. Stark, J. M. Townsend, G. N. Wogan, A. L. Demain, A. Manmade, and A. C. Ghosh, *J. Environ. Path. Toxicol.*, **2**, 313 (1978).

(20) A. C. Ghosh, J. M. Townsend, N. A. Solomon, A. Manmade and A. L. Demain, *ibid.*, (in press).

(21) K. Biemann, F. Gapp and J. J. Seibl, *J. Am. Chem. Soc.*, **81**, 2274 (1959).

(22) K. Biemann, *Chimia*, **14**, 393 (1960).

(23) K. Biemann, in "Biochemical Applications of Mass Spectrometry", 1st Supplementary Volume, G. R. Waller and O. C. Dermer, Eds., J. Wiley and Sons, Inc., New York, N.Y., 1980, p. 46.

(24) R. A. Anderegg, K. Biemann, A. Manmade and A. C. Ghosh, *Biomed. Mass. Spectrom.*, **6**, 129 (1979).