Exophilin A, a New Antibiotic from a Marine Microorganism *Exophiala pisciphila*

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Exophilin A, a new antibacterial compound, was discovered in the culture of the marine microorganism *Exophiala pisciphila* NI10102, which was isolated from a marine sponge *Mycale adhaerens*. The absolute chemical structure of exophilin A was elucidated as a trimer of (3*R*,5*R*)-3,5-dihydroxydecanoic acid by spectroscopic methods and analyses of a degradative product. Exophilin A showed antimicrobial activity against Gram-positive bacteria.

Certain species of fishes are known to utilize live invertebrates as a spawning bed. Their eggs deposited in the invertebrates such as a marine sponge seem to take advantage of predator avoidance, a constant supply of oxygen, and little interference by bacteria. It was reported that a little dragon sculpin *Blepsias cirrhosus* utilized a sponge *Mycale adhaerens* as a spawning bed to protect their eggs from predator and infection[1]. Their eggs deposited in the sponge kept unchanged even if they had been dead. This fact suggests presence of some antimicrobial compounds in the sponge. We supposed that symbiotic microorganisms in the sponge would contribute to production of the antimicrobial compounds. In the course of our screening project for bioactive substances from marine microorganisms, we discovered a new antibiotic compound, named exophilin A (**1**), from mycelia of *Exophiala pisciphila* NI10102 isolated from the sponge *M. adhaerens*. In this article, we describe the taxonomy, fermentation, isolation, physico-chemical properties, and structure elucidation of exophilin A.

Results and Discussion

Taxonomy of the Producing Organism

Morphological characteristics of the producing strain NI10102 are shown below (Fig. 2).

The strain NI10102 could grow on various culture media at 25°C: potato dextrose agar and malt agar. On these agar plates, conidia developed into mature colonies within 10 days, the colonies were initially yeast-like, and rapidly became filamentous, turning dark green to black. During this time, important morphological changes occurred, which included conidia germination, hyphal development, conidiation, melanization, and development of aerial hyphae.

Colonies on potato dextrose agar for 14 days at 25°C reached 10～18 mm in diameter, appeared smooth and floccose. Colony color was grayish white to olivaceous-gray on the surface and the reverse. The boundary region was gray to black. No soluble pigment was observed. Budding cells were absent. Hyphae were 5～20 μm long, 2～10 μm in diameter, smooth cylindrical cell in chain. Conidia were smooth, hyaline, thin-walled, ellipsoidal to globose, rarely separated, and 2～10 × 2～5 μm in size, and were produced by three means: mature hyphae, pre-existing conidia, and annellides.

From the characteristics stated above, the strain

Fig. 1. The structure of exophilin A (**1**).

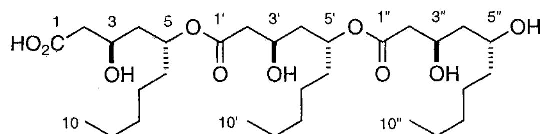
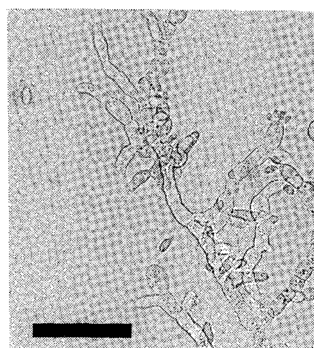


Fig. 2. Light micrograph of strain NI10102 on potato dextrose agar.

Bar represents 50 μm.



NI10102 was identified as *Exophiala pisciphila*[2,3]. This strain is a member of the so-called "black yeasts", a group presenting taxonomic problems, because of their developmental plasticity and the limited number of morphological characteristics available for classification. Annellidic conidiation is a distinctive characteristic, which separates *Exophiala* from other closely related genera.

This strain was deposited in the National Institute Science and Technology, Japan, under the name *Exophiala pisciphila* NI10102 and the accession number is FERM P-14232.

Fermentation

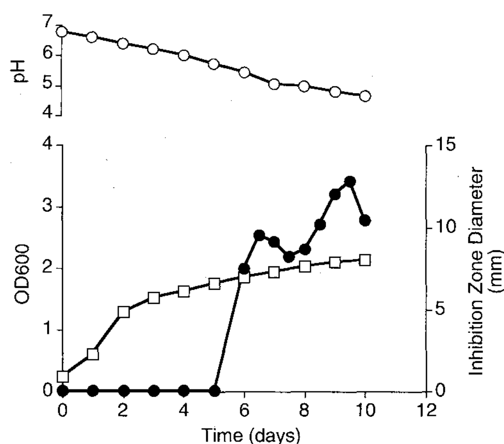
In Fig. 3 are shown time course of pH of the medium, OD at 600 nm, and production of antimicrobial substances evaluated by inhibition zone diameter against *Bacillus subtilis* ATCC 6633. The antibacterial activity of the broth in a 20-liter glass bottle fermenter drastically increased after 6 days and reached its maximum after 10 days of cultivation.

Isolation and Purification

Isolation was carried out with guidance of antimicrobial activity against *Bacillus subtilis* ATCC 6633 by usual paper disk method. The antibacterial compound was purified from the broth culture as described in the Experimental Section. Chloroform-methanol extract of the mycelium was subjected to silica gel column chromatography ($CHCl_3$-MeOH) followed by ODS column chromatography (CH_3CN - H_2O (10 : 1 to 20 : 1) containing 0.05% AcOH) and repeated ODS-HPLC (CH_3CN - H_2O (3 : 1) containing 0.05% AcOH) afforded the active compound, named exophilin A after the producing organism. Pure exophilin A (36.1 mg) was obtained from 15 liters of the whole broth.

Fig. 3. Time course of exophilin A fermentation.

pH (○), OD600 (□), inhibition zone diameter (●).



We initially chose CH_3CN - H_2O (3 : 1) containing 0.05% TFA (trifluoroacetic acid) as solvent system at the final stage purification. The active fraction obtained by the silica gel chromatography was further subjected to ODS-HPLC in this solvent system, no antimicrobial activity was recovered. By comparison of analytical HPLC chromatograms before and after the separation of the ODS-HPLC, the most intense peak (Rt 5.5 minutes) before the separation was almost disappeared and another peak (Rt 3.3 minutes) was enhanced. Changing the solvent system into CH_3CN - H_2O (3 : 1) containing 0.05% AcOH successfully prevented the active substance from this decomposition. We chose AcOH instead of TFA as acid at the final purification. This result indicated exophilin A was labile to TFA.

Structure Elucidation

The physico-chemical properties of exophilin A (**1**) are summarized in Table 1. Exophilin A did not show distinct $(M+H)^+$ ion in positive FAB-MS spectrum. Addition of sodium or potassium ion enabled us to observe $(M+Na)^+$ ion at *m/z* 599 or $(M+K)^+$ at *m/z* 615. Under a negative FAB mode, *m/z* 558, which possibly arose from water elimination, was observable. Therefore, we concluded that the molecular weight of exophilin A was 576 and it had a molecular formula $C_{30}H_{56}O_{10}$ which was established by high-resolution FAB-MS spectra on the basis of $(M+Na)^+$ ion peak (*m/z* 599.3775, $\Delta = +0.4$ mmu). The presence of ester linkages and hydroxyl groups were inferred from the NMR and the IR spectra (3412, 1728 cm^{-1}). Though the ^{13}C NMR spectrum displayed some overlapped signals, 30 carbon-resonances were detected by the HSQC spectrum.

Extensive 2D NMR analyses (HSQC, HMBC, HH-COSY) in $CDCl_3$ - CD_3OD (3 : 1) allowed us to assign three similar partial structures: 3,5-dihydroxydecanoyl moiety. The two of three C-5-positions were presumably acylated, because 5-H and 5'-H protons

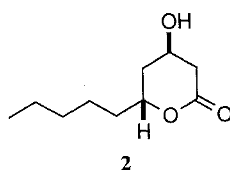
Table 1. Physico-chemical properties of exophilin A.

Appearance	Colorless viscous oil
$[\alpha]_D^{27}$ ($CHCl_3$)	−22.3° (*c* 1.00)
Molecular formula	$C_{30}H_{56}O_{10}$
LR FAB-MS	
Positive	599 $(M+Na)^+$
Negative	558 $(M-H_2O)^-$
HR FAB-MS	
Calcd for $C_{30}H_{56}O_{10}Na$:	599.3771
Found:	599.3775
IR ν_{max} (film) cm^{-1}	3412, 1728

Table 2. NMR data for exophilin A ($CDCl_3$-CD_3OD 3:1).

	δ_C	δ_H		δ_C	δ_H		δ_C	δ_H
1	172.9 s		1′	172.9 s		1″	172.9 s	
2	43.5 t	2.45 m	2′	43.3 t	2.38, 2.48m	2″	43.3 t	2.38, 2.48 m
3	66.5 d[a]	4.05 m	3′	66.7 d[a]	4.05 m	3″	69.0 d	4.10 m
4	41.8 t	1.68, 1.79 m	4′	41.9 t	1.68, 1.79 m	4″	43.7 t	1.54, 1.60 m
5	73.4 d	5.03 m[b]	5′	73.4 d	5.04 m[b]	5″	71.8 d	3.76 m
6	35.0 t	1.56 m	6′	35.0 t	1.56 m	6″	38.4 t	1.40 m
7	25.6 t	1.25 m	7′	25.6 t	1.25 m	7″	25.8 t	1.25 m
8	32.4 t[c]	1.27 m	8′	32.4 t[c]	1.27 m	8″	32.7 t[c]	1.27 m
9	23.3 t[d]	1.26 m	9′	23.4 t[d]	1.26 m	9″	23.4 t[d]	1.26 m
10	14.4 q	0.85 t J=7 Hz	10′	14.4 q	0.85 t J=7 Hz	10″	14.4 q	0.85 t J=7 Hz

a~d Assignment may be interchangeable.



appeared at a lower field (δ_H 5.03 and 5.04) than the rest of one did (5″-H, δ_H 3.76). The HMBC spectrum assisted this assignment: cross-peaks between 5-H and C-1′ and between 5′-H and C-1″ were clearly observed. Thus the planar structure of exophilin A was elucidated as a trimer of 3,5-dihydroxydecanoic acid connected with ester linkage between 1- and 5-position. NMR data are shown in Table 2.

In order to confirm the stereochemistry, we further investigated the degradation product of exophilin A which was obtained as a major peak newly appeared at the final purification by ODS-HPLC (CH_3CN-H_2O (3:1) containing 0.05% TFA) as mentioned above. (+)-(3*R*,5*R*)-3-Hydroxy-5-decanolide (**2**) was the major component of the degradative products. The relative structure of the hydrolysate was confirmed by 2D NMR spectra. The optical rotation of the obtained lactone was $[\alpha]_D^{26}$ +37.6° (*c* 0.31 $CHCl_3$), which was consistent with the reported value: $[\alpha]_D^{20}$ +32.1° (*c* 0.92 $CHCl_3$)[4)] or $[\alpha]_D^{24}$ +38.7° (*c* 1.4 $CHCl_3$)[5)]. Thus the absolute structure of exophilin A was completed as **1**.

Antimicrobial Activity

Antimicrobial spectra of exophilin A were shown in Table 3. Exophilin A showed antimicrobial activity against some Gram-positive bacteria.

Table 3. Antimicrobial spectra of exophilin A.

	MIC (μg/ml)	
	Exophilin A	NFLX[a]
Citrobacter freundii IFO 12681	>100	0.10
Enterobacter cloacae IFO 12935	>100	0.20
Escherichia coli IFO 12734 (NIHJ-JC-2)	>100	≤0.05
Klebsiella pneumoniae IFO 3317	>100	≤0.05
Proteus vulgaris GN-5298	>100	≤0.05
Pseudomonas aeruginosa IFO 3445	>100	0.78
Serratia marcescens IFO 3759	>100	0.20
Streptococcus epidermis IFO 13889	>100	0.39
Enterococcus faecalis IFO 12964	25	3.13
E. faecium IFO 12367	12.5	0.78
Staphylococcus aureus IFO 12732	50	0.20
MRSA4[b]	50	1.56
MRSA69[b]	50	25
		MCZ[c]
Candida albicans IFO-1269	>50	3.13
C. albicans IFM-40009	>50	3.13
Cryptococcus neoformans TIMM-0354	>50	≤0.20
C. neoformans TIMM-0362	>50	≤0.20
Aspergillus fumigatus TIMM-0063	>50	1.56
A. fumigatus IFM-4942	>50	1.56

a NFLX: Norfloxacin.
b MRSA: Methicillin resistant *Staphylococcus aureus*.
c MCZ: Miconazole.

Conclusion

A new antibiotic against Gram-positive bacteria, exophilin A (**1**), was discovered in the culture of *E. pisciphila* NI10102, which was isolated from a marine sponge *M. adhaerens*. Though many bioactive substances were isolated from this sponge to date[6)], there has been no report on substances similar to exophilin A. Interestingly, anti-microalgal substances, halymecins[7)], functionalized trimers or tetramers of 3,5-dihydroxydecanoic acid, were isolated from fungi *Fusarium* sp. and *Acremonium* sp. Exophilin A is expected to have the same activity. The role of exophilin A and the producing microorganism in the sponge is an interesting subject to be elucidated.

Experimental

General

Analytical TLC was performed on E. Merck silica gel plates 60 F_{254} in 0.25 mm-thickness (Art. 5715). Column chromatography was carried out by FL100D for silica gel and Chromatorex-ODS DM1020T for ODS (Fuji Silysia Chemical Ltd.). Analytical HPLC condition was as follows: TSKgel ODS-80Ts (4.6 mm i.d. × 150 mm, TOSOH); CH_3CN - H_2O (4 : 1) containing 0.05% TFA; 0.7 ml/minute; RI detection. NMR spectra were measured on a Varian XL-500 spectrometer (500 MHz for ^1H, and 125 MHz for ^{13}C) at 30°C unless otherwise noted. ^1H chemical shifts were referenced on the basis of residual solvent peaks: $CD_2\mathit{H}OD$ (δ 3.30) in $CDCl_3$ - CD_3OD, $C\mathit{H}Cl_3$ (δ 7.26) in $CDCl_3$. ^{13}C chemical shifts were referenced with respect to the ^{13}C signal of CD_3OD (δ 49.0), $CDCl_3$ (δ 77.0). Specific rotations ($[\alpha]_D$) were obtained on a JASCO DIP-370 digital polarimeter. FAB-MS spectra were taken on a JEOL JMS-SX102 mass spectrometer with 3-nitrobenzyl alcohol as matrix. IR spectra were recorded at a JASCO VALOR III FT-IR spectrometer.

Microorganism

The producing strain NI10102 was isolated from a sponge *Mycale adhaerens* collected off Usujiri (2 ~ 10-meter depth), Hokkaido, Japan. The isolation procedure was as follows: the sponge was cut into pieces and the 2 g-portion was shaken well with 5 ml of membrane-filtered (0.22 μm) sea water. Centrifugation (1000 rpm, 5 minutes) of this mixture afforded the supernatant. It was serially diluted with the filtered sea water (× 100 and × 10000) and 0.1 ml-portion was plated out on agar plates (prepared from glucose 10 g, L-asparagine 0.5 g, KH_2PO_4 0.5 g, bacto-agar 15 g, sea water 1 liter, pH 7.0 before sterilization). The plates were incubated at 25°C for one week and the colonies appeared on the plates were isolated.

Fermentation

The strain NI10102 grew on slants of potato dextrose agar. A loopful of the culture was transferred into a 200-ml Erlenmeyer flask containing 50 ml of seed medium sterilized at 121°C for 15 minutes. The medium composed of glucose 1.0%, L-asparagine monohydrate 0.05%, KH_2PO_4 0.05% in artificial sea water (pH 7.0 before sterilization). The inoculated flask was incubated on a rotary shaker (120 rpm, 4.0 cm-throw) at 25°C for 4 days. The seed culture was transferred into a 2000-ml Erlenmeyer flask containing 500 ml of the same medium, and incubated on a rotary shaker (120 rpm, 4.0 cm-throw) at 25°C for 4 days. All of the resulting cultured broth was transferred into a 20-liter glass bottle fermenter containing 15 liters of the same medium. Fermentation was carried out at 25°C for 10 days without agitation under aeration of 10 liters/minute.

Isolation

The cultured broth (15 liters) of *E. pisciphila* NI10102 was centrifuged at 3000 rpm to obtain the mycelium (50 g), which was extracted with $CHCl_3$ - MeOH (2 : 1) (2 liters) and filtered. To the filtrate was added H_2O (500 ml) and the organic layer was concentrated *in vacuo* to give a brown oily residue. The residue was dissolved in $CHCl_3$ and then subjected to silica gel column chromatography ($CHCl_3$ - MeOH stepwise gradient: 1 : 0, 20 : 1, 10 : 1, 4 : 1, 1 : 1, 0 : 1). The active fractions which were eluted with $CHCl_3$ - MeOH (10 : 1 and 4 : 1) were combined and further purified by repeated ODS column chromatography with CH_3CN - H_2O (10 : 1 then 20 : 1) containing 0.05% AcOH followed by ODS-HPLC (Lichrosorb RP-18, 10 mm i.d. × 250 mm, Merck; CH_3CN - H_2O (3 : 1) containing 0.05% AcOH; 2.0 ml/minute; RI detection) to obtain 36.1 mg of exophilin A (**1**) (Rt 16.0 minutes) (Rf 0.29: $CHCl_3$ - MeOH 10 : 1). Physico-chemical properties and NMR data are shown in Table 1 and 2, respectively.

Antimicrobial Activity

During the purification, antimicrobial activity was tested against *Bacillus subtilis* ATCC 6633 by agar diffusion test using paper disks (6 mm-diameter, thin, ADVANTEC). Bacteria were grown on agar plate containing yeast extract 0.25%, peptone 0.5%, glucose 1.0%, and agar 1.5%. The disks permeated with the test solution were placed on the agar plates, which were incubated at 37°C for 24 hours. Antimicrobial activity was estimated by measuring diameter of inhibition zone formed on the agar plate. Antimicrobial spectra of exophilin A were tested at Fuji Central Research Laboratory, Mochida Pharmaceutical Co., Ltd. (Table 3).

Degradative Product (2)

The active fractions (319 mg), obtained by silica gel column chromatography as described in the isolation procedure of exophilin A (**1**), was subjected to ODS-HPLC (Lichrosorb RP-18, 10 mm i.d. × 250 mm; CH_3CN - H_2O (5 : 1) containing 0.05% TFA; 2.0 ml/minute; RI detection) to afford the exophilin A-containing fraction as a single peak (Rt 12.8 minutes). Solvent was evaporated off at 40°C under reduced pressure to yield 52.9 mg of an oil which was then stored in a glass flask at −20°C for 4 days under nitrogen atmosphere. All the substance in the flask was further purified by medium pressure liquid chromatography on silica gel ($CHCl_3$ - MeOH 50 : 1 to 4 : 1, stepwise gradient) to afford 17.6 mg of (+)-(3*R*,5*R*)-3-hydroxy-5-decanolide (**2**): $[\alpha]_D^{26}$ +37.6° (*c* 0.31 $CHCl_3$); ^1H NMR ($CDCl_3$: recorded at 40°C) δ 4.69 (1H, m), 4.57 (1H, br s), 4.35 (1H, m), 2.72 (1H, dd, J = 18, 5 Hz), 2.61 (1H, ddd, J = 18, 3, 1 Hz), 1.96 (1H, br d), 1.75 (1H, m), 1.72 (1H, m), 1.59 (1H, m), 1.51 (1H, m), 1.40 (1H, m), 1.32 (4H, m), 0.90 (3H, t, J = 7 Hz); ^{13}C NMR ($CDCl_3$) δ 170.8 (s, C-1), 76.0 (d, C-5), 62.5 (d, C-3), 38.6 (t, C-2), 35.9 (t, C-4), 35.5 (t, C-6), 31.5 (t, C-8), 24.5 (t, C-7),

22.5 (t, C-9), 13.9 (q, C-10).

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