Roseocardin, a Novel Cardiotonic Cyclodepsipeptide from *Trichothecium roseum* TT103

AKINOBU TSUNOO*, MASAYUKI KAMIJO, NAOKI TAKETOMO, YOSHIO SATO and KATSUMI AJISAKA

Meiji Institute of Health Science, Meiji Milk Products Co., Ltd., Odawara, Kanagawa 250, Japan

(Received for publication August 20, 1997)

A new cyclodepsipeptide, designated roseocardin, was isolated from the culture broth of *Trichothecium roseum* TT103. Roseotoxin B and destruxins A and B were also isolated during the same procedure. The structure of roseocardin was determined by EI-MS, NMR and X-ray crystallographic analysis. Roseocardin as well as the other cyclodepsipeptides were shown to produce positive inotropic effects on rat heart muscles.

There have been only a few reports in the literature of cardiotonic compounds isolated from microorganisms. The mycelial extract of *Cordyceps sinensis* has a cardiotonic effect on the right atrium of rats.[1,2] However, this effect is not potent enough for the main ingredients to be purified. A substantial cardiac stimulant effect has been found in the culture broth of *Trichothecium roseum* TT103. A new cyclodepsipeptide, roseocardin, as well as roseotoxin B, and destruxins A and B, have been found to possess a cardiotonic action. The structural characteristics of the compounds are unique, differing greatly from those of other known cardiotonic agents. In the present study, we describe the isolation, structural determination and biological activity of roseocardin.

Materials and Methods

Fermentation

The strain TT103 is from our stock cultures which have been collected from various regions of Japan. Spores (2.3×10^5) of the fungi were inoculated into a culture medium (1.5 liters) and cultured at 25°C for 4 days. The medium contained 2 g of yeast extract (DIFCO) and 20 g of molt extract (OXOID) per liter. The pH of the medium was adjusted to 5.5 with conc. HCl.

Isolation

The pooled broth (15 liters) was incubated with 3 liters of HP-20 resin (Mitsubishi Kasei Corporation). The resin was washed with water and then eluted with MeOH. The MeOH was removed *in vacuo* leaving a brown solid (12.6 g). The crude material was dissolved in 400 ml of water and the solution acidified to pH 3.0 with conc. HCl. The solution was extracted three times with the same volume of EtOAc. Evaporation of the solvent gave 1.0 g of a crude residue. The residue was applied to a silica gel column of 50 g (Wakogel C-300, Wako) and washed with EtOAc-hexane, 1 : 1 (v/v), and the active fractions were eluted with EtOAc. Evaporation of the solvent gave 270 mg of a crude residue. The residue was dissolved in MeOH and the cardiotonic fractions were separated by HPLC. The chromatography was carried out on a Capcellpack C18 column (i.d. 20×250 mm, Shiseido) equilibrated with H_2O-CH_3CN-CH_3COOH, 630 : 70 : 0.6 (volume ratio). The acetonitrile gradient was run as follows: 10~40% during 0~10 minutes, 40% during 10~30 minutes and 40~50% during 30~50 minutes. The flow rate was 10 ml/minute and the elutant monitored at UV 220 nm.

NMR Spectra

^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were obtained by Varian Unity 500 NMR spectrometer. Tetramethylsilane was used as an internal standard (0 ppm).

X-Ray Crystallography

Crystals of a fraction IV (see Results) were formed from a MeOH-H_2O solution. A colorless crystal having the approximate dimensions $0.09 \times 0.14 \times 0.36$ mm was mounted on a glass fiber. All measurements were made on an Enraf-Nonius CAD4R diffractometer with graphite monochromated Cu-Kα radiation. Cell constants and an orientation matrix for data collection were obtained from a block diagonal least-squares refinement

using the setting angles from 25 carefully centered reflections in the range $30° < \theta < 32°$. The reflection data were collected at 19°C using the ω-2θ scan technique to a maximum 2θ value of 136°. Scans were performed at a speed of 4°/minute (in omega). A total of 2492 reflections was collected. The structure was solved by the direct method using a *MULTAN78* and the Monte Carlo method. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.25 and -0.14 $e\lambda^{-3}$, respectively. All calculations were performed using *UNICS*-III program system software.

Examination of Effects on Heart Muscles

Male rats of the Sprague-Dawley strain (200~400 g) were decapitated, and the right atria isolated. The preparation was mounted in a horizontal bath of 0.8 ml and perfused at a rate of 3.5~4.0 ml/minute with a salt solution of the following composition (mM): NaCl, 140; KCl, 5; $CaCl_2$, 2.6; $MgCl_2$, 1.3; HEPES, 5; and glucose, 10, as reported previously[1,2]. The perfusion solution was aerated. The pH of the solution was 7.3~7.4. The dissected right atria exhibited automatic contractions. Contractions were recorded isometrically through a strain gauge transducer (IM-300, Physioteck, Tokyo) connected to the preparation. It was examined whether the isolated compounds affected the contraction force (inotropy) and the intercontraction interval (chronotropy).

Results

Isolation

The crude active fractions eluted from the silica gel column were separated by HPLC on a Capcellpack C18 column. Four active fractions (**I**, **II**, **III** and **IV**) which are marked by arrows in Fig. 1 were collected. The acetonitrile in fractions was removed by evaporation. The active compounds were extracted with EtOAc from the aqueous solutions. Evaporation of the solvent gave compounds **I**, **II**, **III** and **IV** in amounts of 12.4, 86.3, 21.0 and 46.1 mg, respectively. The structure of compound **IV** is shown in Fig. 2.

Structural Determination of Compound IV

The ^{13}C NMR, ^{1}H NMR, TOCSY, HMQC, HMBC and NOESY spectra of the compound **IV** were measured in $CDCl_3$. Analyses by ^{13}C NMR and HREI-MS indicated a molecular formula of $C_{31}H_{53}N_5O_7$. All proton and carbon signals were assigned as listed in Table 1. The ^{1}H NMR spectrum of **IV** is shown in Fig. 3.

The sequence of the components was determined by HMBC and NOESY experiments as shown in Fig. 4. The HMBC correlations were observed between H-1 and 31-C, between the NH of Ile and 31-C, between H-7 and 6-C, between H-8 and 6-C, between H-13 and 12-C, between H-14 and 12-C, between the NH of β-Ala and 16-C, and between H-20 and 19-C. Furthermore, NOESY correlations were observed between the NH of Ile and H-2, between the NH of Ile and H-26, between the NH of Ile and H-30, between H-1 and H-7, between H-7 and H-9, between H-7 and H-10, between H-7 and H-11, between H-8 and H-14, between H-13 and H-15, between H-14 and the NH of β-Ala, between the NH of β-Ala and H-17, between the NH of β-Ala and H-18, and between H-20 and H-26. From these results, the structure of compound **IV** was determined as shown in

Fig. 1. HPLC elution profile of cardiotonic compounds.

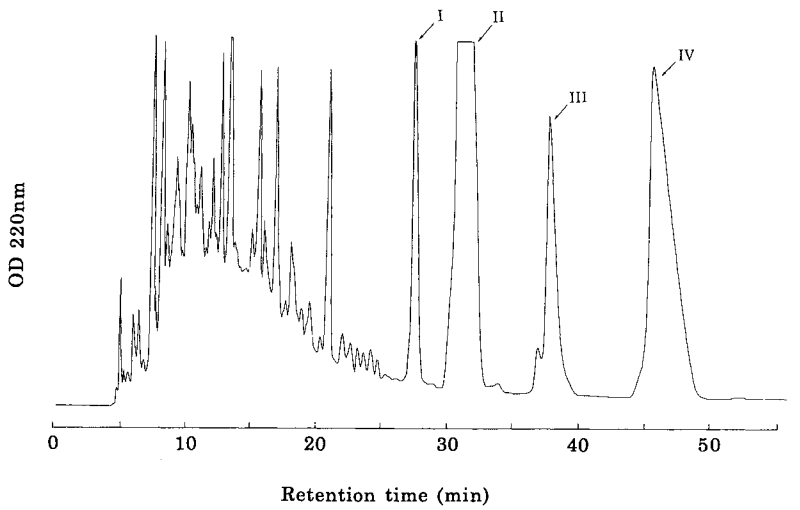


Fig. 2. Structure of roseocardin (**IV**).

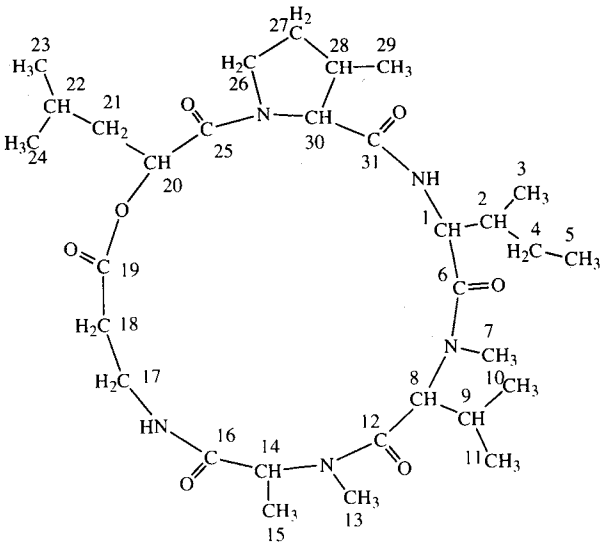


Fig. 2. Compound **IV** has been designated roseocardin.

The physico-chemical properties of **IV** were summarized in Table 2. Compound **IV** was easily soluble in EtOH, MeOH, $CHCl_3$ and DMSO but insoluble in water.

Relative Stereochemistry of Roseocardin (IV)

To confirm the proposed structure of roseocardin (**IV**), X-ray crystallographic analysis was performed. The relative stereochemistry of **IV** was determined as shown in Fig. 5. The crystal data are summarized in Table 3.

Absolute Stereochemistry of Roseocardin (IV)

The absolute configuration of the amino acid residues in **IV** was determined. The complete acid hydrolysates of **IV** were coupled with (+)-1-(9-fluorenyl) ethyl chloroformate and analyzed by column chromatography. The

Table 1. ^{1}H and ^{13}C NMR chemical shifts of roseocardin (**IV**) in $CDCl_3$-d_1.

Moiety	Position	δ_C	δ_H
Ile	1	53.6	4.84 dd
	2	37.4	1.92 m
	3	15.0	0.85 d
	4	24.5	1.45 m, 1.28 m
	5	11.7	0.86 t
	6	173.4	
	1-NH		7.07 d
MeVal	7	30.7	3.22 s
	8	58.4	4.96 d
	9	27.3	2.32 dq
	10	19.5	0.93 d
	11	19.8	0.89 d
	12	171.0	
MeAla	13	28.5	2.73 s
	14	55.8	5.17 q
	15	15.2	1.31 d
	16	169.6	
ß-Ala	17	33.5	4.05 m, 3.08 m
	18	34.0	2.68 m, 2.57 m
	19	173.7	
	17-NH		8.26 d
2-Hydroxyisocaproic acid	20	72.2	4.91 dd
	21	39.0	1.96 ddd, 1.85 m
	22	24.2	1.38 ddd
	23	23.9	1.00 d
	24	21.7	0.95 d
	25	169.8	
3-Methyl-Pro	26	44.9	3.86 dt, 3.57 dt
	27	30.6	2.10 m, 1.70 m
	28	35.7	2.79 m
	29	19.2	1.11 d
	30	65.2	4.27 d
	31	170.8	

Fig. 3. ^1H NMR spectrum of roseocardin (**IV**) (500 MHz, $CDCl_3$).

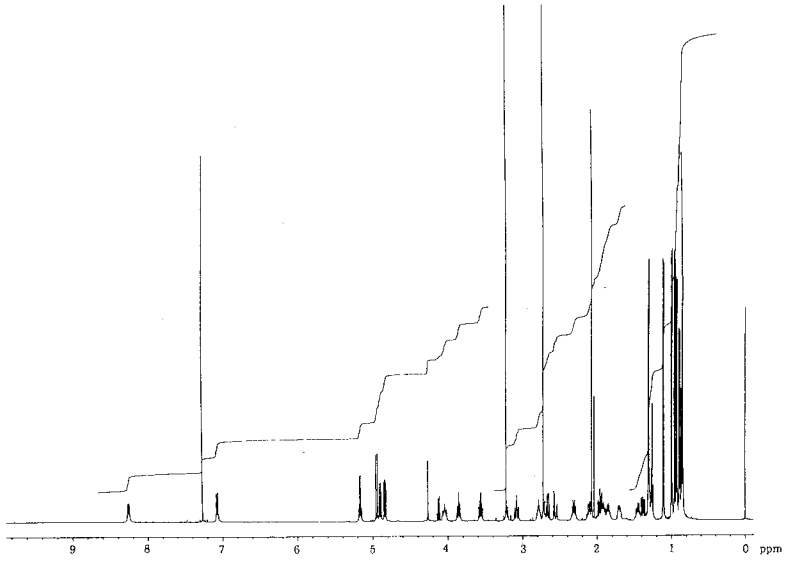
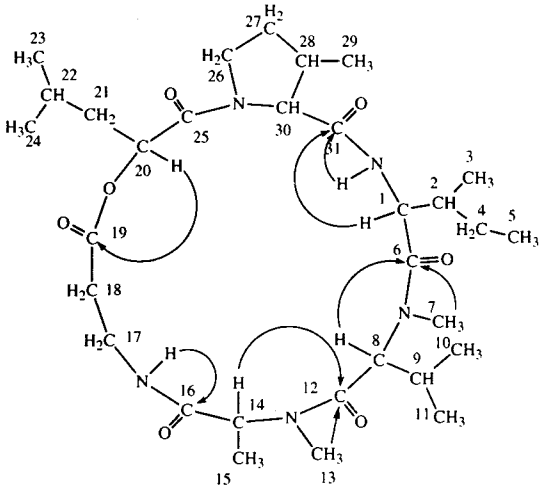


Fig. 4. HMBC (a) and NOESY (b) correlations for roseocardin (**IV**).

(a) HMBC correlations



(b) NOESY correlations

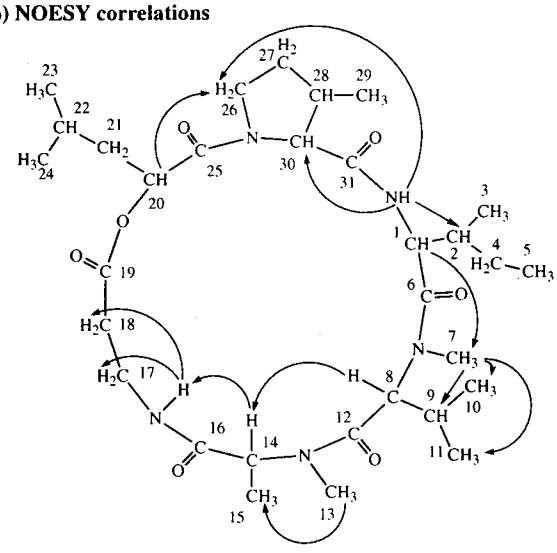


Table 2. Physico-chemical properties of roseocardin (**IV**).

Appearance	Colorless crystal
Molecular formula	$C_{31}H_{53}N_5O_7$
Molecular weight	607.79
HREI-MS	
Found:	607.3964
Calcd:	607.3942
$[\alpha]_D^{27}$ (MeOH)	-206.4 (c 1.00)
UV λmax (MeOH) nm	208

Table 3. Crystal data of roseocardin (**IV**).

Molecular formula	$C_{31}H_{53}N_5O_7$
Molecular weight	607.78
Crystal system	Orthorhombic
Crystal color	Colorless
Space group	$P2_12_12_1$
Unit cell dimensions	
a (λ)	14.836 (1)
b (λ)	21.121 (1)
c (λ)	11.240 (1)
Unit cell volume U ($λ^3$)	3521.8 (4)
Z value	4
D_{calc} ($Mg\ m^{-3}$)	1.146
μ (mm^{-1})	0.63

results indicated that the complete acid hydrolysates of **IV** contained L-Ile and L-MeVal. Thus, the absolute structure of **IV** was determined to be cyclo(-L-Ile-L-MeVal-L-MeAla-β-Ala-2(*R*)-hydroxy-4-methylpentanoyl-*trans*-3-methyl-L-Pro-).

Structure Elucidation of Other Compounds

The structures of the other active compounds were also determined by ^{13}C NMR, ^{1}H NMR, TOCSY and HMQC. The NMR chemical shifts are listed in Table 4. These spectra were similar to those of roseocardin. SI-MS of compounds **I** and **III** displayed molecular ions at *m/z* 578.4 ($M+H^+$) and 594.1 ($M+H^+$), respectively. The HREI-MS of compound **II** displayed molecular ion at *m/z* 591.1. By the assignment of protons and carbons, the compounds **I**, **II** and **III** were identified as destruxin A, roseotoxin B and destruxin B (Fig. 6), respectively. These compounds have previously been isolated from *Trichothecium roseum*[3,4] and *Metarhizium anisopliae*.[5~8]

Inotropic and Chronotropic Effects on Heart Muscles

Biological activities of roseocardin on automatically contracting right atria were examined to determine whether the compound had an inotropic effect to increase the contractile force of a right atrium, and whether it had a chronotropic effect to change the intercontraction interval. As summarized in Table 5, roseocardin

Fig. 5. Crystallized structure of roseocardin (**IV**).

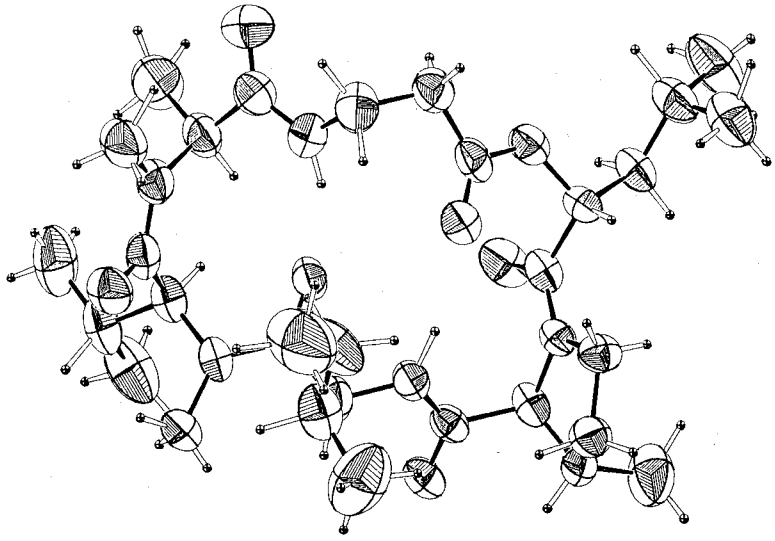
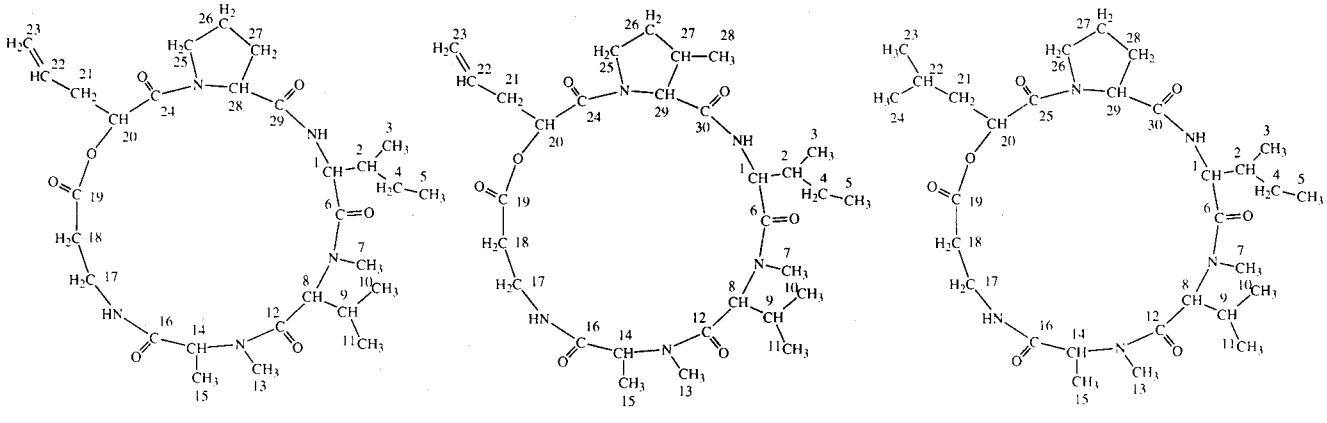


Fig. 6. Structures of roseotoxin B, and destruxins A and B.



Destruxin A (I) **Roseotoxin B (II)** **Destruxin B (III)**

Table 4. ^{1}H and ^{13}C NMR chemical shifts of roseotoxin B (**II**), destruxin A (**I**) and destruxin B (**III**) in $CDCl_3$-d_1.

Moiety	Position	Roseotoxin B (**II**)		Destruxin A (**I**)		Destruxin B (**III**)	
		δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Ile	1	53.4	4.85 dd	53.6	4.87 dd	53.7	4.86 dd
	2	37.4	1.89 m	37.4	1.90 m	37.5	1.84 m
	3	15.3	0.86 d	15.1	0.86 d	15.4	0.85 d
	4	24.5	1.43 dq, 1.26 m	24.3	1.42 dq, 1.26 m	24.5	1.43 m, 1.36 m
	5	11.2	0.85 t	11.2	0.85 t	11.4	0.84 t
	6	173.5		173.5		*	
	1-NH		7.05 d		7.14 d		7.17 d
MeVal	7	30.7	3.20 s	29.0	3.22 s	30.8	3.21 s
	8	58.0	4.97 d	58.0	4.95 d	58.1	4.93 d
	9	27.1	2.31 dq	27.1	2.32 m	27.3	2.31 m
	10	19.9	0.93 d	19.5	0.93 d	20.0	0.88 d
	11	19.5	0.89 d	19.9	0.89 d	19.6	0.92 d
	12	171.0		171.0		*	
MeAla	13	28.0	2.72 s	28.0	2.72 s	28.1	2.71 s
	14	55.5	5.15 q	55.4	5.15 q	55.5	5.16 q
	15	15.1	1.31 d	15.3	1.30 d	15.2	1.29 d
	16	169.6		168.8		*	
ß-Ala	17	33.1	4.04 m, 3.07 t	33.2	4.04 m, 3.07 t	33.2	4.04 m, 3.07 m
	18	34.4	2.67 ddd, 2.55 dd	34.4	2.67 ddd, 2.49 dd	34.4	2.66 m, 2.56 m
	19	173.5		173.5		*	
	17-NH		8.26 d		8.20 d		8.19 d
2-Hydroxy acid	20	72.5	4.86 d	72.6	4.84 dd	71.9	4.87 dd
	21	35.1	2.63 dd, 2.59 dd	34.8	2.62 m, 2.57 m	38.9	1.93 m, 1.38 m
	22	130.9	5.79 m	131.3	5.80 m	24.4	1.84 m
	23	119.8	5.23 dd, 5.18 dd	119.3	5.20 qq, 5.15 m	23.4	0.98 d
	24	169.0		169.6		21.5	0.93 d
3-Methyl-Pro (**II**) or Pro (**I, III**)	25	45.0	3.85 td, 3.61 td	46.6	3.89 td, 3.48 m	*	
	26	30.7	2.07 m, 1.66 m	24.0	1.94 m, 1.60 m	46.5	3.90 m, 3.41 m
	27	36.0	2.78 t	30.7	2.04 m, 1.65 m	24.1	2.05 m, 1.93 m
	28	18.8	1.08 d	60.7	4.66 d	28.9	2.48 m, 1.93 m
	29	67.2	4.38 s	170.9		60.7	4.66 d
	30	170.8				*	

* Not assigned.

Table 5. Inotropic and chronotropic effects of the cyclodepsipeptides.

Test compounds[a]	Contractile force[b]	Intercontraction interval[b]
Roseocardin	2.06	1.11
Roseotoxin B	2.69	1.04
Destruxin A	1.74	1.06
Destruxin B	1.53	1.04

[a] Concentration tested, 20µM.

[b] Relative value to the control before application of the test compounds.

(20 μM) increased the contractile force of the right atrium and prolonged the intercontraction interval. Similarly, roseotoxin B and destruxins A and B at 20 μM caused positive inotropic and negative chronotropic effects.

Discussion

Many cyclodepsipeptides displaying a variety of biological activities have been reported. Among these, destruxins are known to possess insecticidal,[5,8] antiviral[9] activities and cytotoxity toward leukemia cells.[10] In the present study we have shown that the newly isolated roseocardin as well as three related cyclodepsipeptides display positive inotropic and negative chronotropic effects on mammalian hearts. This discovery could result in the development of a class of cardiotonic agent since these compounds are structurally unrelated to the cardiac stimulants in current use.

Acknowledgments

The authors thank KIMIKO KOBAYASHI of the Institute of Physical and Chemical Research for X-ray analysis of roseocardin, AKEMI SAKAMURA for technical assistance and Dr. RICHARD WALTON for reading the manuscript.

References

1) TSUNOO, A.; N. TAKETOMO, M. KAMIJO, A. YAMASHITA, N. KINJO & N.-L. HUAN: Pharmacological effects of the mycelial extract of cultured *Cordyceps sinensis* on airways and aortae of the rat. *In* Science and Cultivation of Edible Fungi. *Ed.*, T. J. ELLIOTT, pp. 425~431, A. A. Balkema, Rotterdam, Brookfield, 1995

2) TSUNOO, A.; N. TAKETOMO, H. TSUBOI, M. KAMIJO, A. NEMOTO, H. SASAKI, M. UCHIDA, A. YAMASHITA, N. KINJO & N.-L. HUANG: *Cordyceps sinensis*: its diverse effects on mammals *in vitro* and *in vivo*. Proc. 3rd. Int. Symp. Mycol. Soc. Japan. 1~10, 1995

3) ENGSTROM, G. W.; J. V. DELANCE, J. L. RICHARD & A. L. BAETZ: Purification and characterization of roseotoxin B, a toxic cyclodepsipeptide from *Trichothecium roseum*. J. Agr. Food Chem. 23: 244~253, 1975

4) SPRINGER, J. P.; R. J. COLE, J. W. DORNER, R. H. COX, J. L. RICHARD, C. L. BARNES & D. VAN DER HELM: Structure and comformation of roseotoxin B. J. Am. Chem. Soc. 106: 2388~2392, 1984

5) GUPTA, S.; D. W. ROBERTS & J. A. A. RENWICK: Insecticidal cyclodepsipeptides from *Metarhizium anisopliae*. J. Chem. Soc. Perkin Trans. I: 2347~2357, 1989

6) NAGANAWA, H.; T. TAKITA, A. SUZUKI, S. TAMURA, S. LEE & N. IZUMIYA: Conformational studies of destruxins, insecticidal cyclodepsipeptides. Agr. Biol. Chem. 40: 2223~2229, 1976

7) PAÏS, M.; B. C. DAS & P. FERRON: Depsipeptides from *Metarhizium anisopliae*. Phytochemistry. 20: 715~723, 1981

8) TAMURA, S. & N. TAKAHASHI: Destruxins and piericidins. *In* Naturally occuring insecticides. *Eds.*, M. JACOBSON & O. G. CROSBY, pp. 499~539, Marcel Dekker, New York, 1971

9) YEH, S. F.; W. PAN, G.-T. ONG, A.-J., CHIOU, C.-C. CHUANG, S.-H. CHIOU & S.-H. WU: Study of structure-activity correlation in destruxins, a class of cyclodepsipeptides possessing suppressive effect on the generation of hepatitis B virus surface antigen in human hepatoma cells. Biochem. Biophys. Res. Comm. 229: 65~72, 1996

10) MOREL, E.; M. PAIS, M. TURPIN & M. GUYOT: Cytotoxity of cyclopepsipeptides on murine lymphocytes and on L 1210 leukemia cells. Biomed. Pharmacother 37: 184~185, 1983