Cladospolide D, a New 12-Membered Macrolide Antibiotic Produced by *Cladosporium* sp. FT-0012

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A new antibiotic termed cladospolide D was isolated along with the known cladospolides A and B from the fermentation broth of *Cladosporium* sp. FT-0012 by solvent extraction, ODS column chromatography and preparative HPLC. The structure of cladospolide D was deduced to be (*E*)-2-dodecen-5-hydroxy-11-olide-4-one. Cladospolide D showed antifungal activity against *Pyricularia oryzae* and *Mucor racemosus*.

During our screening for novel bioactive compounds of microbial origin, we have isolated three cladospolide-related compounds, members of 12-membered macrolide family, from the culture broth of *Cladosporium* sp. FT-0012 (Fig. 1). Although two compounds were identified as cladospolides A and B, the other was found to be a new compound, termed cladospolide D (Fig. 2). Cladospolides A and B were originally isolated from the culture broth of *Cladosporium* sp. as a root growth inhibitor and a promoter of lettuce seedlings, respectively[1~4]. The structure of cladospolide D was elucidated by various spectral analyses including NMR experiments.

In this paper, the taxonomy of the producing strain, fermentation, isolation, biological properties and structure elucidation of cladospolide D are described.

Materials and Methods

General Experimental Procedures

Fungal strain FT-0012 was isolated from a sponge sample collected in Pohnpei island, Federated State of Micronesia, and was used for production of cladospolides. HPLC was carried out using the Waters (600E) system.

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. Optical rotations were

Fig. 1. Scanning electron micrograph of spore chains of strain FT-0012 grown on potato dextrose agar for 7 days.

Bar represents 10 μm.

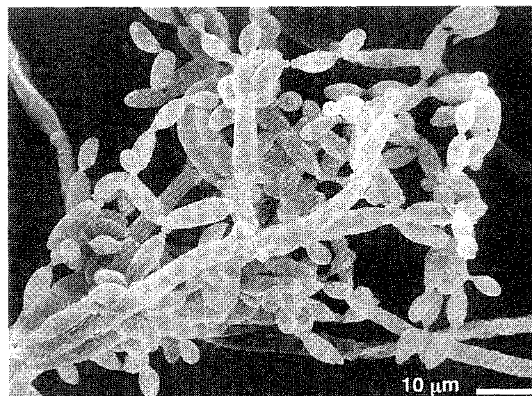

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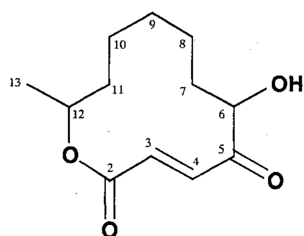
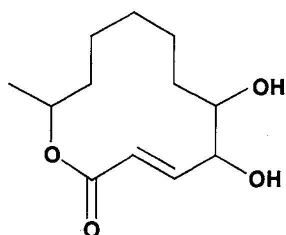
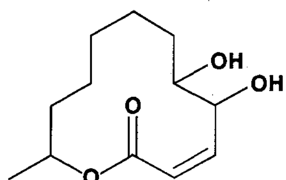
Fig. 2. Structures of cladospolides.







obtained with a JASCO DIP-370 digital polarimeter. Melting points were measured with a Yanaco micro melting point apparatus. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer. HPLC was carried out using the JASCO (TRI ROTAR V) system. Gas chromatography was carried out using the Shimadzu (GC-14A) system with a flame ionization detector (FID).

Taxonomic Studies

For the identification of the fungus, potato dextrose agar (Difco), malt extract agar, CZAPEK's agar, corn meal agar (Difco) and YpSs agar (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 2.0%, pH 6.0) were used. The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL).

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (6 mm, ADVANTEC). Bacteria were grown on Mueller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

The effect of cladospolide D on the growth of several microoganisms was determined using 96-well microplates[5,6] and the following media: peptone 0.5% and meat extract 0.5% (pH 7.0) for *Xanthomonas oryzae* and *Staphylococcus aureus*; and glucose 1.0% and yeast extract 0.5% (pH 6.0) for *Pyricularia oryzae*. Microorganisms were inoculated in each well at about $2\times10^4\sim1\times10^5$ cells/ml and incubated in the presence of the drug (0~150 μg/ml). The growth was measured as OD_{600} after a 24-hour incubation at 27°C for *X. oryzae*, after a 24-hour incubation at 37°C for *S. aureus*, or after a 48-hour incubation at 27°C for *P. oryzae*.

Results and Discussion

Characteristics of the Producing Strain FT-0012

Strain FT-0012 was isolated from a sponge sample collected in Pohnpei island. Morphological properties were examined after incubation at 25°C for 7 days on potato-dextrose agar (Fig. 1), malt extract agar, corn meal agar and Miura's medium. This organism grew moderately on various agar media, and formed olive gray to grayish brown colonies (diameter of colonies, 40~50 mm). The colony surface was velvety to floccose. Reverse of the colonies was brownish gray to olivaceous brown. The conidiophores (2.7~5.0×140~400 μm in size) were born from the vegetative hyphae, erect, pigmented, and unbranched. The blastconidia (2.5~3.0×3.5~5.5 μm in size) were ellipsoidal to lemon-shaped, conidial chains were very fragile and broken up readily into units, and scars of blastconidia were observed.

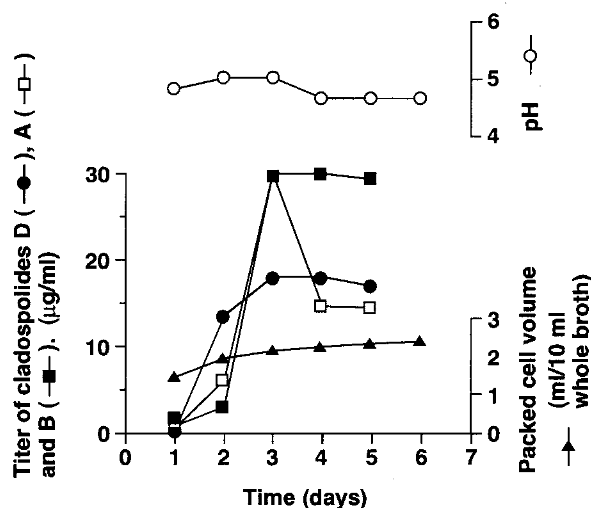
From these morphological characteristics, strain FT-0012 was considered to belong to the genus *Cladosporium*.

Fermentation

A slant culture of strain FT-0012 grown on YpSs agar was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml (50% sea water) of a seed medium (glucose 2.0%, yeast extract (Oriental Yeast Co.) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Daigo Nutritive Chemicals) 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker (210 rpm) for 3 days at 27°C.

One ml of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml (50% sea water) of the production medium (potato dextrose broth (Difco) 2.4%). The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. The production of cladospolides A, B and D was measured by HPLC (Senshu PEGASIL ODS (6.0×50 mm), 40% aq CH_3CN, UV at 220 nm, 1.5 ml/minute). Under these conditions, cladospolides A, B and D were eluted with retention times of 6.0, 7.0 and 12.0 minutes, respectively. The production of cladospolide D was observed at day 1 after inoculation, and reached a maximum at day 3.

Fig. 3. Time course of cladospolides production by *Cladosporium* sp. FT-0012 in a 500-ml Erlenmeyer flask.

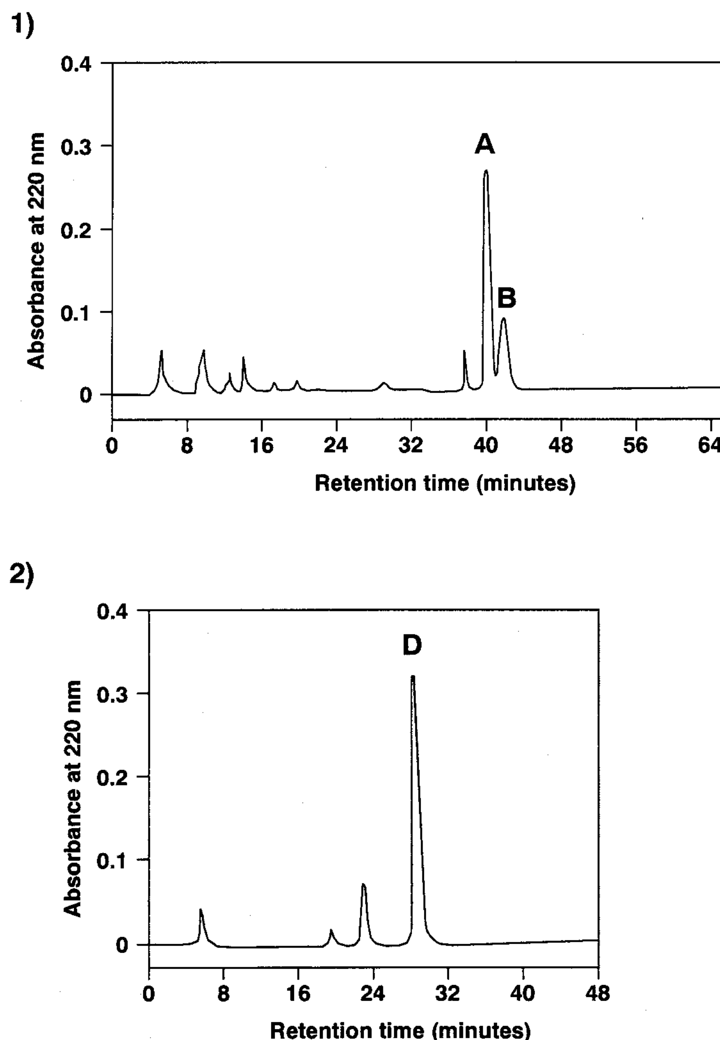


Isolation

The 6-day old whole broth (4 liters) was centrifuged at 3000 rpm for 15 minutes. The supernatant was extracted

Fig. 4. A chromatographic profile of cladospolides separated by preparative HPLC.

Column, YMC pack D-ODS-AM (20×250 mm), detection, UV at 220 nm; flow rate, 6.0 ml/minute; solvent, 1) 25% aq CH_3CN and 2) 40% aq CH_3CN.



with ethyl acetate (4 liters). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a red oil (445 mg). The mycelium was extracted with 600 ml of acetone. After centrifugation, the extracts were concentrated to remove acetone, and the remaining aqueous solution was extracted with ethyl acetate (600 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a red oil (485 mg). Both extracts were combined and subjected to an ODS column (Senshu SS 1020T, 110 g). The materials were eluted stepwise with 10%, 20%, 30%, 40%, 50%, 65%, 80% and 100% CH_3CN (300 ml each), and each 30 ml of the elution was successively collected. From the antimicrobial activity of each elute, two fractions, Fr. 1 (the 33rd~36th fractions), and Fr. 2 (the 41st~43rd fractions), were pooled.

Fr. 1 was concentrated and extracted with ethyl acetate to give a brown powder (152 mg), which was purified by preparative HPLC (YMC pack D-ODS-AM (20×250 mm), 25% aq CH_3CN, UV at 220 nm, 6.0 ml/minute). Under these conditions, cladospolides A and B were eluted with retention times of 40 and 42 minutes, respectively (Fig. 4), each of which was concentrated and extracted with ethyl acetate to give cladospolides A (11.7 mg) and B (7.07 mg) as white powders.

Fr. 2 was concentrated and extracted with ethyl acetate to give a brown powder (20.6 mg), which was purified by preparative HPLC (YMC pack D-ODS-AM (20×250 mm), 40% aq CH_3CN, UV at 220 nm, 6.0 ml/minute). Under these conditions, cladospolide D was eluted as a peak with a retention time of 28 minutes (Fig. 4) to yield pure cladospolide D (6.15 mg) as a colorless oil.

Structure of Cladospolide D

The physico-chemical property is summarized in Table 1. The molecular formula of cladospolide D was determined to be $C_{12}H_{18}O_4$ on the basis of HREI-MS measurement. The ^{13}C NMR spectrum ($CDCl_3$) showed 12 resolved peaks (Table 2), which were classified into one methyl, five methylene, two *O*-methine, two sp^2 methine, and two carbonyl carbons by analysis of the DEPT spectra. The ^1H NMR spectrum displayed 17 proton signals (Table 2). To fulfill the molecular formula of cladospolide D, the presence of one hydroxyl group was suggested, which was

Table 1. Physico-chemical properties of cladospolides D, A and B.

	Cladospolide D	Cladospolide A	Cladospolide B
Appearance	Colorless oil	White powder	White powder
Molecular formula	$C_{12}H_{18}O_4$	$C_{12}H_{20}O_4$	$C_{12}H_{20}O_4$
Molecular weight	226	228	228
FAB-MS (*m/z*) Positive	227 $[M+H]^+$	229 $[M+H]^+$	229 $[M+H]^+$
	249 $[M+Na]^+$	251 $[M+Na]^+$	251 $[M+Na]^+$
Negative	225 $[M-H]^-$	227 $[M-H]^-$	227 $[M-H]^-$
HRFAB-MS (*m/z*) (positive)			
MF+H	$C_{12}H_{19}O_4$	$C_{12}H_{21}O_4$	$C_{12}H_{21}O_4$
Calcd:	227.1283	229.1440	229.1440
Found:	227.1280	229.1429	229.1431
UV $\lambda_{max}^{CH_3OH}$ nm (ε)	204 (22400)	217 (10700)	211 (5700)
IR ν_{max}^{KBr} (cm^{-1})	3585, 2935, 2859, 1722, 1633, 1463, 1380, 1288, 1168	3470, 3340, 2925, 2850, 1710, 1640, 1270, 1160	3300, 2950, 2870, 1709, 1638, 1460, 1290, 1280, 1210
$[\alpha]_D^{28}$ (*c* 0.1, CH_3OH)	+56°	-30°	+27°
Solubility			
Soluble:	CH_3OH, $CHCl_3$, CH_3CN, Acetone, EtOH, EtOAc	CH_3OH, $CHCl_3$, CH_3CN, Acetone, EtOH, EtOAc	CH_3OH, $CHCl_3$, CH_3CN, Acetone, EtOH, EtOAc
Insoluble:	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane
Color reaction			
Positive:	50% H_2SO_4	50% H_2SO_4	50% H_2SO_4
Negative:	Ninhydrin reagent	Ninhydrin reagent	Ninhydrin reagent

Table 2. NMR chemical shifts of cladospolide D.

Carbon No.	^{13}C chemical shifts ppm[a)]	^{1}H chemical shifts ppm[b)]
C-2	165.4	
C-3	130.9	6.31 (1H, d,J =13.5 Hz)
C-4	133.3	6.41 (1H, d,J =13.5 Hz)
C-5	203.5	
C-6	73.46	4.66 (1H., dd$J$ =8.0, 4.0 Hz)
C-7	31.03	1.94 (1H, m), 1.44 (1H, m)
C-8	21.58	1.70 (1H, m), 1.35 (1H, m)
C-9	21.46	1.40 (1H, m), 1.33 (1H, m)
C-10	22.97	1.32 (1H, m), 1.46 (1H, m)
C-11	33.15	1.66 (2H, m)
C-12	71.50	5.23 (1H, td,J =6.5, 1.5 Hz)
C-13	20.59	1.31 (3H, d,J =6.5 Hz)

a)The sample was dissolved in $CDCl_3$. Chemical shifts are shown with reference to $CDCl_3$ as 77.7 ppm. b) Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm.

Fig. 5. COSY, HOHAHA and HMBC experiments of cladospolide D.

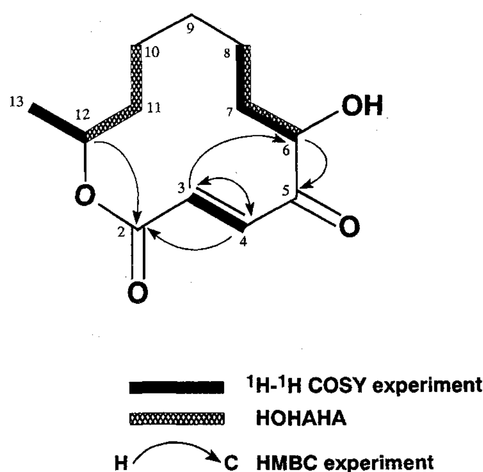
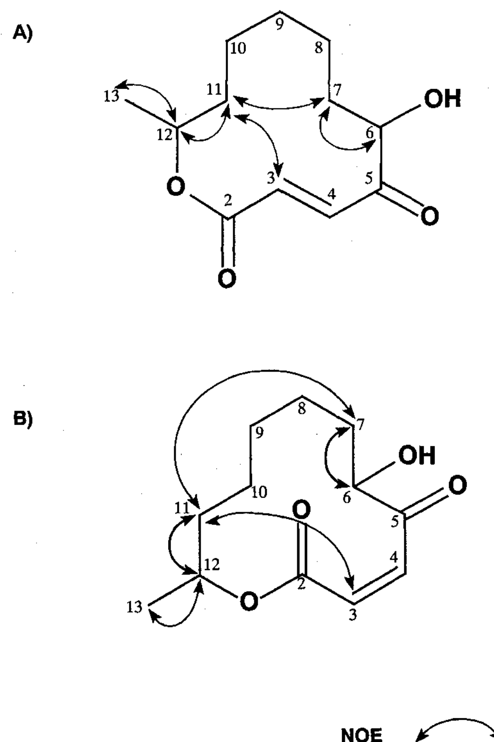


Fig. 6. NOE experiments of cladospolide D.



supported by the fragment ion peak of m/z 209 $[M+1-H_2O]^+$ in the FAB-MS spectrum. The connectivity of proton and carbon atoms was established by the ^{13}C-^{1}H HMQC spectrum (Table 2). Analyses of ^{1}H-^{1}H COSY and ID homonuclear Hartmann-Hahn (HOHAHA) spectra revealed the three partial structures (Fig. 5). Taking into further consideration the chemical shifts including the C-9 (δ 21.46), H_2-9 (δ 1.33, 1.40) in comparison with those of cladospolides[4)] and the degree of unsaturation, it was concluded that cladospolide D is a 12-membered macrolide. From the molecular modeling for cladospolide D, two types (A) like cladospolide A and (B) like cladospolide B of geometry for C-3 are proposed as illustrated in Fig. 6. Observation of NOE between H_2-11 (δ 1.66) and H-3 (δ 6.31)/H_2-7 (δ 1.94) revealed that the A type of geometry with E configuration is reasonable for cladospolide D.

Taken together, the structure of cladospolide D was elucidated to be (E)-2-dodecen-5-hydroxy-11-olide-4-one (Fig. 2).

Biological Properties

Biological Activities

First, antimicrobial activity was tested using paper disks at a concentration of 1000 μg/ml (10 μg/disk). Cladospolide D was active against *Pyricularia oryzae* KB180 (diameter of inhibition zone: 16 mm) and *Mucor racemosus* KF223 (IFO 4581) (12 mm). No or very weak antimicrobial activity was observed against other microorganisms as listed in Table 3.

The inhibitory effect on the growth of the four microorganisms was confirmed in a liquid culture using 96-

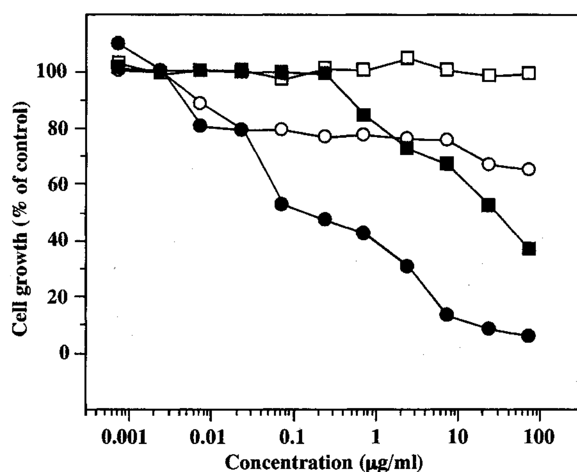
Table 3. Antimicrobial activity.

Test organism	a) Paper disk method			b) Liquid culture method		
	Diameter of inhibition zone (mm)[a] at 10 μg/6 mm disk			IC_{50} (μg/ml)		
	Cladospolides			Cladospolides		
	D	A	B	D	A	B
Staphylococcus aureus KB210	0	0	0	>100	>100	>100
Micrococcus luteus PCI 1001	0	0	0	NT	NT	NT
Bacillus subtilis KB27	14	0	0	>100	>100	>100
Mycobacterium smegmatis ATCC 607	0	0	0	NT	NT	NT
Xanthomonas campestris pv. oryzae	0	14	0	>100	17	>100
Acholeplasma laidlawii KB174	0	0	0	>100	>100	>100
Escherichia coli NIHJ	0	0	0	NT	NT	NT
Escherichia coli NIHJJC-2 IFO 12734	0	0	0	NT	NT	NT
Pseudomonas aeruginosa P-3	0	0	0	NT	NT	NT
Bacteroides fragilis ATCC 23745	0	0	0	NT	NT	NT
Pyricularia oryzae KB180	16	0	0	29	29	16
Candida albicans KF1	0	0	0	>100	>100	>100
Saccharomyces cerevisiae KF26	0	0	0	>100	>100	>100
Aspergillus niger KB103	0	0	0	>100	>100	>100
Mucor racemosus IFO 4581	11.5	0	0	0.15	>100	>100

[a] Determined by the agar diffusion test using paper discs of 6mm diameter (ADVANTEC).

Fig. 7. Inhibition of microbial growth by cladospolide D.

M. racemosus (●), *P. oryzae* (■), *B. subtilis* (○), and *X. oryzae* (□) were grown in each medium containing various concentrations of cladospolide D, and growth was plotted as percent of control growth in the absence of the inhibitor.



well microplates. The growth of *M. racemosus* and *P. oryzae* was inhibited by cladospolide D in a dose-dependent fashion (Fig. 7) with IC_{50} values of 0.15 and 29 μg/ml, respectively. The results are comparable to those by the paper disk method.

Acknowledgment

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