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Nafuredin, a Novel Inhibitor of NADH-fumarate Reductase, Produced by *Aspergillus niger* FT-0554

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A novel compound, nafuredin, was isolated as an inhibitor of anaerobic electron transport (NADH-fumarate reductase). It was obtained from culture broth of *Aspergillus niger* FT-0554 isolated from a marine sponge. The structure was elucidated as an epoxy-δ-lactone with an attached methylated olefinic side chain on the basis of spectral analysis.

NADH-fumarate reductase (NFRD) is a terminal electron transport system involved in a unique energy metabolic pathway found in many anaerobic organisms such as helminth[1~3]. This electron transport system is used to generate ATP in the absence of oxygen, which is different from an aerobic system[4]. It allows helminths to live in a microaerobic lumen with the host inside. Thus an inhibitor of NFRD is expected to be a selective anthelmintic. Though several compounds were reported to have NFRD inhibiting activity[5,6], they are weak or non-specific inhibitors. In the course of our screening for NFRD inhibitors, we found a novel compound, nafuredin (**1**, Fig. 1), from the culture broth of *A. niger* FT-0554 isolated from a marine sponge. Compound **1** inhibited NFRD of *Ascaris suum* (pig roundworm) at nM concentrations, while it showed very weak inhibition against a mammalian enzyme. Details of the inhibition and *in vivo* studies were described previously[7]. In this paper we describe the fermentation, isolation, structure elucidation, and biosynthesis of **1**.

Results

Taxonomy of Producing Strain FT-0554

The producing fungal strain was isolated from a marine sponge collected in Palau Islands, Republic of Palau.

Fig. 1. Structure of nafuredin (**1**).

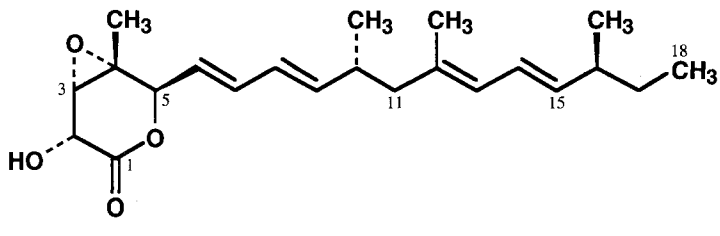

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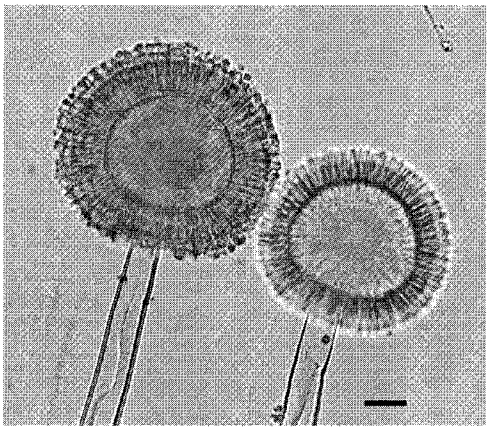
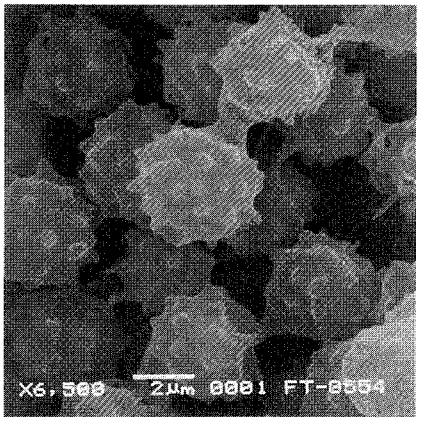
Fig. 2. Photomicrograph of aspergilla of strain FT-0554.



Bar represents 20 μm.

Fig. 3. Scanning electron microscope of conidia.



Bar represents 2 μm.

Morphological properties were examined after incubation at 25°C for 7 days on Czapek yeast extract agar, Czapek yeast extract agar with 20% sucrose, malt extract agar, and potato dextrose agar (Difco). This strain grew rapidly to form dark brown to black sporulated colonies with a diameter of 70~80 mm. Reverse color of the colonies was pale yellow to light olive gray on each medium. The colonies were granular to floccose on each medium. Soluble pigment was not produced. Morphological observations were carried out under a microscope (Fig. 2) and a scanning electron microscope (Fig. 3). Conidial heads were radiate and dark brown to black in color. Conidiophores were borne from substrate hyphae, 500~2,500×12~24 μm in size, smooth-walled hyphae, and hyaline to slightly brown in color. Vesicles were spherical and 35~60 μm in width. Aspergilla were almost biseriate formed metulae and phialides; metulae were covering the entire surface of the vesicle, measuring 8.5~11.5×2.5~3.5 μm, and phialides were flask-shaped and 5.5~8.5×2.8~3.3 μm in size (Fig. 2). Conidia were globose with echinulate walls, measuring 3.0~4.5 μm (Fig. 3).

From the above characteristics, strain FT-0554 was identified as *Aspergillus niger*[8] and named *Aspergillus niger* FT-0554. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-6443.

Fermentation of Nafuredin

A slant culture of the strain FT-0554 was scraped off with sterilized water and filtered to obtain spore suspension (10^7 spores/ml). One milliliter of the suspension was inoculated into each of one hundred 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of potato dextrose broth (Difco) 2.4% and natural seawater (34‰ salinity) 50%, and cultured on a rotary shaker (210 rpm) at 27°C for 7 days. Compound **1** was produced mainly in mycelia. Addition of the natural seawater enhanced the production of **1**. Improvement study of the fermentation will be published elsewhere.

Isolation

The isolation procedure of **1** is outlined in Fig. 4. The mycelial cake, collected by filtering whole broth (10 liters), was extracted with acetone, which was then removed from the extract by evaporation. This subsequent aqueous extract was further partitioned with an equal volume of *n*-hexane, and the organic layer was evaporated to dryness *in vacuo* to afford an oily residue (775 mg). The residue was chromatographed on a silica gel column by eluting with mixture of *n*-hexane and ethyl acetate (stepwise gradient). Active fractions eluted with *n*-hexane - ethyl acetate (10 : 2, v/v) were evaporated, and finally **1** was obtained as a white powder (18 mg) by precipitation in *n*-hexane.

Fig. 4. Isolation procedure of **1**.

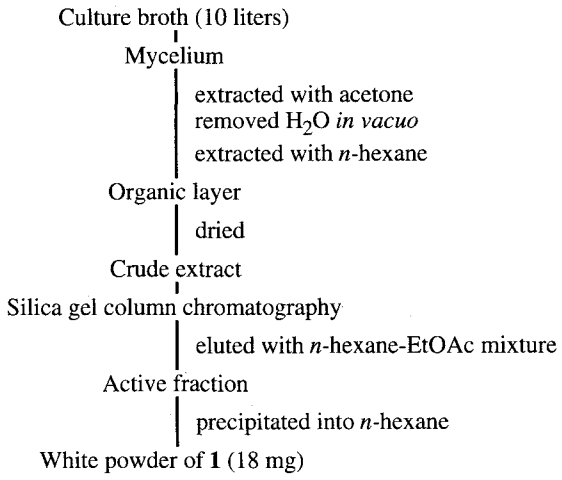


Table 1. Physico-chemical properties of **1**.

Appearance	White powder
Melting point	105°C
$[\alpha]_D^{25}$	+89.9° (*c* 0.1, $CHCl_3$)
Molecular formula	$C_{22}H_{32}O_4$
HR-FAB-MS (m/z)	found 361.2374 $(M+H)^+$
	calcd 361.2379 (for $C_{22}H_{33}O_4$)
UV λ_{max}^{EtOH} nm (ε)	253 (23,400)
IR ν_{max} (KBr) cm^{-1}	3444, 2962, 2924, 1736, 1182, 1120, 995
Solubility	
soluble	$CHCl_3$, EtOAc, EtOH
slightly soluble	*n*-hexane
insoluble	H_2O
Color reaction	H_2SO_4, I_2

Physico-chemical Properties

Physico-chemical properties of **1** are summarized in Table 1. Compound **1** showed strong absorption at 253 nm in UV spectrum. The IR spectrum of **1** suggested the presence of a hydroxyl group (3444 cm^{-1}) and a δ-lactone ring (1736 cm^{-1}). The molecular formula of **1** was established as $C_{22}H_{32}O_4$ by HR-FAB-MS of *m/z* 361.2374 $[M+H]^+$ (calcd 361.2376 for $C_{22}H_{33}O_4$). Chemical shifts in the ^1H and ^{13}C NMR of **1** are shown in Table 2.

Structure Elucidation

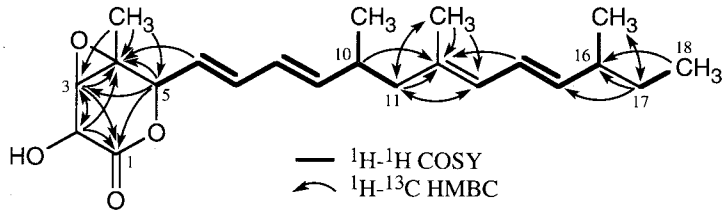
Analysis of the ^1H NMR, ^{13}C NMR, DEPT, and HMQC spectra revealed the presence of three quaternary carbons (one ester carbonyl carbon, one olefinic carbon, and one oxycarbon) and nineteen proton-bearing carbons (seven olefinic methine, three oxymethine, two methine, two methylene, and five methyl). A methylated olefinic hydrocarbon chain (C-5~C-18) was deduced from the ^1H-^1H COSY and HMBC analysis as shown in Fig. 5. The δ-lactone portion of the structure was elucidated mainly by HMBC. Cross peaks from a singlet proton of 4-CH_3 to oxygen-bearing three carbons, C-3, C-4, and C-5, elongated the hydrocarbon chain to C-3 with branching at 4-CH_3. Moreover cross peaks between H-2 and C-4, and between H-3 and C-1 revealed the alignment of C-1 to C-3. Finally the δ-lactone ring was deduced based on a cross peak observed between H-5 and C-1, and their respective chemical shifts. Two adjacent carbons (C-3 and C-4) were indicative of an epoxy ring from the index of hydrogen deficiency of **1** and the $^1J_{CH}$ values of C-2 (141 Hz) and C-3 (193 Hz). Acetylation of **1** afforded monoacetyl-**1** (**2**), which confirmed the presence of 2-OH, and thus the planar structure of **1** was elucidated.

The geometrical isomerisms of two dienes were elucidated as all *E* by the vicinal coupling constants of protons and the chemical shift of 12-CH_3 (δ_c 16.5)[9]. The relative configurations of the δ-lactone ring of **1** were

Table 2. ^1H and ^{13}C data of **1** (in $CDCl_3$).

Position	δ_C (mult)	δ_H [int, mult, *J* (Hz)]
1	170.6 (s)	
2	68.0 (d)	4.57 (1H, s)
3	58.5 (d)	3.52 (1H, s)
4	58.2 (s)	
5	80.2 (d)	4.94 (1H, d, 7.9)
6	122.0 (d)	5.49 (1H, dd, 7.9, 15.2)
7	137.9 (d)	6.38 (1H, dd, 10.2, 15.2)
8	126.0 (d)	6.02 (1H, dd, 10.2, 15.2)
9	145.2 (d)	5.80 (1H, dd, 6.9, 15.2)
10	34.8 (d)	2.43 (1H, m)
11	47.2 (t)	2.0 (1H, m), 2.1 (1H, m)
12	133.7 (s)	
13	127.0 (d)	5.76 (1H, d, 10.9)
14	124.6 (d)	6.18 (1H, dd, 10.9, 15.2)
15	138.9 (d)	5.46 (1H, dd, 7.6, 15.2)
16	38.6 (d)	2.1 (1H, m)
17	29.8 (t)	1.31 (2H, m)
18	11.8 (q)	0.86 (3H, t, 7.3)
4-CH_3	17.8 (q)	1.47 (3H, s)
10-CH_3	19.4 (q)	0.97 (3H, d, 3.6)
12-CH_3	16.5 (q)	1.71 (3H, s)
16-CH_3	20.2 (q)	1.00 (3H, d, 3.6)

Fig. 5. Selected HMBC and ^{1}H-^{1}H COSY correlation of **1**.

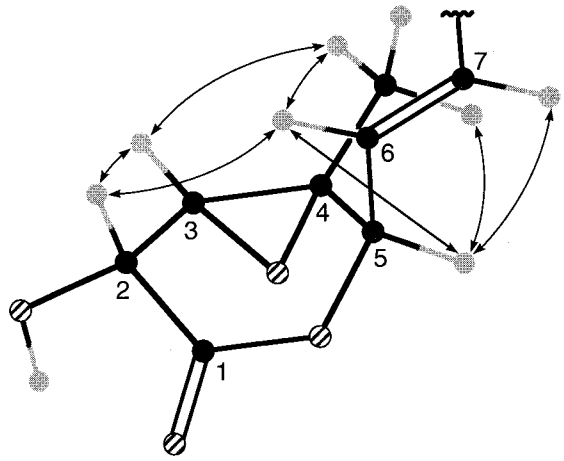


deduced from the NOESY experiment as shown in Fig. 6. The NOEs between 2-H and 3-H, between 3-H and 4-CH_3, between 4-CH_3 and 6-H, and between 6-H and 2-H, suggest that 2-H, 3-H, 4-CH_3, and C-6 are on the same face (all *cis* configuration).

Consequently, the structure of **1** was elucidated as (2*R**,3*S**,4*S**,5*R**,6*E*,8*E*,12*E*,14*E*)-3,4-epoxy-2-hydroxy-4,10,12,16-tetramethyl-6,8,12,14-octadecatetraeno-5-lactone. Absolute configuration of **1** (Fig. 1) will be reported by TAKANO *et al.*[10]. Natural compounds having β,γ-epoxy-δ-lactone were only found in clerodane furanoditerpene group, such as palmarin[11] and fibraurin[12], but their δ-lactones are parts of fused rings. So **1** is the first natural compound having monocyclic β,γ-epoxy-δ-lactone.

We studied the biosynthesis of this biologically and structurally interesting compound by ^{13}C NMR spectra of ^{13}C labelled **1**. The labelling patterns for **1** as results of the labelling experiments with [1-^{13}C] and [2-^{13}C]acetates and [methyl-^{13}C]methionine are shown in Fig. 7. The data indicated that C-1~C-18 carbons were derived from nine acetates and branched four methyl carbons were derived from methionines (Fig. 7).

Fig. 6. Selected correlations in NOESY experiment of **1**.



Experimental

General

Morphological observations were carried out using Olympus Vanox-S AH-2 microscope and JEOL JSM-5600 scanning electron microscope.

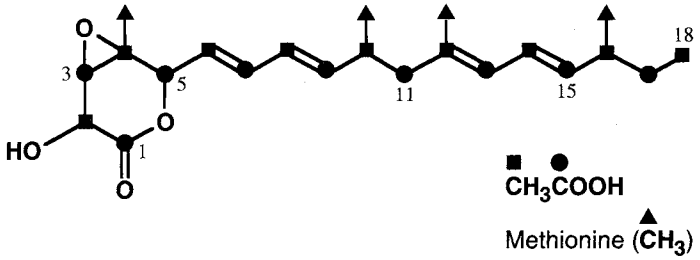
NMR spectra were recorded on a JEOL JNM-EX270 spectrometer ($^{2\text{-}3}J_{CH}$=8 Hz in HMBC). Chemical shifts are shown in δ values (ppm) relative to $CDCl_3$ at 7.26 ppm for ^{1}H NMR and at 77.0 ppm for ^{13}C NMR. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively. Optical rotation was recorded on a JASCO model DIP-181 polarimeter. Melting point was measured with a Yanaco micro melting point apparatus MP-S3.

Acetylation of 1

A mixture of **1** (3.0 mg) and acetic anhydride (0.5 ml) in pyridine (0.5 ml) was kept at room temperature for 1 hour. The reaction mixture was concentrated and partitioned between EtOAc and water at pH 3. The organic layer was dried over Na_2SO_4 and concentrated to yield a white powder of **2** (acetylnafuredin, 3.2 mg).

2: HR-FAB-MS (*m/z*) found 425.2300 $(M+Na)^+$, calcd 425.2304 (for $C_{24}H_{34}O_5Na$); IR ν_{max} (KBr) cm^{-1} 3440, 2960, 2930, 1770, 1230; ^{1}H NMR (270 MHz) δ 6.39 dd (1H, *J*=10.2, 15.0 Hz, H-7), 6.18 dd (1H, *J*=11.0, 15.0 Hz, H-14), 6.03 dd (1H, *J*=10.2, 15.2 Hz, H-8), 5.82 dd (1H, *J*=6.9, 15.2 Hz, H-9), 5.77 d (1H, *J*=11.0 Hz, H-13), 5.70 s (1H, H-2), 5.53 dd (1H, *J*=7.9, 15.0 Hz, H-6), 5.47 dd (1H, *J*=7.9, 15.0 Hz, H-15), 4.92 d (1H, *J*=7.9 Hz, H-5), 3.42 s

Fig. 7. Incorporation of ^{13}C-acetates and ^{13}C-methionine into **1**.



Each labelled compound (0.5 mg/ml of sodium [1-^{13}C]acetate, 0.5 mg/ml of sodium [2-^{13}C]acetate, and 0.25 mg/ml of [methyl-^{13}C]methionine) was fed two days after the fermentation. Marked carbons were enriched at least two times for [1-^{13}C]acetate, two times for [2-^{13}C]acetate, and thirty times for [methyl-^{13}C]methionine.

(1H, H-3), 2.43 m (1H, H-10), 2.24 s (3H, 2Ac-CH_3), 2.0~2.2 m (3H, H_2-11 and H-16), 1.71 s (3H, 12-CH_3), 1.48 s (3H, 4-CH_3), 1.31 dq (2H, J=7.1, 7.3 Hz, H_2-17), 0.994 d (3H, J=6.6 Hz, 16-CH_3), 0.986 d (3H, J=6.6 Hz, 10-CH_3), 0.86 t (3H, J=7.3 Hz, H_3-18); ^{13}C NMR (67.5 MHz) δ 169.7 s (C-1), 164.0 s (2Ac-C=O), 145.1 d (C-9), 138.7 d (C-15), 138.0 d (C-7), 133.6 s (C-12), 126.9 d (C-13), 126.0 d (C-8), 124.6 d (C-14), 122.1 d (C-6), 79.6 d (C-5), 67.7 (C-2), 58.3 d (C-3), 56.9 s (C-4), 47.1 t (C-11), 38.5 d (C-16), 34.7 d (C-10), 29.7 t (C-17), 20.5 q (2Ac-CH_3), 20.1 q (16-CH_3), 19.3 q (10-CH_3), 17.6 q (4-CH_3), 16.4 q (12-CH_3), 11.7 q (C-18).

Acknowledgements

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