VOL. 57 NO. 1, JAN. 2004 THE JOURNAL OF ANTIBIOTICS pp. 24 — 28

Paeciloxazine, a Novel Nematicidal Antibiotic from *Paecilomyces* sp.

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(Received for publication September 3, 2003)

A novel nematicidal antibiotic, paeciloxazine has been isolated from the culture broth of a fungus *Paecilomyces* BAUA3058 strain. This compound, whose structure was determined by spectroscopic methods, has a pyrrolobenzoxazine skeleton.

Paeciloxazine has moderate cidal activity against *Rhabditis pseudoelongata* and is weakly active against some insects.

It is well known that the macrocyclic lactone derivatives are effective against many nematodes and ectoparasites. However, resistance to the macrocyclic lactone derivatives is expanding continuously[1]. For the purpose of finding new nematicidal agents, a screening of fungi for the production of nematicidal compound was carried out.

In the screening program, we have found that the extract of culture of the *Paecilomyces* BAUA3058 strain was active against *Rhabditis pseudoelongata.* An active metabolite was isolated and characterized by spectroscopic methods.

In this paper, we describe producing strain, fermentation, isolation, physico-chemical properties, structure elucidation and biological activities of this compound.

Materials and Methods

Characterization of the Producing Strain

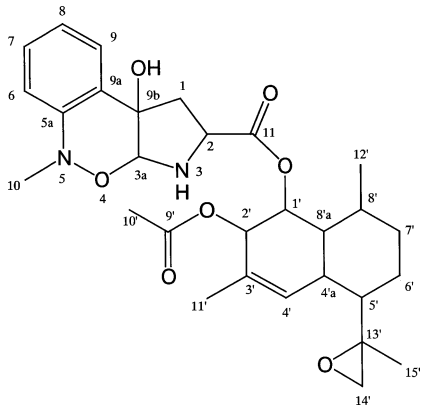
For identification of the fungi, the following media were used; potato dextrose agar, malt agar and Czapek dox agar. Morphology was observed under an optical microscope (Olympus BH-2).

Fermentation

A slant culture of strain BAUA3058 grown on PDA was used to inoculate into a 24 mm i.d. test tube containing 10 ml of a sterile seed medium consisting of potato extract 10%, V8 vegetable juice 10%, soluble starch 2.0%, soybean flour 1.5%, glucose 1.2%, malt extract 0.5%, $MgSO_4$ 0.05%. The pH was adjusted to 6.5 before autoclaving. The test tube was shaken on a reciprocal shaker at 25°C and 200 rpm for 72 hours.

A half ml of the first seed culture was transferred to 100 ml Erlenmeyer Flasks containing 10 ml of a sterile

Fig. 1. Structure of paeciloxazine.



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producing medium consisting of glucose 5.0%, soybean flour 0.5%, corn steep liquor 0.5%, yeast extract 0.3%, urea 0.08%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.001%, $ZnSO_4 \cdot 7H_2O$ 0.001%, $MnCl \cdot 4H_2O$ 0.001%, CoCl 0.001%. The pH was adjusted to 6.5 before autoclaving. Fermentation was carried out for 120 hours at 25°C and 200 rpm on a rotary shaker.

Isolation

The culture broth (1.6 liters) was extracted with EtOAc (1.6 liters) and the organic layer was concentrated under reduced pressure. This extract was applied onto a silica gel column (Wakogel C-200, 25 mm i.d.×400 mm), and then it was eluted with a mixture of hexane - EtOAc (100 : 70, v/v). All fractions were monitored by TLC and/or HPLC. Evaporation of the active fractions yielded 444.9 mg of crude paeciloxazine. 100 mg of crude paeciloxazine was dissolved in DMSO and applied to preparative HPLC (Shiseido CAPCELL PAK C_{18} UG120 10 mm i.d.×250 mm). The column was eluted with MeCN - Water (50 : 50, v/v) at a flow rate of 5 ml/minute. The fractions containing paeciloxazine (R.T. 22.5 minutes) were collected and concentrated *in vacuo* to dryness. 85.8 mg of paeciloxazine was obtained as white powder.

General Methods

UV spectra were determined on a HITACHI U-3200 spectrophotometer. Melting point data were obtained with a BÜCHI 535 Melting point apparatus. ^1H and ^{13}C NMR spectra were recorded with a JEOL JNM-ALPHA500 spectrometer. ESI-MS spectra were obtained with a FINNIGAN LCQ^{DUO} apparatus. HRESI-MS spectra were measured on a JOEL JMS-700 mass spectrometer; exact mass was drawn from comparison with PEG600. HPLC was performed on a Shimadzu LC-10AD system.

Biological Activities

Nematicidal activity of paeciloxazine toward *Rhabditis pseudoelongata*[2] was measured as follows: suspension of adult worms from a five days old culture was diluted with NIPSF medium to about 200～300 nematodes/ml. 100 μl of the suspension was incubated with test compounds at 25°C in the dark. As a standard, ivermectin[3] was used. Nematicidal activity was recorded after four days.

Insecticidal activity of paeciloxazine toward *Culex pipiens pallens* was observed as follows: suspension of L_2 larvae was diluted with distilled water to about 50～100 larvae/ml. 100 μl of the suspension was incubated with test compounds at 25°C. As a standard, imidacloprid[4] was used. Insecticidal activity was monitored after 24 hours.

Insecticidal activity against *Plutella xylostella* was observed as follows: test compounds were dissolved in the solution consisting of emulsifier (Sorpol®-2564, TOHO chemical industry) 5%, DMSO 1%, spreader (Neoesterin®, KUMIAI chemical industry) 0.02%. A leaf of a cabbage was cut by the round shape with a diameter of 5 cm and soaked in the test compound solution for 20 minutes. After the leaf got dry, ten L_3 larvae were placed on it. Insecticidal activity was monitored after two days.

Results

Producing Strain

The producing strain BAUA3058 was isolated from a soil sample collected in Ibaraki Prefecture, Japan.

Strain BAUA3058 exhibited good growth at 20～25°C on potato dextrose agar. Colonies on potato dextrose agar were velvetinous and white to pale gray. The reverse side of the culture was beige. Formation of conidia was moderate. Colonies on malt agar were velvetinous and white. The reverse side of the colonies was pale beige. Formation of conidia was not observed. Colonies on Czapek dox agar were velvetinous and white. The reverse side of the culture was red brown. Formation of conidia was not observed.

Morphological observation was conducted under a microscope. When strain BAUA3058 was grown on potato dextrose agar, conidiophores were formed from the aerial hyphae. Two phialides were born from conidiophore. Conidia were produced in chains from the tip of phialide, and were globose or subglobose, pale gray and their surface was smooth.

Based on cultural and microscopic characteristics described above, the strain BAUA3058 was considered to belong to the genus *Paecilomyces.* The strain has been deposited to International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Japan, as *Paecilomyces* sp. BAUA3058 with the accession No. FERM P-19116.

Preparation of Paeciloxazine

The isolation and purification scheme used to obtain paeciloxazine is outlined in Fig. 2. Paeciloxazine was extracted from the culture broth with EtOAc, and was subsequently purified by silica gel chromatography and preparative HPLC. These purification processes gave pure Paeciloxazine, and the purity was checked by HPLC analysis.

Fig. 2. Isolation and purification procedure of paeciloxazine.

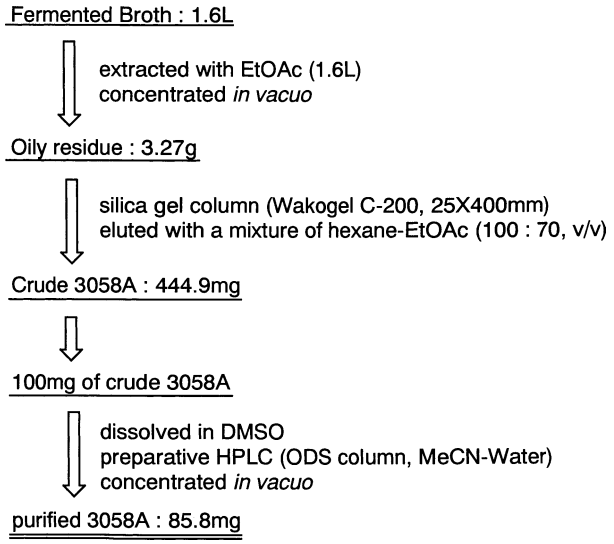


Table 1. Pysico-chemical properties of paeciloxazine.

Appearance	White powder
Molecular formula	$C_{29}H_{38}N_2O_7$
ESI-MS (*m/z*)	527 $[M+H]^+$, 549 $[M+Na]^+$
HRESI-MS	
found	527.2777 $[M+H]^+$
calcd.	527.2757 $[M+H]^+$ for $C_{29}H_{39}N_2O_7$
Melting point	158°C
UV λ_{max} nm in MeOH (ε)	248 (7400), 285 (1400)

Physico-chemical Properties

Physico-chemical properties of paeciloxazine were summarized in Table 1. It was readily soluble in methanol, chloroform, ethyl acetate and dimethylsulfoxide and practically insoluble in water. In the electrospray ionization MS (ESI-MS) positive ion mode measurement, paeciloxazine showed the peak of *m/z* 527 $[M+H]^+$ and 549 $[M+Na]^+$. The molecular formula of paeciloxazine was determined to be $C_{29}H_{38}N_2O_7$ on the basis of HRESI-MS measurement and NMR spectral analyses.

Structure Elucidation

The structure of paeciloxazine was determined by the analyses of 1D and 2D NMR spectra and MS spectra.

Table 2. ^1H and ^{13}C NMR data of paeciloxazine in $CDCl_3$.

position	δ_H	δ_C
1	2.50 (2H, m)	41.9
2	4.03 (1H, d, *J*=8.2Hz)	58.2
3	3.77 (1H, br s)	
3a	5.17 (1H, br s)	94.5
5a		148.2
6	6.71 (1H, d, *J*=7.9Hz)	112.1
7	7.21 (1H, dd, *J*=7.3, 7.9Hz)	128.3
8	6.97 (1H, dd, *J*=7.0, 7.3Hz)	121.9
9	7.43 (1H, d, *J*=7.0Hz)	127.2
9a		129.3
9b		76.5
9b-OH	2.21 (1H, s)	
10	3.10 (3H, s)	41.1
11		174.4
1'	5.26 (1H, s)	73.2
2'	5.09 (1H, s)	70.6
3'		130.8
4'	5.62 (1H, s)	126.8
4'a	2.94 (1H, br s)	32.7
5'	1.82 (1H, m)	44.0
6'	0.94 (2H, m)	21.8
	1.51	
7'	0.94 (2H, m)	35.1
	1.67	
8'	1.67 (1H, m)	27.6
8'a	1.67 (1H, m)	43.6
9'		170.1
10'	2.09 (3H, s)	20.8
11'	1.72 (3H, s)	20.4
12'	0.97 (3H, d, *J*=5.2Hz)	19.4
13'		57.8
14'	2.63 (2H, d, *J*=4.7Hz)	51.2
	2.85	
15'	1.47 (3H, s)	21.7

The ^{13}C NMR and DEPT spectra of paeciloxazine displayed 29 signals composed of four methyl carbons, one *N*-methyl carbon, four methylene carbons, eight methine carbons, five sp^2 methine carbons, three sp^2 quaternary carbons, two quaternary carbons, and two ester carbons. The ^1H NMR spectrum showed two D_2O exchangeable protons.

Analyses of DQFCOSY and HMQC spectra of paeciloxazine showed following four structural fragments (**a** to **d**) as shown in Fig. 3. The presence of the epoxy ring was indicated by the chemical shift of C-14′ methylene (δ_C 51.2) and the characteristic coupling constants: $^1J_{CH}$=172.2 Hz. The connection of these structural fragments was deduced from the observation of the HMBC correlations as shown in Fig. 4.

The correlations from H-14′ (δ_H 2.63, 2.85) to C-13′ (δ_C 57.8), and from H-15′ (δ_H 1.47) to C-5′ (δ_C 44.0) and C-14′ revealed that the 2-methyl-oxiranyl moiety is bonded to C-5′. Methyl proton H-10′ (δ_H 2.09) showed correlation to

Fig. 3. Partial structures of paeciloxazine.

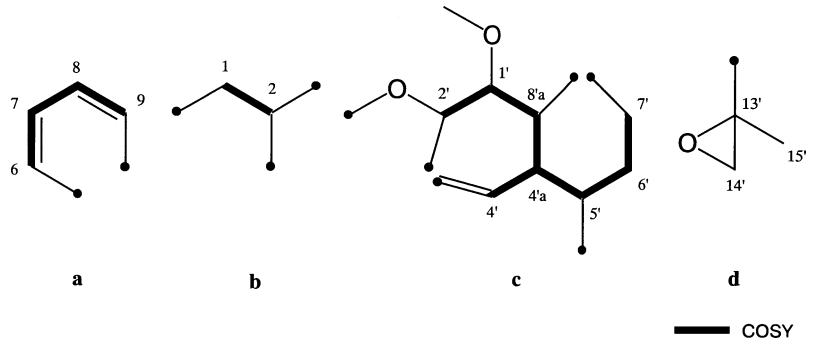
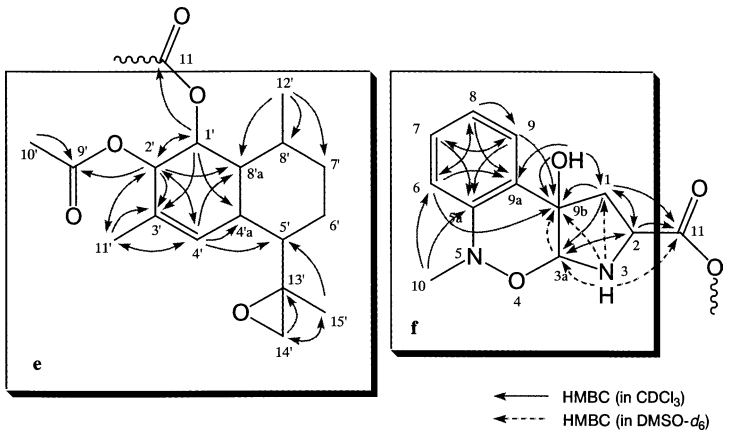


Fig. 4. HMBC correlations of paeciloxazine.



C-9′ (δ_C 170.1). Moreover, correlation from H-2′ (δ_H 5.09) to C-9′ was observed. These correlations revealed that the acetoxy group is bonded to C-2′ (δ_C 70.6). Methyl proton H-11′ (δ_H 1.72) showed correlations to C-2′, C-3′ (δ_C 130.8) and C-4′ (δ_C 126.8). Moreover, correlations from H-12′ (δ_H 0.97) to C-7′ (δ_C 35.1), C-8′ (δ_C 27.6) and C-8′a (δ_C 43.6) were observed. Therefore, the structure of **e** was elucidated as shown in Fig. 4.

In examining sp^2 methine protons, we found that correlations from H-6 (δ_H 6.71) to C-8 (δ_C 121.9) and C-9a (δ_C 129.3), from H-7 (δ_H 7.21) to C-5a (δ_C 148.2) and C-9 (δ_C 127.2), from H-8 (δ_H 6.97) to C-6 (δ_C 112.1) and C-9a, and from H-9 (δ_H 7.43) to C-5a and C-7 (δ_C 128.3), respectively. These data revealed that the presence of an *o*-phenylene skeleton. Moreover, the correlations from H-6 to C-9b (δ_C 76.5), from H-9 to C-9b, and from 9b-OH (δ_H 2.21) to C-1 (δ_C 41.9), C-9a and C-9b revealed connection of structures **a** with **b**, and 9b-OH is bonded to C-9b. In the HMBC experiment in $CDCl_3$, amine proton H-3 (δ_H 3.77) did not show any HMBC cross peak. However, in the HMBC experiment in DMSO-d_6, H-3 showed correlations to C-1, C-3a (δ_C 94.5), C-9b and C-11 (δ_C 174.4). Moreover, correlations from H-1 (δ_H 2.50) to C-3a, from H-2 (δ_H 4.03) to C-3a were observed. These data suggested the presence of a pyrrolidine ring, as shown in Fig. 4. The *N*-methyl proton H-10 (δ_H 3.10) showed correlation with C-5a and C-6. These data revealed *N*-methyl is bonded to C-5a. However, H-10 did not show other HMBC cross peak. The structure of **f** was deduced to have a composition of $C_{11}H_{13}N_2O_2$ by considering the molecular formula and the structure of **e**. Moreover, the methine carbon chemical shift value of C-3a suggested that it is further connected to an oxygen atom. From these results, the structure of **f** was found to be a pyrrolobenzoxazine skeleton as shown in Fig. 4.

The correlations from H-1 to C-11, from H-2 to C-11,

Table 3. Biological activities of paeciloxazine.

Species	MIC (μg/ml)		
	paeciloxazine	Ivermectin	Imidacloprid
Rhabditis pseudoelongata	50	0.125	N.T.
Culex pipiens pallens	25	N.T.	0.625

Table 4. Insecticidal activities of paeciloxazine against *Plutella xylostella*.

species	Concentration (μg/ml)	Mortality (%)
Plutella xylostella	125	30
	250	50
	500	100

from H-3 to C-11, and from H-1′ (δ_H 5.26) to C-11 were observed. These observations revealed that structures **e** and **f** are bound *via* an ester. In the INADEQUATE experiment, all of spin coupling except connection between C-2 and C-11 were observed.

Based on all of the observations described above, the structure of paeciloxazine was elucidated as shown in Fig. 1.

Biological Activities

Biological activities of paeciloxazine are shown in Table 3 and 4. Nematicidal activity of paeciloxazine toward *Rhabditis pseudoelongata* was tested. The compound was active at a concentration of 50 μg/ml. Ivermectin, which is known to have a potent activity against various nematodes, was active at a concentration of 0.125 μg/ml.

Insecticidal activity of paeciloxazine was assayed. Paeciloxazine was active at a concentration of 25 μg/ml against *Culex pipiens pallens*, and killed all of the larvae of *Plutella xylostella* at a concentration of 500 μg/ml.

Discussion

The structure of paeciloxazine was determined as shown in Fig. 1. This compound has a pyrrolobenzoxazine skeleton as already reported for UK-88051[5], CJ-12662 and CJ-12663[6]. 1D-NMR spectra of paeciloxazine were very similar to those of these compounds, which support the existence of a pyrrolobenzoxazine skeleton in paeciloxazine. However, structures of these pyrrolobenzoxazines were elucidated by crystal X-ray analysis. The configurations at C-2, C-3a, C-9b, C-1′, C-2′, C-4′a, C-5′, C-8′, C-8′a and C-13′ of paeciloxazine remain to be determined.

It was also reported that UK-88051, CJ-12662 and CJ-12663 exhibit nematicidal and insecticidal activities. These reports and our results suggested pyrrolobenzoxazine compounds were active against both nematodes and insects. Moreover, paeciloxazine adhering to the cabbage leaf killed the larvae of *Plutella xylostella.* This evaluation system imitates the usual insecticide spraying. Therefore, the paeciloxazine class of compounds may serve as a useful leads for development of new nematicidal and insecticidal agents.

Acknowledgements

The authors wish to thank Dr. KIYOSHI ISONO, research advisor of Institute of Biotechnology Applied to Soil Eumycetes for his helpful advice.

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