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A γ-Lactone Form Nafuredin, Nafuredin-γ, also Inhibits Helminth Complex I

Kazuro Shiomi, Hideaki Ui, Hideaki Suzuki, Hiroko Hatano, Tohru Nagamitsu, Daisuke Takano, Hiroko Miyadera, Tetsuo Yamashita, Kiyoshi Kita, Hideto Miyoshi, Achim Harder, Hiroshi Tomoda, Satoshi Ōmura

Abstract Nafuredin, a δ-lactone antibiotic, is a fungal metabolite showing selective helminth NADH-fumarate reductase inhibition, and whose target had been revealed as complex I. We found that nafuredin is easily converted to nafuredin-γ by weak alkaline treatment. The structure of nafuredin-γ was elucidated as a γ-lactone form of nafuredin with keto-enol tautomerism. Nafuredin-γ shows similar complex I inhibitory activity as nafuredin, and it also possesses anthelmintic activity *in vivo*.

Keywords nafuredin, complex I, NADH-fumarate reductase, anthelmintic antibiotic

Introduction

Microorganisms produce many useful antiparasitic agents [1~5]. We have carried out screening for inhibitors of NADH-fumarate reductase (NFRD) from microbial metabolites to find new class of anthelmintics. Differences in energy metabolisms between hosts and helminths are attractive targets for treatment of helminthiasis. NFRD is a part of electron transport system of a unique energy metabolism found in many anaerobic organisms such as helminths, and it is composed of complex I and complex II. Electrons from NADH are accepted by rhodoquinone through complex I (NADH-rhodoquinone oxidoreductase), and then transferred to fumarate through complex II (rhodoquinol-fumarate reductase). This electron transport is used to generate ATP in the absence of oxygen, which is different from aerobic one.

Therefore, we have screened NFRD inhibitors using mitochondria of *Ascaris suum* (pig roundworm), and obtained a novel inhibitor, nafuredin (**1**), from the cultured broth of *Aspergillus niger* FT-0554 isolated from a marine sponge [6, 7]. Compound **1** inhibited NFRD of *A. suum* at nanomolar levels, while it showed very weak inhibition to the mammalian enzyme [6]. Its target was revealed as complex I, and it also showed anthelmintic activity against *Haemonchus contortus* (barber pole worm) in *in vivo* trials using sheep. Thus, **1** is a new potential lead compound as a novel anthelmintic. Its structure is an epoxy-δ-lactone with

K. Shiomi (Corresponding author)**, H. Ui, T. Nagamitsu, D. Takano:** School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, E-mail: shiomik@pharm.kitasato-u.ac.jp
S. Ōmura (Correspoding author)**, H. Suzuki, H. Hatano, H. Tomoda:** The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, E-mail: omura-s@kitasato.or.jp
H. Miyadera, T. Yamashita, K. Kita: Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

H. Miyoshi: Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan
A. Harder: Bayer HealthCare AG, Animal Health-Research & Development-Parasiticides, Agricultural Center Monheim, D-40789 Monheim, Germany
H. Tomoda, S. Ōmura: Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
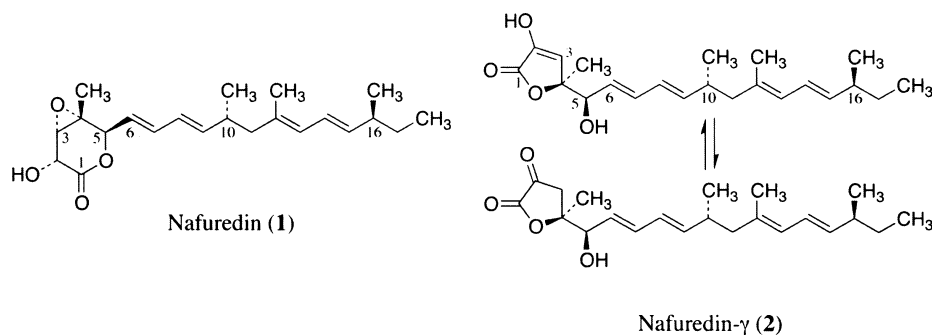
Fig. 1 Structures of nafuredin (**1**) and nafuredin-γ (**2**).

a methylated olefinic side chain [7]. The absolute configuration of **1** was elucidated by comparing ozonolysis products of **1** and their corresponding synthetic compounds [8], and total synthesis of **1** has been also achieved [9].

In the course of total synthesis of **1**, we found that **1** is easily converted to another compound **2** by weak alkaline treatment, and **2** also showed NFRD inhibitory activity. So we studied its structure and elucidated it as γ-lactone tautomers. The compound **2** was named nafuredin-γ. Here, we report the structure elucidation, enzyme inhibitory activity, and anthelmintic activity of **2**.

Results and Discussion

When $CaCO_3$ was added to methanol solution of **1**, **1** was readily converted to **2**. HR-FAB-MS of **2** revealed that its molecular formula is $C_{22}H_{32}O_4$, which was the same as that of **1**. The structure of **2** was elucidated by NMR study (Table 1 and Fig. 2). The side chain signals of **1** (from C-6 to C-18) were also observed in **2**, and it was confirmed by TOCSY and HMBC experiments. Many proton signals (from 3-H to 9-H) and carbon signals (from C-1 to C-13 and C-15) of **2** were duplicated, which suggested that the lactone moiety of **2** may have tautomerism.

We elucidated the structure of one tautomer (tautomer B) first. The TOCSY result revealed that a hydroxymethine ($\delta_{C\text{-}5}$ 77.0, $\delta_{5\text{-}H}$ 4.22, and $\delta_{5\text{-}OH}$ 5.11) is connected to C-6. ^1H-^{13}C long-range couplings of 3-H_2 (δ_H 2.55, 2.88)/C-4 (δ_C 84.9), 3-H_2/4-CH_3 (δ_C 23.3), 3-H_2/C-5, 4-CH_3 (δ_H 1.48)/C-3 (δ_C 40.6), 4-CH_3/C-4, 4-CH_3/C-5, and 5-H/C-4 were observed by HMBC, which indicated the alignment of CH_2–$C(CH_3)$–CHOH. Long-range couplings from 3-H_2 to C-1 (δ_C 161.5) and C-2 (δ_C 193.7) proved that C-1, C-2, and C-3 are aligned. Chemical shifts in ^{13}C NMR indicated that C-1 is an ester carbonyl carbon and C-4 is an oxycarbon, and they are suggested to be connected via

oxygen to form γ-lactone. Thus, the structure of tautomer B of **2** was elucidated as 5-hydroxy-4,10,12,16-tetramethyl-2-oxo-6,8,12,14-octadecatetraeno-4-lactone.

The structure of lactone moiety of tautomer A was similar to that of tautomer B. Long-range couplings by HMBC indicated the alignment of =CH–C(CH₃)–CHOH as shown in Fig. 2. C-3 (δ_C 121.5) of tautomer A is a methine instead of a methylene of tautomer B. A long-range coupling was observed between H-3 (δ_H 6.17) and C-1 (δ_C 169.0). Though C-2 signal (δ_C 144.2) was very broad and no correlation was observed, the chemical shifts of C-1 to C-4 suggested that they form γ-lactone. Thus, the structure of tautomer A of **2** was elucidated as 2,5-dihydroxy-4,10,12,16-tetramethyl-2,6,8,12,14-octadeca-pentaeno-4-lactone. Therefore, **2** was deduced to have keto-enol tautomerism at C-2. The structure of **2** was confirmed by its total synthesis [10].

The postulated conversion mechanism from **1** to **2** is shown in Fig. 3. An enol (**a**) would be formed under basic condition at first, and then the opening reaction of the epoxide would occur. The resulting *t*-alcohol (**b**) that cannot be isolated would successively afford γ-lactone (**2**) by translactonization.

Biological Activities

The IC_{50} values of **1** and **2** against *A. suum* NFRD were 9.7 and 6.4 nM, respectively. Since **1** was shown to inhibit helminth complex I selectively [6], we evaluated the inhibition of *A. suum* complex I by **2**. Compound **2** inhibited about two and ten times more potently than **1** against NADH-ubiquinone reductase and NADH-rhodoquinone reductase, respectively (Table 2). The inhibition of **2** against bovine liver complex I was very weak, and **2** did not inhibit *A. suum* complex II (succinate-ubiquinone reductase) at 1,000 nM as same as **1**. Therefore inhibitory activity of **2** against complex I was similar or

Table 1 ¹H and ¹³C NMR data of **2**

Position	Tautomer A (enol form)		Tautomer B (keto form)	
	¹³C[a]	¹H[a]	¹³C[a]	¹H[a]
1	169.0 s		161.5 s	
2	144.2 s		193.7 s	
3	121.5 d	6.17 s (1H)	40.6 d	2.55 d (1H, J=18.7 Hz)
				2.88 d (1H, J=18.7 Hz)
4	86.2 s		84.9 s	
4-CH₃	21.3 q	1.32 s (3H)	23.3 q	1.48 s (3H)
5	76.6 d	4.08 m (1H)	77.0 d	4.22 m (1H)
5-OH		4.32 br d (1H, J=4.0 Hz)		5.11 br d (1H, J=3.6 Hz)
6	130.0 d	5.58 dd (1H, J=7.2, 15.1 Hz)	128.7 d	5.61 dd (1H, J=7.2, 15.1 Hz)
7	133.8 d	6.25 dd (1H, J=10.5, 15.1 Hz)	135.0 d	6.34 dd (1H, J=10.5, 15.1 Hz)
8	128.6 d	6.02 dd (1H, J=10.5, 15.4 Hz)	128.3 d	6.05 dd (1H, J=10.5, 15.4 Hz)
9	141.4 d	5.61 dd (1H, J=7.2, 15.4 Hz)	142.4 d	5.67 dd (1H, J=7.2, 15.4 Hz)
10	35.6[b] d	2.40 m (1H)	35.5[b] d	2.40 m (1H)
10-CH₃	20.1[b] q	0.91 d (3H, J=6.9 Hz)	20.0[b] q	0.91 d (3H, J=6.9 Hz)
11	48.1[b] t	1.93 m (1H), 2.05 m (1H)	48.2[b] t	1.93 m (1H), 2.05 m (1H)
12	134.74[b] s		134.66[b] s	
12-CH₃	16.5 q	1.67 s (3H)	16.5 q	1.67 s (3H)
13	127.7[b] d	5.74 d (1H, J=10.7 Hz)	127.6[b] d	5.74 d (1H, J=10.7 Hz)
14	125.9 d	6.18 dd (1H, J=10.7, 15.1 Hz)	125.9 d	6.18 dd (1H, J=10.7, 15.1 Hz)
15	138.8[b] d	5.39 dd (1H, J=8.0, 15.1 Hz)	138.9[b] d	5.39 dd (1H, J=8.0, 15.1 Hz)
16	39.3 d	2.01 m (1H)	39.3 d	2.01 m (1H)
16-CH₃	20.5 q	0.93 d (3H, J=6.6 Hz)	20.5 q	0.93 d (3H, J=6.6 Hz)
17	30.4 t	1.25 m (2H)	30.4 t	1.25 m (2H)
18	12.0 q	0.80 t (3H, J=7.4 Hz)	12.0 q	0.80 t (3H, J=7.4 Hz)

[a] The acetone-d_6 signals (2.00 ppm of ¹H and 29.725 ppm of ¹³C) were used as references.

[b] Each chemical shift is exchangeable for the one of the corresponding carbon of tautomer.
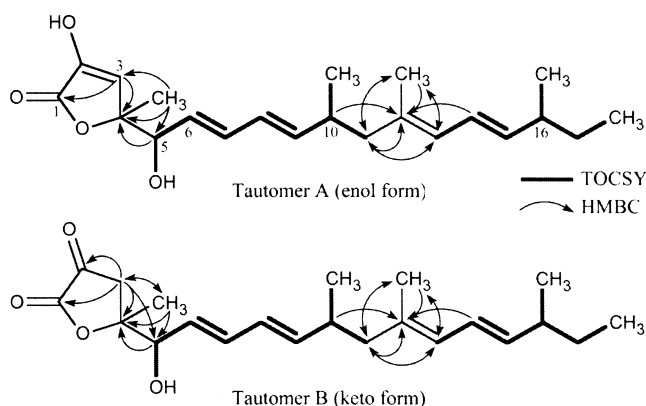
Fig. 2 TOCSY and selected HMBC correlations of **2**.

As **1** showed anthelmintic activity *in vivo* after a single oral treatment with 2 mg/kg [6], we analyzed the efficacy of **2** using *H. contortus* infected sheep. Here we used two treatments with **1** and **2** because **2** had no activity during the single treatment schedule. The first treatment was performed with an oral dosage of 2 mg/kg, given once. The second treatment with **1** was conducted one week after the first treatment, and the second treatment with **2** was performed at the same dosage after an interval of three weeks. Anthelmintic activity of **1** and **2** (two sheep treated each) against *H. contortus* is shown in Table 3. Although **2** did not suppress the egg output of female worms after the first treatment in contrast to **1** (reference 6 and Table 3), it reduced the number of faecal eggs about 92% eleven days after the second treatment. The result after the second treatment is nearly similar to that of **1**, and **2** is also expected to be an anthelmintic.

We are interested in studying whether **1** is converted into more potent than that of **1**, and the inhibition selectivity was also similar to **1**.

Fig. 3 The postulated conversion mechanism from **1** to **2**.
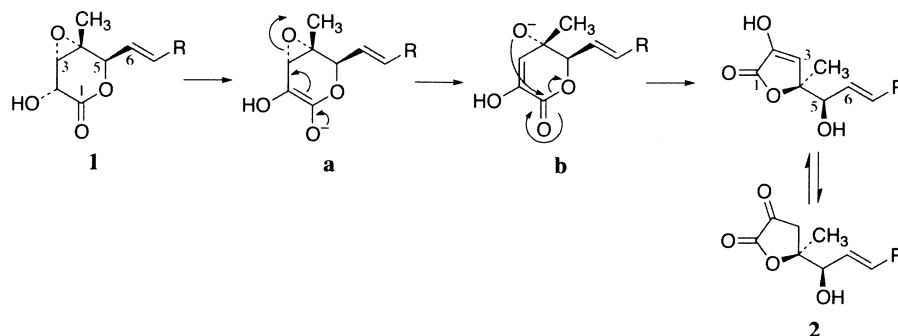
Table 2 Inhibition of complex I by **1** and **2**

Complex I	IC$_{50}$ (nM)	
	1	**2**
NADH-ubiquinone reductase (*A. suum*)	8.0	4.0
NADH-rhodoquinone reductase (*A. suum*)	24	2.3
NADH ubiquinone reductase (bovine liver)	100,000	>10,000

Fig. 4 Conversion of nafuredin (**1**) into nafuredin-γ (**2**) in neutral buffer.

Table 3 Effects of treatment with **1** or **2** on the faecal egg counts in sheep infected with *Haemonchus contortus*

	Number of eggs per gram of faeces[a]	
	1	**2**
Before treatment	6692 ± 50	5400 ± 1791
After the first treatment	1667 ± 2348	5676 ± 1325
After the second treatment[b]	11.1 ± 27	433 ± 935

[a] Values are means of faecal egg counts ± S.D.

[b] Second treatment with **1** was conducted one week after the first treatment and with **2** three weeks after the first treatment.

2 during enzyme assay. As **1** is hardly soluble in water, highly dilute solution of **1** (17 μM) in the assay buffer was incubated at 37°C. The buffer was used for the screening of NFRD inhibitors. After 30 minutes, the solution was extracted with ethyl acetate, concentrated, and analyzed by HPLC. No electron transport enzyme was added in this solution, because **1** and **2** were not extracted when the solution contained the enzyme. They were suggested to be adsorbed by the enzyme.

About 10% of **1** was converted to **2** as shown in Fig. 4. Since only a part of **1** was converted in this study, it is not
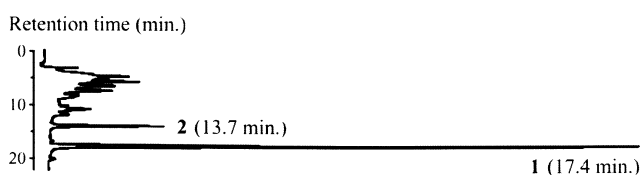
likely that **1** is converted into **2** and shows inhibitory activity. Therefore, both **1** and **2** may inhibit complex I directly. However, it is not certain whether **1** is converted to **2** when **1** is bound to complex I. It is necessary to continue the studies to know the inhibition mechanism.

Annonins are γ-lactone compounds isolated from seeds of *Annona squamosa* and known as complex I inhibitors [11]. Annonin group compounds are called acetogenins, and their inhibitory activity against complex I, cytotoxicity, and insecticidal activity are well studied [12, 13]. Acetogenins are characterized by an α,β-unsaturated γ-lactone with long aliphatic side chain at α position. The side chain contains 0~3 tetrahydrofuran rings and some hydroxyl moieties. Some acetogenins are rearranged to form γ-lactones with side chain at γ position, which are the same as **2**. However, **2** shows selective inhibition against helminth complex I, and the cytotoxicity of **2** is very weak compared to acetogenins. Therefore, structure difference between **2** and acetogenins may affect largely to their biological activities.

Experimental

General

NMR spectra were recorded on a Varian Inova 600 spectrometer ($^{2-3}J_{CH}$=8 Hz in HMBC). Chemical shifts are shown in δ values (ppm) relative to acetone-d_6 at 2.00 ppm

for ^{1}H NMR and at 29.725 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.

Conversion of 1 to 2

Compound **1** (10.0 mg) was dissolved in 0.28 ml of MeOH, and 2.0 mg of $CaCO_3$ was added in the solution. After the solution was stirred at room temperature for 30 minutes, 10 ml of EtOAc and 10 ml of saturated NaCl aq soln was added and mixed. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to yield a white powder of **2** (9.3 mg).

Nafuredin-γ (**2**): white powder; $[\alpha]_D^{26}$ +4.6° (*c* 1.0, CHCl$_3$); mp 101~103°; molecular formula $C_{22}H_{32}O_4$; HR-FAB-MS (*m/z*) found 383.2201 (M+Na)$^+$, calcd 383.2198 (for $C_{22}H_{32}O_4Na$); IR ν_{max} (KBr) cm^{-1} 3375, 2960, 2926, 1755, 1738, 1660, 1456, 1261, 1074, 1024, 800.

Biological Studies

NFRD activity was assayed using mitochondrial fraction of *A. suum*. *A. suum* muscle (11 g) was homogenized in 35 ml of homogenize buffer (0.14 M sucrose, 5 mM EDTA, 2.5 mM dithiothreitol, 0.15% bovine serum albumin, pH 7.4) and centrifuged at 1,000 *g* for 10 minutes to remove cell debris. The supernatant was further centrifuged at 10,000 *g* for 30 minutes and resulted mitochondrial precipitate was resuspended in assay buffer (120 mM sodium phosphate, pH 7.0). The assay buffer (80 μl) containing 0.35 mM NADH, 7.2 mM disodium fumarate, and 10 μl of DMSO solution of test compound was preincubated for 5 minutes at 37°C. The reaction was initiated by the addition of 10 μl of the mitochondrial fraction (0.3 mg protein/ml) and the incubation was carried out for 10 minutes at 37°C. The NFRD activity was measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm.

NADH-ubiquinone and NADH-rhodoquinone reductase assays were performed as described previously [6]. The following quinones were used for the assays: ubiquinone 1 for *A. suum* NADH-ubiquinone reductase, ubiquinone 2 for bovine liver NADH-ubiquinone reductase, and decylrhodoquinone for *A. suum* NADH-rhodoquinone reductase.

Method for sheep study was reported previously [6].

Incubation of Compound 1 in Assay Buffer

3 μg of **1** (8.3 nmole) was dissolved in 50 μl of DMSO, and 450 μl of the assay buffer (120 mM sodium phosphate buffer, pH 7.0) was added. It was incubated for 30 minutes at 37°C, and then extracted with EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. It was dissolved in 50 μl of 75% acetonitrile and 10 μl of the solution was injected to HPLC: Column, Pegasil ODS (Senshu Scientific Co.), i.d. 4.6×250 mm; mobile phase, 75% acetonitrile; temperature, 30°C; flow rate, 1 ml/minute; detection, UV 240 nm. Compounds **1** and **2** were eluted at 17.4 and 13.7 minutes, respectively.

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