Verticipyron, a New NADH-fumarate Reductase Inhibitor, Produced by *Verticillium* sp. FKI-1083

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Abstract A new NADH-fumarate reductase inhibitor, verticipyron, was isolated from the cultured broth of a fungus, *Verticillium* sp. FKI-1083. The structure was established as (*E*)-2-methoxy-3,5-dimethyl-6-(3-methyl-2-undecenyl)-4*H*-pyran-4-one. Verticipyron exhibited an IC₅₀ value of 0.88 nM against NADH-fumarate reductase of *Ascaris suum*. Verticipyron inhibited both *Ascaris* and bovine heart complex I, and its synthetic analogue, 8,9-dihydro-8-hydroxyverticipyron, showed good selectivity against *Ascaris* complex I.

Keywords verticipyron, electron transport enzyme inhibitor, NADH-fumarate reductase

Introduction

Several electron transport inhibitors are in common practical use. A complex II inhibitor, siccanin, produced by *Helminthosporium siccans*, is used clinically for dermatophytosis, and analogues of a complex III inhibitor, strobilurin A, produced by *Strobilurus tenacellus*, are used for phytopathogenic fungi. In the course of

screening for anthelmintic antibiotics, we have been interested in the differences in energy metabolisms between the host and helminths [1]. The NADH-fumarate reductase (NFRD) system, which is found in many anaerobic organisms, is part of a special respiratory system in parasitic helminths [2]. The system is composed of complex I (NADH-rhodoquinone oxidoreductase) and complex II (rhodoquinol-fumarate oxidoreductase). Electrons from NADH are accepted by rhodoquinone through complex I, and then transferred to fumarate through complex II. Though this anaerobic electron transport system is inefficient, it can provide ATP in the absence of oxygen. During our screening for inhibitors of NFRD using *Ascaris suum* (roundworm) mitochondria, we obtained nafuredin, atpenins, and paecilaminol from cultured broth of fungi. Nafuredin is a selective inhibitor of helminth complex I which showed anthelmintic activity *in vivo* [3, 4]. Atpenins are the most potent complex II inhibitors, useful as tools for biochemical studies, though the inhibition is non-selective between helminths and mammals [5]. Paecilaminol is the first amino alcohol that has NFRD inhibitory activity [6].

Further screening for NFRD inhibitors led to the

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Fig. 1 Structures of verticipyrene (1) and related γ -pyrones (2–8).

isolation of a new compound, verticipyrene (1, Fig. 1), produced by a cultured broth of *Verticillium* sp. FKI-1083 [7]. The structure of 1 contains 2-methoxy-3,5-dimethyl- γ -pyrone similar to that of actinopyrone A (3) [8, 9], aureothin (4) [10–12], neo-aureothin (spectinabilin, 5) [13–15], kalkipyrene (6) [16], and podopyrones (7, 8) [17–19]. In this report, we describe the taxonomy of the producing strain and the fermentation, isolation, structure elucidation, and biological properties of 1. Selective inhibition of *A. suum* complex I by a synthetic analogue, 8,9-dihydro-8-hydroxyverticipyrene, (2) is also shown.

Results and Discussion

Taxonomy and Producing Strain FKI-1083

Strain FKI-1083 (Fig. 2) was isolated from a soil sample collected on Yakushima Island, Kagoshima Prefecture, Japan. The strain was taxonomically determined as genus *Verticillium* sp. The strain has been deposited at International Patent Organism Depository, the National

Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, as FERM BP-7804.

Fermentation and Isolation of Verticipyrene

A stock culture of the strain FKI-1083 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium. After incubation on a rotary shaker (200 rpm) at 27°C for 3 days, one milliliter of the seed culture was transferred into each of twenty 500-ml Erlenmeyer flasks containing 100 ml of a production medium. The fermentation was carried out on a rotary shaker (200 rpm) at 27°C for 7 days.

Mycelia were collected from the cultured broth (2 liters) by centrifugation. The pellet was treated with methanol, and the extracted methanol solution was evaporated *in vacuo* to remove methanol. The aqueous extract was partitioned with ethyl acetate, and the organic layer was concentrated to dryness *in vacuo* to afford a crude material (1.21 g). This was applied on a silica gel column (Merck Art. 7734) and washed with *n*-hexane-ethyl acetate (3 : 1). Active fractions eluted with *n*-hexane-ethyl acetate (1 : 1)

Fig. 2 Photomicrograph of phialide and conidia of *Verticillium* sp. FKI-1083. Bar represents 20 μm .

were concentrated to yield a crude material (81.0 mg), which was then chromatographed over another silica gel column. The column was packed with *n*-hexane - ethyl acetate (2:1) and active fractions were eluted with *n*-hexane - ethyl acetate (2:1). The elution afforded a colorless oil of **1** (61.1 mg)

Structure Elucidation of Verticipyrone

Physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was established as $\text{C}_{20}\text{H}_{32}\text{O}_3$ by HR-FAB-MS.

Chemical shifts of **1** in the ^1H and ^{13}C NMR are shown in Table 2. Analysis of the ^1H NMR, ^{13}C NMR, DEPT, and HMQC spectra revealed the presence of six quaternary, one methine, eight methylene, and five methyl carbons. A γ -pyrone ring was established on the basis of HMBC data (Fig. 3). A methoxy group 2-OCH₃ (δ_{H} 4.00) showed correlation to a polarized olefinic carbon C-2 (δ 164.5), and the C-2 correlated with a vinyl methyl 3-CH₃ (δ_{H} 1.80). The 3-CH₃ had further correlations to another polarized olefinic carbon C-3 (δ 100.1) and a conjugated carbonyl C-4 (δ 183.1). The C-4 carbonyl showed correlation to a vinyl methyl 5-CH₃ (δ_{H} 1.94), and the 5-CH₃ correlated with the second set of polarized olefinic carbons, C-5 (δ 118.7) and C-6 (δ 160.1). Therefore, a 2-methoxy-3,5-dimethyl- γ -pyrone ring was suggested, and UV absorption at 250 nm and IR absorption at 1670 cm^{-1} (C=O) are consistent with the previous data for related compounds [9, 11, 14–19]

The structure of 3-methyl-2-undecenyl side chain (C-7~C-17) attached to C-6 was deduced by ^1H - ^1H COSY and HMBC as shown in Fig. 3. The geometrical isomerism of the olefin was elucidated as *E* by the chemical shift of 9-CH₃ (δ_{C} 16.3) [20]. Correlations between H₂-16 (δ 1.28) and H₃-17 (δ 0.88) in ^1H - ^1H COSY and HMBC among 15,

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

Appearance	Colorless oil
Molecular formula	$\text{C}_{20}\text{H}_{32}\text{O}_3$
Molecular weight	320.47
HR-FAB-MS (<i>m/z</i>)	
found	343.2247 (M+Na) ⁺
calcd	343.2249 for $\text{C}_{20}\text{H}_{32}\text{O}_3\text{Na}$
UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ)	204 (20,500), 215 (sh, 13,720), 250 (10,460)
IR ν_{max} (KBr) cm^{-1}	2927, 2854, 1732, 1670, 1605, 1464, 1408, 1379, 1325, 1250, 1165
Solubility	
Soluble	CHCl_3 , EtOAc, MeOH
Insoluble	H_2O , <i>n</i> -hexane
Color reaction	
Positive	H_2SO_4 , I_2

Table 2 ^1H and ^{13}C data of **1**^a

Position	δ_{C} (mult)	δ_{H} (int, <i>J</i> (Hz))
2	164.5 s	
3	100.1 s	
4	183.1 s	
5	118.7 s	
6	160.1 s	
7	30.8 t	3.40 d (2H, 7.2)
8	118.2 d	5.26 t (1H, 7.2)
9	140.7 s	
10	40.5 t	2.06 t (2H, 7.6)
11	28.8 t	1.43 m (2H)
12	30.5 t	1.26 m (8H)
13	30.4 t	
14	30.2 t	
15	33.0 t	
16	23.7 t	1.28 m (2H)
17	14.4 q	0.88 t (3H 7.2)
2-OCH ₃	56.4 q	4.00 s (3H)
3-CH ₃	7.0 q	1.80 s (3H)
5-CH ₃	10.0 q	1.94 s (3H)
9-CH ₃	16.3 q	1.76 s (3H)

^a NMR spectra were recorded on a Varian Inova 600 spectrometer. Chemical shifts are shown in δ value (ppm) relative to CD_3OD at 3.30 ppm for ^1H NMR and at 49.8 ppm for ^{13}C NMR.

16, and 17 positions suggested the terminal linear structure of the hydrocarbon chain. Though the remaining two methylenes could not be assigned because protons from H₂-12 to H₂-15 showed almost the same chemical shifts, they should be included into the hydrocarbon chain. Thus,

the structure of **1** was elucidated as (*E*)-2-methoxy-3,5-dimethyl-6-(3-methyl-2-undecenyl)-4*H*-pyran-4-one (Fig. 1).

Biological Activities of Verticipyrene

We evaluated inhibitory activities of **1** against each complex using submitochondrial particles of *A. suum* and bovine heart (Table 3). Compound **1** inhibited NFRD from *A. suum* with an IC_{50} value of 0.88 nM and the inhibition was specific to NADH-rhodoquinone oxidoreductase (complex I). It also inhibited NADH-ubiquinone oxidoreductase (complex I) from bovine heart to the same extent as *A. suum* enzymes. However, the IC_{50} values differed greatly between NFRD and NADH-rhodoquinone oxidoreductase of *A. suum* and between NADH oxidase and NADH-ubiquinone oxidoreductase of bovine heart. This may be due to the difference of quinone concentration. Endogenous quinones were used for NFRD and NADH oxidase assays, while rhodoquinone and ubiquinone were added exogenously for NADH-quinone oxidoreductase assays. Therefore, the latter assays contained higher concentration of quinones, and competitive (or partially competitive) inhibitors may have reduced their inhibition.

We have accomplished the total synthesis of **1** and prepared some analogues of **1** [21]. Among them, 8,9-dihydro-8-hydroxyverticipyrene (**2**) showed potent inhibition against *Ascaris* complex I (IC_{50} =2.0 nM).

Fig. 3 Selected 1H - 1H COSY and HMBC correlations of **1**.

The inhibition of **2** against bovine heart complex I was 100 times less potent, and **2** exhibited good selectivity to *Ascaris* complex I. The similarity of the IC_{50} values between NFRD and NADH-rhodoquinone oxidoreductase of *A. suum* suggested that **2** may not be competitive to rhodoquinone at *Ascaris* complex I inhibition. Further studies are required to clarify the inhibitory mechanisms of these compounds.

Compound **1** is produced by a fungus and has 6-substituted-2-methoxy-3,5-dimethyl-4*H*-pyran-4-one. Such compounds are found from not only fungi but also cyanobacteria, actinomycetes, and plants. Among them, aureothin (**4**) was reported to have a complex I inhibitory activity [12]. We found that neo-aureothin (**5**) inhibited NFRD at IC_{50} value of 15 nM. A γ -pyrone with a side chain seems to be essential to inhibit complex I, but interestingly, **4** is not competitive to the structurally-related substrate, quinone [12].

Nematocidal and insecticidal activities of **1** were studied by a microplate assay using the free-living nematode *Caenorhabditis elegans* and brine shrimp *Artemia salina*. Minimum growth inhibitory concentrations of **1** against *C. elegans* and *A. salina* were 20 μ g/ml and 2.0 μ g/ml, respectively. Kalkipyrene (**6**) was reported to show similar toxicity against brine shrimp (LD_{50} =1 μ g/ml) [16]. As shown in Table 4, **1** exhibited moderate antimicrobial activity against Gram-positive bacteria. Actinopyrene A (**3**) exhibited weak antimicrobial activities against some Gram-positive bacteria and dermatophytes, in addition to coronary vasodilating activities in anesthetized dogs [9].

Experimental

General

NMR spectra were recorded on a Varian Inova 600

Table 3 Inhibition of NFRD and NADH oxidase by **1** and **2**

Origin	Enzyme	Complex	IC_{50} [nM]	
			1	2
<i>A. suum</i>	NADH-fumarate reductase	I+II	0.88	1.5
	NADH-rhodoquinone oxidoreductase	I	49	2.0
	Rhodoquinol-fumarate oxidoreductase	II	>100,000	>100,000
Bovine heart	NADH oxidase	I+III+IV	1.3	20
	NADH-ubiquinone oxidoreductase	I	46	200
	Succinate-ubiquinone oxidoreductase	II	>100,000	>100,000
	Ubiquinol-cytochrome c oxidoreductase	III	26,000	80,000

Table 4 Antimicrobial activity of **1**

Microorganisms	MIC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i> ATCC6538P	6.25
<i>Bacillus subtilis</i> ATCC6633	6.25
<i>Micrococcus luteus</i> ATCC9341	6.25
<i>Mycobacterium smegmatis</i> ATCC607	12.5
<i>Escherichia coli</i> NIHJ	>100
<i>Escherichia coli</i> IFO12734	>100
<i>Pseudomonas aeruginosa</i> IFO3080	>100
<i>Xanthomonas campestris</i> pv. <i>oryzae</i> KB88	>100
<i>Candida albicans</i> KF1	>100
<i>Saccharomyces cerevisiae</i> KF26	>100
<i>Aspergillus niger</i> ATCC6275	100
<i>Mucor racemosus</i> IFO4581	100
<i>Acholeplasma laidlawii</i> PG8 KB174	>50
<i>Bacteroides fragilis</i> ATCC23745	>50

spectrometer ($^2\text{-}^3J_{\text{CH}}=8\text{ Hz}$ in HMBC). Chemical shifts are shown in δ values (ppm) relative to CD_3OD at 3.30 ppm for ^1H NMR and at 49.8 ppm for ^{13}C NMR. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. The UV and IR spectra were measured with Shimadzu UV-240 spectrophotometer and Horiba FT-210 Fourier transform infrared spectrometer, respectively.

Taxonomic Studies of the Producing Organism

Morphological observations of the verticipyrene-producing strain were carried out using an Olympus Vanox-S AH-2 microscope.

Media

The seed medium consisted of glucose 2.0%, Polypepton (Nihon Pharmaceutical Co.) 0.5%, yeast extract (Oriental Yeast Co.) 0.2%, KH_2PO_4 0.1%, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 0.05%, and agar 0.1%, pH 5.7. The production medium consisted of potato dextrose broth (Difco) 2.4%, malt extract (Difco) 1.5%, $\text{MgPO}_4\cdot 8\text{H}_2\text{O}$ 0.5%, and agar 0.1%, pH 6.0.

Biological Studies

Submitochondrial particles were prepared from adult *A. suum* and bovine heart and used for electron transport enzyme assays. The enzyme assays were performed as described previously [3]. The assay method for nematocidal and insecticidal activities was reported previously [22]. The antimicrobial activity was measured by agar dilution method.

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