DACTYLFUNGINS, NOVEL ANTIFUNGAL ANTIBIOTICS PRODUCED BY *Dactylaria parvispora*

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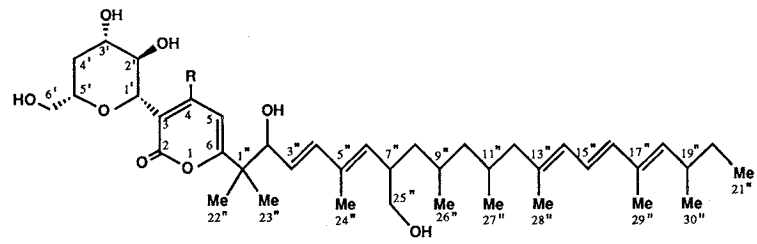
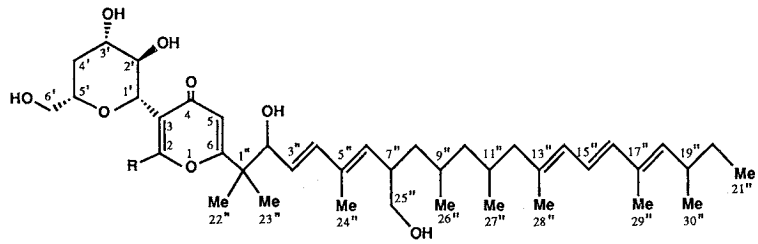
Novel antifungal antibiotics, designated as dactylfungins A (**1**) and B (**3**), were isolated from the culture broth of *Dactylaria parvispora* D500. Dactylfungins A and B were found to be new substances containing an α-pyrone and a γ-pyrone ring, respectively, which conjoined with a polyalcohol moiety and a long side chain, based on NMR spectral analyses. The antibiotics were active against *Candida pseudotropicalis* and other fungi, with an MIC value at less than 10 μg/ml.

In the course of our screening program for new antibiotics, two new antifungal antibiotics were isolated from the culture broth of *Dactylaria parvispora* strain D500 and designated as dactylfungins A (**1**) and B (**3**) (Fig. 1). This report describes the taxonomy of the producing organism, fermentation and isolation procedures as well as physico-chemical and biological properties of the new antibiotics.

Fig. 1. Structures of dactylfungins A (**1**) and B (**3**).



1 R=OH
2 R=OMe



3 R=OH
4 R=OMe

Identification of the Producing Fungus

Strain D500 was isolated from a dead leaf sample collected at Chiba Prefecture, Japan. The fungus was identified as *D. parvispora*, from the characteristics described below.

Microscopic Characteristics

Colonies on Potato-Dextrose agar (PDA) for 2 weeks at 27°C attaining 1 cm diameter, flat, dark yellowish brown to dull yellowish orange; the reverse yellowish brown to dull orange. Vegetable haphae hyaline, septate, unbranched, up to 1.8~2.8 μm wide. Aerial hyphae abundant. Conidiophores arising from vegetative hyphae or aerial hyphae, usually unbranched, septate, up to 146 μm long, 4.6~6.3 μm wide, at first pale brown, thin-walled, later becoming dark-brown and thick-walled. Conidiogenous cells, flexuous, cylindrical, septate, hyaline to pale brown, with scattered short cylindrical denticles. Conidia hyaline, smooth, ellipsoid to obovoid, usually 1- to 3-septa, 7.8~22.0 × 2.8~5.3 μm.

Physiological Properties

Growing temperature on LcA (Miura medium): 15~30°C. Optimum growing temperature: 20~26°C. Growing pH: 2 to 10. Optimum growing pH: 4 to 7.

The producing fungus (D500) is characterized by the following features: 1) Conidiophores are brown in color, unbranched; 2) conidiogenous cells have scattered short cylindrical denticles and 3) conidia-type is sympodulospore. Thus, the strain belongs to *Pleurophragmium* Sect. in the genus *Dactylaria*.

According to DE HOOG[1], 11 species are described under the *Pleurophragmium* Sect. These species are identified by the difference in the conidium-ontogeny, the shape and size of conidia. The cultural and morphological characteristics of the producing strain agreed well with the description of *Dactylaria parvispora* given by DE HOOG[1].

Fermentation

The culture of *D. parvispora* D500 from a Potato-Dextrose agar slant was inoculated into 200-ml Erlenmeyer flasks containing 40 ml of a medium consisting of maltose syrup 4%, soybean oil 0.3%, soybean protein 2%, Pharmamedia (Traders Oil Mill Co., Texas) 1%, soluble vegetable protein 0.5%, $CaCO_3$ 0.3%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CoCl_2 \cdot 6H_2O$ 0.001%, $NiCl_2$ 0.001% (pH 6.0 before sterilization). The culture was incubated on a rotary shaker at 210 rpm for 5 days at 27°C. Four ml of the seed culture were inoculated into each of one hundred 500-ml Erlenmeyer flasks containing 80 ml of the same medium. The culture was continued at the same condition for another 7 days. The production of dactylfungins was monitored by bioassay of antifungal activity against *Trigonopsis variabilis* M9031.

Fig. 2. Isolation procedure for dactylfungins A and B.

Culture broth (6.5 liters)
| centrifuged
Mycelial cake
| acetone
Extract
| concd
| water-saturated *n*-butanol
Butanol layer
| concd
| silica gel column chromatography
Chloroform-methanol (4 : 1 ~ 2 : 1) fraction
| concd
| HPLC (ODS, 20 × 250 mm, 90% acetonitrile, 10 ml/minute)
Dactylfungin A (minor compound)
and
Dactylfungin B (640 mg)

Isolation

The purification scheme of dactylfungins A and B is shown in Fig. 2. The culture broth (6.5 liters) was centrifuged and the mycelial cake was extracted with 4 liters acetone. The organic layer was then concentrated and further extracted with water-saturated *n*-butanol. The extracts were concentrated to yield 12 g of crude residue. The residue was mixed with 15 g of silica gel and dried at room temperature. The dried silica gel was placed on the top of a silica gel column (5 × 15 cm) packed with chloroform. The column was eluted stepwise with chloroform, chloroform - methanol (4 : 1), chloroform - methanol (2 : 1), chloroform - methanol (1 : 1) and methanol. The active fractions (chloroform - methanol, 4 : 1 ~ 2 : 1) were collected and concentrated to dryness *in vacuo* to give a crude powder (2.2 g). Dactylfungins A and B were separated by preparative HPLC using a ODS column (2 × 25 cm, YMC Co., Ltd.) with a mobile phase of acetonitrile - water (90 : 10). Fractions containing each compound were collected and concentrated *in vacuo* to yield amorphous powder of dactylfungins A (minor compound) and B (640 mg).

Physico-chemical Properties

The physico-chemical properties of dactylfungins A and B are shown in Table 1. The antibiotics were obtained as a colorless amorphous powder, easily soluble in methanol and dimethyl sulfoxide, slightly soluble in chloroform, but insoluble in water, acetone, ethyl acetate and hexane. They were unstable under acidic conditions.

The UV spectra of dactylfungins A and B showed maximum absorption at 237, 278 and 288 nm indicating the presence of diene and triene functions in their molecule. The IR spectra were unremarkable except for the presence of a relatively intense OH stretch at 3400 cm^{-1}, and a broad absorption band from 1680 to 1650 cm^{-1} of dactylfungin A and an absorption band at 1645 cm^{-1} of dactylfungin B which probably attributed to a carbonyl or olefinic functions.

The SI-MS (positive mode) showed molecular ion peaks at *m/z* 723 $(M+Na)^+$, 739 $(M+K)^+$ for both of dactylfungins A and B, indicating a molecular weight of 700. These results are in agreement with the molecular ion peak at *m/z* 699 $(M-H)^-$ in the SI-MS in the negative mode. The molecular formulae were established to be $C_{41}H_{64}O_9$ based on mass, NMR and elemental analyses.

Structure Determination

Structure of Dactylfungin B

Structure determination was started by using the major component B. Figs. 3 and 4 show the ^1H and

Table 1. Physico-chemical properties of dactylfungins A (**1**) and B (**3**)

	A (**1**)	B (**3**)
MP	120 ~ 122°C (dec)	173 ~ 174°C (dec)
$[\alpha]_D^{25}$	−17.2° (*c* 1.0, MeOH)	−23.4° (*c* 1.0, MeOH)
UV λ_{max}^{MeOH} nm (ε)	216 (36,800), 237 (38,100), 278 (43,000), 288 (sh, 35,200)	216 (35,500), 237 (37,500), 278 (44,800), 288 (sh, 36,200)
IR ν_{max}^{KBr} cm^{-1}	3400, 2950, 2930, 2870, 2850, 1680, 1650, 1580, 1500, 1460, 1380, 1060, 1040, 960, 770	3400, 2950, 2920, 2870, 1645, 1610, 1580, 1450, 1440, 1370, 1260, 1070, 1040, 1020, 970
SI-MS (*m/z*)		
Positive:	723 $(M+Na)^+$, 739 $(M+K)^+$	723 $(M+Na)^+$, 739 $(M+K)^+$
Negative:	699 $(M-H)^-$	699 $(M-H)^-$
Molecular formula	$C_{41}H_{64}O_9$	$C_{41}H_{64}O_9$

sh: Shoulder.

Fig. 3. ^{1}H NMR spectrum of dactylfungin B (**3**).

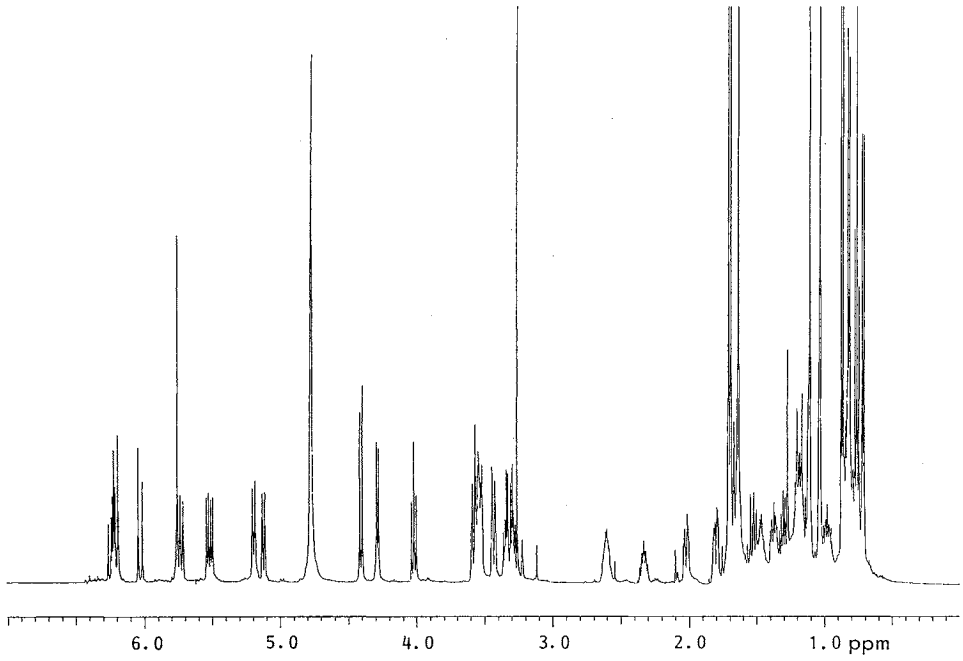
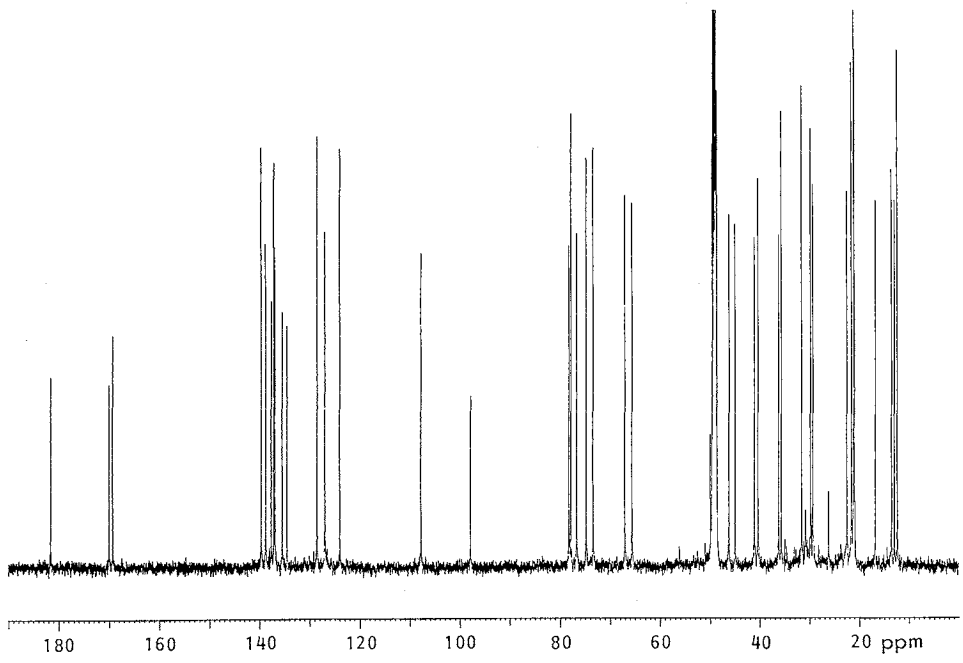


Fig. 4. ^{13}C NMR spectrum of dactylfungin B (**3**).



^{13}C NMR spectra of B which are summarized in Tables 2 and 3. These NMR data suggested the presence of the following carbon units for dactylfungin B:

Table 2. ^{1}H NMR chemical shifts of dactylfungins A (**1**) and B (**3**) in CD_3OD (δ: ppm)

No.	A	B
5	5.99 (1H, s)	5.75 (1H, s)
1′	4.38 (1H, d, J=10 Hz)	4.40 (1H, d, J=10 Hz)
2′	3.90 (1H, dd, J=10, 10 Hz)	4.02 (1H, dd, J=10, 10 Hz)
3′	3.52 (1H m)	3.53 (1H, m)
4′	1.46 (1H, ddd, J=10, 10, 12 Hz),	1.65 (1H, ddd, J=10, 10, 12 Hz),
	1.86 (1H, ddd, J=2.5, 5, 10 Hz)	1.80 (1H, ddd, J=2.5, 5, 10 Hz)
5′	3.51 (1H, m)	3.52 (1H, m)
6′	3.45 (2H, d, J=6 Hz)	3.42 (1H, dd, J=4, 11 Hz),
		3.58 (1H, m)
2″	4.24 (1H, d, J=7.5 Hz)	4.29 (1H, d, J=7.5 Hz)
3″	5.49 (1H, dd, J=7.5, 15 Hz)	5.49 (1H, dd, J=7.5, 15 Hz)
4″	6.20 (1H, d, J=15 Hz)	6.20 (1H, d, J=15 Hz)
6″	5.20 (1H, d, J=10 Hz)	5.18 (1H, d, J=10 Hz)
7″	2.61 (1H, m)	2.61 (1H, m)
8″	0.97 (1H, m),	0.97 (1H, m),
	1.36 (1H, m)	1.36 (1H, m)
9″	1.44 (1H, m)	1.44 (1H, m)
10″	0.83 (1H, m),	0.83 (1H, m),
	1.18 (1H, m)	1.18 (1H, m)
11″	1.62 (1H, m)	1.62 (1H, m)
12″	1.51 (1H, dd, J=9, 12 Hz),	1.51 (1H, dd, J=9, 12 Hz),
	2.01 (1H, dd, J=5, 12 Hz)	2.01 (1H, dd, J=5, 12 Hz)
14″	5.71 (1H, d, J=11 Hz)	5.71 (1H, d, J=11 Hz)
15″	6.23 (1H, dd, J=11, 15 Hz)	6.23 (1H, dd, J=11, 15 Hz)
16″	6.02 (1H, d, J=15 Hz)	6.02 (1H, d, J=15 Hz)
18″	5.12 (1H, d, J=10 Hz)	5.12 (1H, d, J=10 Hz)
19″	2.32 (1H, ddd, J=5, 7, 13 Hz)	2.32 (1H, ddd, J=5, 7, 13 Hz)
20″	1.17 (1H, ddd, J=5, 7, 13 Hz),	1.17 (1H, ddd, J=5, 7, 13 Hz),
	1.25 (1H, m)	1.25 (1H, m)
21″	0.75 (3H, t, J=7.0 Hz)	0.75 (3H, t, J=7.0 Hz)
22″	1.07 (3H, s)	1.07 (3H, s)
23″	1.13 (3H, s)	1.13 (3H, s)
24″	1.67 (3H, s)	1.67 (3H, s)
25″	3.26 (1H, dd, J=6, 11 Hz),	3.26 (1H, dd, J=6, 11 Hz),
	3.31 (1H, dd, J=6, 11 Hz)	3.31 (1H, dd, J=6, 11 Hz)
26″	0.82 (3H, d, J=6.5 Hz)	0.82 (3H, d, J=6.5 Hz)
27″	0.65 (3H, d, J=6.5 Hz)	0.65 (3H, d, J=6.5 Hz)
28″	1.62 (3H, s)	1.62 (3H, s)
29″	1.72 (3H, s)	1.72 (3H, s)
30″	0.86 (3H, d, J=6.5 Hz)	0.86 (3H, d, J=6.5 Hz)

$$9\times\text{-}CH_3,\ 5\times\text{-}CH_2\text{-},\ 4\times\text{-}\overset{|}{C}H\text{-},\ 8\times=CH\text{-},\ 4\times=\overset{|}{C}$$

$$2\times\text{-}CH_2\text{-}O,\ 5\times\text{-}\overset{|}{C}H\text{-}O,\ 1\times\text{-}\overset{|}{\underset{|}{C}}\text{-},\ 1\times\text{-}COO\text{-},\ 2\times=\overset{|}{C}\text{-}O$$

The following units, A, B and C (Fig. 5), as the partial structures of dactylfungin B were elucidated by detailed analysis of ^{1}H and ^{13}C NMR data including 2D NMR.

Unit A (C-2～C-6)

In the ^{1}H NMR spectrum (Fig. 3), a singlet olefinic proton was seen at δ 5.75 (5-H), which showed long range coupling to C-3 (δ 97.7), C-4 (δ 181.4) and C-6 (δ 169.1) (Fig. 6). These ^{1}H and ^{13}C chemical

shifts of C-2 (δ 169.6), C-3, C-4, C-5 (δ 107.6) and C-6 were characteristic for those of the γ-pyrone. The presence of γ-pyrone was also supported by comparison with published NMR data for γ-pyrone-type substances[2,3], and by the IR absorption band at 1645 cm^{-1}. These consideration enabled the determination of the structure of unit A.

Unit B (C-1′~C-6′)

The ^{13}C NMR shifts of C-1′ (δ 76.5), C-2′ (δ 73.2), C-3′ (δ 74.6), C-5′ (δ 78.1) and C-6′ (δ 65.5) suggested the presence of a polyalcohol moiety. An AB multiplet was observed for 4′-H_2 at δ 1.65, 1.80, which showed long range coupling to C-2′, C-3′, C-5′ and C-6′ (Fig. 6). Long range coupling was also observed of the doublet proton at δ 4.40 (1′-H) to C-2′, C-3′ and C-5′ (Fig. 6). Furthermore, ^1H-^1H COSY experiments showed cross peaks between 1′-H and 2′-H, 2′-H and 3′-H, 3′-H and 4′-H_2, 4′-H_2 and 5′-H, as well as 5′-H and 6′-H_2. These NMR data enabled the structure elucidation of the polyalcohol ring.

Table 3. ^{13}C NMR chemical shifts of dactylfungins A (**1**) and B (**3**) in CD_3OD (δ: ppm).

No.	A	B	No.	A	B
2	167.5	169.6	11″	29.7	29.8
3	99.6	97.7	12″	48.7	48.7
4	171.6	181.4	13″	137.5	137.5
5	101.9	107.6	14″	128.3	128.3
6	172.4	169.1	15″	123.8	123.8
1′	75.7	76.5	16″	136.9	136.9
2′	73.4	73.2	17″	134.3	134.3
3′	74.2	74.6	18″	139.5	139.5
4′	36.4	36.2	19′	35.6	35.6
5′	78.3	78.1	20″	31.6	31.6
6′	65.6	65.5	21″	12.4	12.4
1″	45.4	44.7	22′	22.8	22.5
2″	78.2	78.1	23″	20.6	20.9
3″	126.3	126.8	24″	13.0	13.0
4″	139.0	138.6	25″	66.6	66.7
5″	135.2	135.3	26″	21.6	21.6
6″	137.1	136.7	27″	20.9	20.9
7″	40.2	40.2	28″	16.8	16.8
8″	41.0	41.0	29″	13.6	13.5
9″	29.3	29.3	30″	21.2	21.2
10″	46.0	46.1			

Fig. 5. Partial structures of dactylfungin B (**3**).

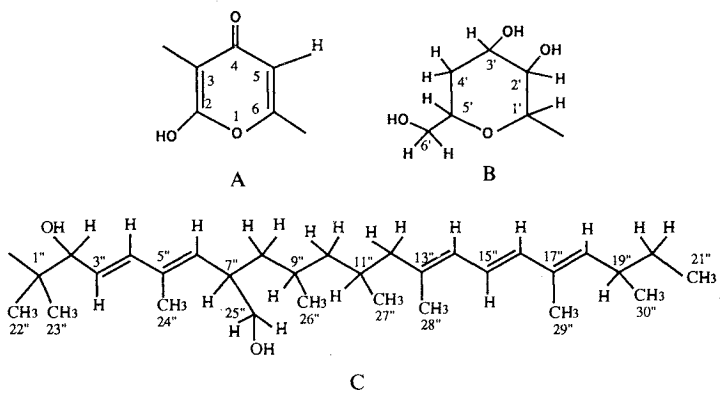
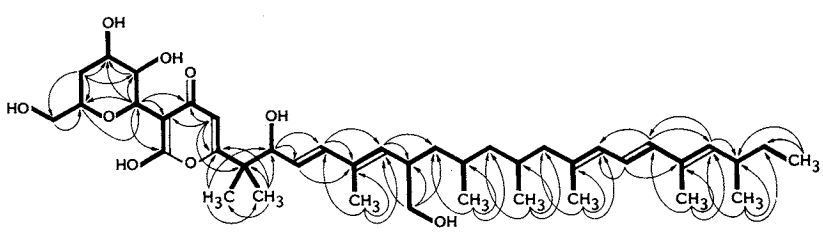


Fig. 6. Summary of observed long range ^1H-^{13}C coupling (HMBC) for dactylfungin B (**3**).



Unit C (C-1″~C-30″)

Assignment of these atoms at the side chain was unambiguously done from the COSY, HOHAHA and HMBC experiments (Fig. 6). The large coupling constants between 3″-H and 4″-H (J=15.0 Hz), 15″-H and 16″-H (J=15.0 Hz) as well as the NOE-enhancements in the 3″-H and 15″-H signal intensity upon irradiation of 24″-H_3 and 28″-H_3 or 29″-H, respectively, indicated that all of these olefinic linkages are orientated in *E*-configurations.

Connection of the units A, B and C was carried out on the basis of a HMBC experiment. Long range couplings were observed for the methine proton of 1′-H to C-2, C-3 and C-4, and the methyl protons of 22″- and 23″-H_3 to C-6 (Fig. 6). Therefore, units A and B, and A and C were joined between C-3 and C-1′, and C-6 and C-1″, respectively. Relative stereochemistry of the polyalcohol ring was assigned based on the observation that axial-axial couplings fall in the range of 10~11 Hz while axial-equatorial and equatorial-equatorial couplings are typically smaller[4]. Furthermore, NOE experiments revealed the NOE network from 1′-H through 3′-H to 5′-H.

From all of the above results, the structure of dactylfungin B was determined as **3** shown in Fig. 1.

Structure of Dactylfungin A

Detailed NMR experiments including 2D NMR experiments were carried out on dactylfungin A, in the similar manner as described above in B. As summarized in Tables 2 and 3, ^{1}H and ^{13}C NMR spectra of A were extremely similar to those of B except the chemical shifts of proton (5-H δ 5.99) and carbons (C-2 δ 167.5, C-3 δ 99.6, C-4 δ 171.6, C-5 δ 101.9 and C-6 δ 172.4) which were characteristic for those of an α-pyrone[2,5,7~9]. Thus, the structure of dactylfungin A was determined as **1** (Fig. 1).

Methylation of dactylfungin B with CH_2N_2 gave a mixture of two compounds which were separated by HPLC. Spectral analysis of the two compounds showed them to be almost identical except for: 1) Position of C=O resonance in ^{13}C NMR spectra (172.1 vs. 183.2) and 2) position of a single proton resonance in ^{1}H NMR spectra (6.40 vs. 6.18). By comparing with literatures[2,3,6,9], these two compounds were identified as the methyl ethers of α-pyrone **2** and γ-pyrone **4** (Fig. 1), respectively. These results further confirmed the structure of dactylfungins A (**1**) and B (**3**).

Biological Properties

The minimum inhibitory concentrations (MIC) of dactylfungin B against bacteria and fungi were determined by the agar dilution method, and the results are given in Table 4. The antibiotic was inactive against bacteria. It showed antimicrobial activity against fungi, such as *Candida pseudotropicalis* and *Saccharomyces cerevisiae* with an MIC value at less than 10 μg/ml range. When tested in mice by the ip route, acute LD_{50} value for dactylfungin B was more than 200 mg/kg. A similarly antifungal activity was observed for dactylfungin A. Further studies on biological activities of dactylfungins A and B are in progress.

Table 4. Antimicrobial activities of dactylfungin B[a].

	MIC (μg/ml)
Micrococcus luteus ATCC 9341	>100
Staphylococcus aureus 209P	>100
Pseudomonas aeruginosa M-8152	>100
Escherichia coli NIHJ	>100
Candida albicans M9001	>100
C. pseudotropicalis M9035	3.1
Cryptococcus neoformans M9010	6.2
Debaryomyces hansenii M9011	3.1
Hansenula schneggi IAM 4269	100
Saccharomyces cerevisiae SHY3	6.2
Schizosaccharomyces pombe M9025	3.1
Trigonopsis variabilis M9031	3.1

[a] MIC assay was carried out in an agar dilution method in a YA medium (containing yeast extract 0.55% and agar 2%) for the bacteria and a PYGA medium (containing Polypeptone 0.55%, yeast extract 0.27%, glucose 0.55% and agar 1.65%) for the fungi.

Discussion

The structure of the novel antibiotic dactylfungins A and B were determined based on analysis of the spectroscopic data presented in this paper. The components contain an α- and a γ-pyrone ring, respectively, which connect with a long side chain and a polyalcohol moiety. There are natural products that possess pyrone function[2,3,5,7~9], but there is no report of component that contains a polyalcohol moiety at the pyrone ring. Additionally, the side chain of MK2266 contains diene and triene groups. Thus, dactylfungins A and B represent a new class of antibiotic which have not previously been reported.

Experimental

NMR spectra were recorded on a Bruker AM500 spectrometer with TMS as internal standard. Mass spectra were obtained on a Hitachi G-3000 spectrometer. IR spectrum was recorded on a JASCO FT/IR-8000 spectrometer. UV spectrum was obtained on a Shimadzu UV-3100S spectrometer. Optical rotation was measured with a JASCO DIP-370 polarimeter.

Methylation of Dactylfungin B (**3**)

Dactylfungin B (20 mg) in MeOH was reacted with CH_2N_2 to give a mixture of two compounds which were separated by preparative HPLC (ODS column using a mobile phase of MeOH - H_2O, 8:2) to give **2** (4 mg) and **4** (5 mg). **2** was a colorless oil. FAB-MS: 737 $(M+Na)^+$. UV λ_{max} nm: 237, 278, 288 (sh). IR ν_{max} (KBr) cm^{-1}: 3380, 2950, 2920, 2830, 1680, 1550, 1460, 1380. ^1H NMR (in CD_3OD) δ 6.40 (1H, s, 5-H), δ 3.90 (3H, s, 4-OMe). ^{13}C NMR (in CD_3OD) δ 172.1, 173.7, 172.1 (C-2, 4, 6). **4** was a colorless oil. FAB-MS: 737 $(M+Na)^+$. UV λ_{max} nm: 239, 278, 288 (sh). IR ν_{max} (KBr) cm^{-1}: 3380, 2950, 2920, 2830, 1650, 1560, 1465. ^1H NMR (in CD_3OD) δ 6.18 (1H, s, 5-H), δ 4.10 (3H, s, 4-OMe). ^{13}C NMR (in CD_3OD) δ 171.0, 183.2, 167.1 (C-2, 4, 6).

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