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Ukulactones A and B, new NADH-fumarate reductase inhibitors produced by *Penicillium* sp. FKI-3389

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ABSTRACT

Screening for NADH-fumarate reductase inhibitors led to the isolation of the new polyketide compounds, ukulactones A and B (**1** and **2**, Fig. 1) from a culture broth of *Penicillium* sp. FKI-3389. The structure of ukulactone A was elucidated as a methylated derivative of prugosene A1, which was produced by *Penicillium rugulosum* and NOESY experiment revealed ukulactone B was a stereoisomer of ukulactone A. Ukulactone A showed potent inhibitory activity against NADH-fumarate reductase of the roundworm *Ascaris suum* in vitro.

1. Introduction

NADH-fumarate reductase (NFRD), consisting of mitochondrial complexes I and II, is an electron transport system involved in a unique energy metabolic pathway found in many anaerobic organisms, such as helminths.[1] This system is used to generate ATP in the absence of oxygen and allows helminths to live in anaerobic circumstances inside the host. Therefore, a selective inhibitor of NFRD is expected to be a good anthelmintic. We have screened for NFRD inhibitors in culture broths of fungi using helminth (*Ascaris suum*) mitochondria, and found some potent inhibitors, such as nafuredin,[2] atpenins,[3] verticipyrone,[4] and paecilaminol.[5] During this screening we obtained new NFRD inhibitors, ukulactones A and B (**1** and **2**, Fig. 1), from the culture broth of the terrestrial fungus *Penicillium* sp. FKI-3389. In this report, we describe the isolation, structural elucidation, and biological activities of these two ukulactones.

2. Results and discussion

2.1. Isolation and structure elucidation of ukulactones A (1) and B (2)

A solid culture of *Penicillium* sp. FKI-3389 was extracted with EtOAc and the extract was purified by silica gel column chromatography and HPLC. From 3 kg of the solid broth, 515 mg of **1** and 3.5 mg of **2** were isolated.

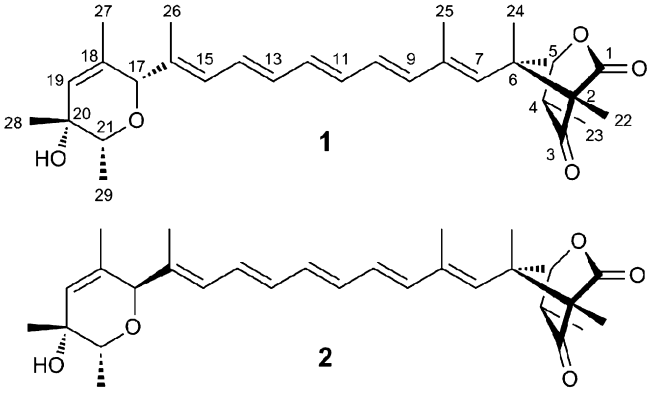

Fig. 1. Structures of ukulactones A (**1**) and B (**2**).

Ukulactone A (**1**) was obtained as a yellow syrup. The molecular formula was elucidated to be $C_{29}H_{38}O_5$ by HRFABMS (observed $[M]^+$ at 466.2722, calcd $[M]^+$ 466.2719). The characteristic absorption maxima at 325, 340, and 358 nm in the UV spectrum, strongly suggested that **1** has a pentaene in the structure. The IR absorptions indicated the presence of hydroxyl (3730 cm^{-1}) and carbonyl (1795 and 1745 cm^{-1}) groups.

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

Position	**1**[a]		**2**[b]	
	δ_H (Int., mult., *J* in Hz)	δ_C mult.	δ_H (Int., mult., *J* in Hz)	δ_C mult.
1		171.4 s		171.4 s
2		70.1 s		70.1 s
3		207.6 s		207.6 s
4	2.65 (1H, qd, 7.4, 2.2)	44.3 d	2.66 (1H, qd, 7.4, 2.2)	44.3 d
5	5.15 (1H, d, 2.2)	85.2 d	5.16 (1H, d, 2.2)	85.2 d
6		57.9 s		57.9 s
7	5.21 (1H, s)	127.7 d	5.22 (1H, s)	127.8 d
8		138.8 s		138.8 s
9	6.12 (1H, d, 14.4)	136.1 d	6.13 (1H, d, 14.4)	136.2 d
10	6.32 (1H, dd, 14.4, 10.9)	133.5 d	6.38 (1H, dd, 14.4, 10.9)	134.2 d
11	6.27 (1H, dd, 14.4, 10.9)	132.7 d	6.24 (1H, dd, 14.4, 10.9)	132.8 d
12	6.37 (1H, dd, 14.4, 10.9)	134.3 d	6.31 (1H, dd, 14.4, 10.9)	133.6 d
13	6.30 (1H, dd, 14.4, 10.9)	129.5 d	6.28 (1H, dd, 14.4, 10.9)	129.5 d
14	6.47 (1H, dd, 14.4, 10.9)	129.1 d	6.49 (1H, dd, 14.4, 10.9)	129.2 d
15	6.16 (1H, d, 10.9)	130.5 d	5.82 (1H, d, 10.9)	128.9 d
16		136.3 s		134.9 s
17	4.36 (1H, s)	85.2 d	4.30 (1H, s)	80.7 d
18		136.3 s		134.8 s
19	5.68 (1H, s)	130.8 d	5.66 (1H, s)	130.1 d
20		67.0 s		67.3 s
21	3.47 (1H, q, 6.3)	77.2 d	3.51 (1H, q, 6.3)	71.0 d
22	1.21 (3H, s)	4.8 q	1.21 (3H, s)	4.8 q
23	1.18 (3H, d, 7.4)	11.6 q	1.19 (3H, d, 7.4)	11.6 q
24	1.40 (3H, s)	16.8 q	1.40 (3H, s)	16.8 q
25	1.89 (3H, s)	14.3 q	1.90 (3H, s)	14.4 q
26	1.70 (3H, s)	12.0 q	1.90 (3H, s)	16.2 q
27	1.47 (3H, s)	18.5 q	1.62 (3H, s)	20.1 q
28	1.14 (3H, s)	23.5 q	1.13 (3H, s)	23.7 q
29	1.22 (3H, d, 6.3)	14.2 q	1.15 (3H, d, 6.3)	14.0 q

[a] Recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C).
[b] Recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C).

The ^{1}H and ^{13}C NMR data of **1** are shown in Table 1. Analysis of the ^{1}H and ^{13}C NMR, DEPT, and HSQC spectra revealed the presence of eight methyl groups, one sp^3 methine carbon, three quaternary carbons, three oxymethine carbons, nine sp^2 methine carbons, three sp^2 quaternary carbons, one carboxyl group, and one carbonyl group. The COSY correlations showed the presence of a spin system of seven olefin protons (from H-9 to H-15) in a pentaene moiety suggested by UV spectrum. The configurations of the olefins were assigned to be *trans* by their coupling constants (J=14.4 and 10.9 Hz). In the pentaene moiety, NOE correlations between H-7 and H-9 and between H-14 and H-26 were observed (Fig. 2). In addition, ^{13}C chemical shifts of the olefin methyl carbons C-25 (δ 14.3) and C-26 (δ 12.0) suggested the configuration of the pentaene moiety was all-*trans*.[6]

HMBC correlations from methyl proton H-25 to C-7, C-8, and C-9 and from H-26 to C-15, C-16, and C-17 were observed (Figs. 3 and 5), thus two methyl moieties were attached to C-8 and C-16 of the pentaene substructure of **1**, respectively. By the HMBC correlations in Fig. 3, another partial structure was deduced to be 4,6,7-trimethyl-2-oxabicyclo[2.2.1]heptane-3,5-dione contained in shimalactones A and B,[7] prugosenes A1–A3,[8] coccidiostatin A[9] and wartmannilactones E, F, and H.[10] The ^{1}H and ^{13}C chemical shifts of this substructure were in good accordance with those of shimalactones A and B in $CDCl_3$.[7] The HMBC correlations from the methyl proton H-24 revealed that this bicyclo unit connected to C-7 of the pentaene (Fig. 3). We conducted a NOESY experiment to analyze relative configuration of the bicyclo unit. The observed NOE correlations are shown in Fig. 4. The data indicated the relative configuration of this unit was (2*S*,4*R*,5*S*,6*S*) the same as prugosenes A1–A3.[8]

HMBC correlations (Fig. 5) clarified **1** had the 3,6-dihydro-3-hydroxy-2,3,5-trimethyl-2*H*-pyran ring also contained in prugosene A1[8] and wortmannilactones E and F.[10] The relative conformation of the dihydropyran ring was (17*S*,20*R*,21*R*), indicated by NOE correlations from H-17 to H-21, H-26 and H-27, and those from H-28 to H-19 and H-21 (Fig. 6). Therefore, ukulactone A was deduced to be an 8-methyl derivative of prugosene A1.

Ukulactone B (**2**) was also obtained as a yellow syrup. The molecular formula was elucidated to be $C_{29}H_{38}O_5$, the same value as ukulactone A (**1**) by HRFABMS. UV and IR spectra were almost identical, but the sign of $[\alpha]_D$, which was opposite to that of **1**, suggested **2** is a stereoisomer of **1**. As compared with ^{1}H and ^{13}C

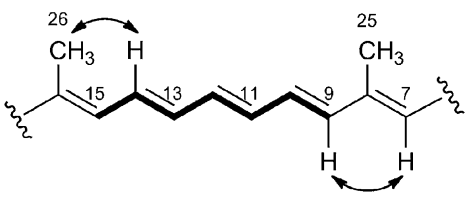

Fig. 2. COSY (bold line) and key NOESY (arrow) correlations of pentaene unit of **1**.

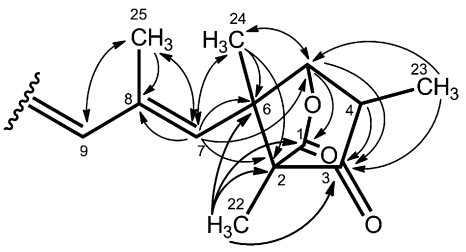

Fig. 3. HMBC correlations of the trimethyloxabicyclo[2.2.1]heptane unit of **1**.

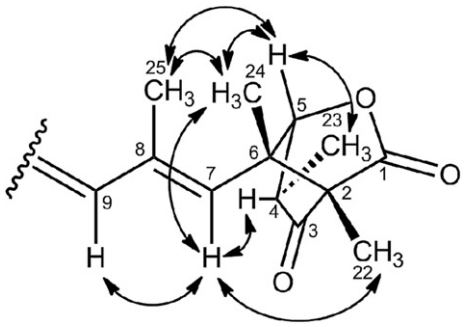

Fig. 4. NOESY correlations of the trimethyloxabicyclo[2.2.1]heptane unit of **1**.

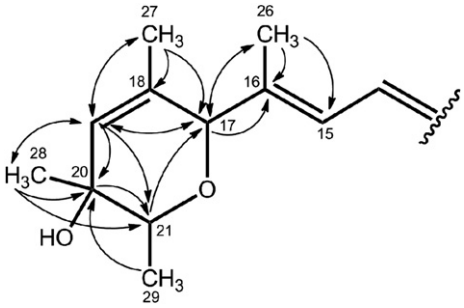

Fig. 5. HMBC correlations of the dihydropyran unit of **1**.

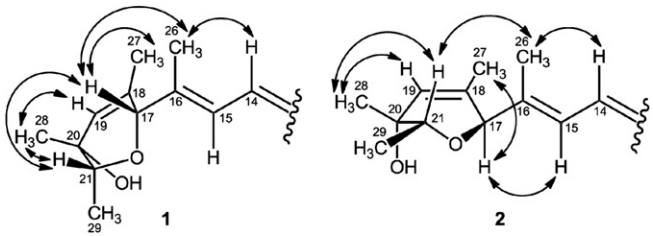

Fig. 6. NOESY correlations of the dihydropyran unit of **1** and **2**.

NMR spectra of **1**, the chemical shifts of signals assigned to the dihydropyran ring were changed from **1** (Table 1), although the HMBC correlations showed **1** and **2** had the same planar structure. These results suggested **2** had a different configuration in the dihydropyran ring moiety compared with **1**. This was elucidated by a NOESY experiment. The NOEs observed in the dihydropyran ring of **2** are shown in Fig. 6. Compared with the data of **1**, the NOE correlation between H-17 and H-21 is not observed and the correlation between H-15 and H-17 was only observed in **2**. Therefore, the relative configuration was defined as (17*R*,20*R*,21*R*). We also confirmed the 2-oxabicyclo[2.2.1]heptane unit had the same configuration (2*S*,4*R*,5*S*,6*S*) as that of **1** by analysis of NOESY data. From the above data **2** was elucidated to be the C-17 epimer (17*R*-epimer) of **1**. While the difference between two compounds was only the conformation of C-17, **2** was much more labile than **1**.

3. Biological activities

Inhibitory activities of **1** and **2** against electron transport system enzymes were evaluated using submitochondrial particles of *A. suum* and bovine heart (Table 2). Compound **1** inhibited NADH-fumarate reductase (NFRD, composed of complexes I and II) from *A. suum* with an IC_{50} value of 2.4 nM, although the inhibition against mammal enzymes NADH oxidase (constituted with complexes I, III, and IV) and succinate-cytochrome *c* reductase (constituted of complexes II and III) from bovine heart was weak, with IC_{50} values of 9000 nM and 68,000 nM, respectively. Next we examined the selectivity of inhibitory activity of **1** against each complex. Compound **1** inhibited NADH-rhodoquinone reductase (complex I) activity with an IC_{50} value of 55 nM, but it did not affect rhodoquinol-fumarate reductase (complex II) activity at 100 μM. On the other hand, **1** showed only weak inhibitory activities against each complex of bovine heart. Therefore **1** is concluded to be a selective inhibitor of helminth complex I, similar to nafuredin.[2a]

Table 2
Inhibition of electron transport enzymes by **1** and **2**

	Enzyme	Complex	IC_{50} (nM)	
			1	**2**
A. suum	NADH-fumarate reductase	I+II	2.4	470
	NADH-rhodoquinone reductase	I	55	NT
	Rhodoquinol-fumarate reductase	II	>100,000	NT
Bovine heart	NADH oxidase	I+III+IV	9000	16,000
	Succinate-cytochrome *c* reductase	II+III	68,000	30,000
	NADH-ubiquinone reductase	I	28,000	NT
	Succinate-ubiquinone reductase	II	>100,000	NT
	Ubiquinol-cytochrome *c* reductase	III	32,000	NT

NT: not tested.

The IC_{50} value of the epimer **2** against NFRD was 470 nM, about 200 times weaker than **1**. However **2** inhibited NADH oxidase and succinate-cytochrome *c* reductase at the IC_{50} values of 16,000 nM and 30,000 nM, respectively, which were similar to those of **1**. These data suggested **1** had more helminth specific-NFRD inhibitory activity compared to **2**. The specificity might be due to the conformation of the dihydropyran ring, which would be considerably changed by altering the configuration of C-17.

Furthermore, we obtained prugosene A1 from culture broth of another fungus *Penicillium* sp. FKI-5329, and measured the NFRD inhibitory activity.[11] The IC_{50} values of prugosene A1 against NFRD and NADH oxidase were 13 nM and 8600 nM, respectively, similar to those of **1**. The data suggested that the (17*S*)-epimer was more potent inhibitor and the conformation of dihydropyran ring was much more important than 2-oxabicyclo[2.2.1]heptane unit for *Ascaris* enzyme-specific inhibition. In addition, the results indicated the methyl moiety at C-8 did not affect the inhibitory activity.

We evaluated the cytotoxicity of **1** using human T-lymphocyte Jurkat cells by MTT method.[12] Morphological change and growth inhibition were observed by **1** at high concentration. The IC_{50} value of growth inhibition was 21 μM, about 8800 times weaker than that of NFRD inhibition (2.4 nM). Antimicrobial activities of **1** were tested by an agar dilution method.[13] As well as prugosenes,[8] **1** did not show antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi at 100 μg/mL (214 μM). Therefore, **1** and prugosene A1 are expected to be selective and potent NFRD inhibitors.

4. Experimental

4.1. General

UV spectra were measured by Hitachi UV/Vis spectrometer U-2810. Optical rotations were recorded by means of JASCO DIP-1000 polarimeter. FT-IR spectra were conducted on Horiba FT-170. NMR spectra were recorded on 300 MHz (Varian XL-300), 400 MHz (Varian XL-400), and 600 MHz (Varian Inova 600) NMR spectrometer. For calibration of ^{13}C and ^{1}H chemical shifts, the carbon signal and residual proton signal of $CDCl_3$ were used ($CDCl_3$: δ_H 7.26 ppm and δ_C 77.0 ppm). FABMS and HRFABMS spectra were measured on JEOL JMS-AX505HA. Normal phase HPLC analysis was

performed using Pegasil silica column (4.6φ×250 mm) applying $CHCl_3$/acetone (100:1). Reverse phase HPLC analysis was performed using Pegasil ODS column (4.6φ×250 mm) applying CH_3CN/H_2O (65:35). Adults *A. suum* and bovine heart were purchased from Tokyo Shibaura Zouki Co. Ltd.

4.2. Organisms

The fungus *Penicillium* sp. FKI-3389 was isolated from a soil sample collected in Hilo, Hawaii, USA. By the sequence data of ITS rDNA and morphological characteristics, the fungus FKI-3389 was identified to be a member of the *Penicillium* genus. The ITS sequence of the strain FKI-3389 was deposited at the DNA Data Bank of Japan with accession number AB455515. The fungus FKI-3389 was deposited at the NITE Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan as NITE BP-244.

4.3. Fermentation

One loopful of the strain FKI-3389 grown on LcA slant (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, and agar 1.5%, pH 6.0) was inoculated into 500-mL Erlenmeyer flask containing 100 mL of a seed culture medium (glucose 2%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, polypeptone 0.5%, KH_2PO_4 0.1%, and agar 0.1%, pH 6.0) and incubated on a rotary shaker at 27 °C for 2 days. A 1-mL of the seed culture was inoculated into each of sixty 500-mL Erlenmeyer flasks containing a production medium (50 g of water-sodden rice). Fermentation was carried out statically at 27 °C for 13 days.

4.4. Isolation

Moldy rice (3 kg) was extracted with 3.0 L of EtOAc. After the rice was removed by filtration the extract was concentrated in vacuo to afford a brown oil (14 g). The oil was applied to a silica gel column (48φ×450 mm) and washed with $CHCl_3$. Active materials eluted with $CHCl_3$/MeOH (100: 1) were concentrated to yield a crude material (2.7 g), which was then chromatographed over another silica gel column (30φ×330 mm). After washing with $CHCl_3$, the active materials were eluted with $CHCl_3$/MeOH (100:0 to 100:1). The active materials (1.9 g) were purified by preparative HPLC (column, Pegasil silica, 20φ×250 mm; mobile phase, $CHCl_3$/acetone (100:1); flow rate, 7.0 mL/min; detection, UV at 360 nm). Ukulactones A (**1**) and B (**2**) were eluted at 11 and 17.5 min, respectively. Each fraction was concentrated to dryness in vacuo to afford ukulactones A (**1**, 515 mg) and B (**2**, 3.5 mg) as yellow syrups.

*4.4.1. Ukulactone A (**1**).* Yellow syrup; $[\alpha]_D^{25}$ −54.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 243 (sh, 3.38), 308 (sh, 4.29), 325 (4.60), 340 (4.78), 358 (4.76) nm; IR ν_{max} (NaCl) 3730, 3595, 2927, 2858, 1795, 1745 cm^{-1}; HRFABMS *m*/*z* 466.2722 ($[M]^+$, 466.2719 calcd for $C_{29}H_{38}O_5$); ^1H and ^{13}C NMR, see Table 1.

*4.4.2. Ukulactone B (**2**).* Yellow syrup; $[\alpha]_D^{25}$ +87.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 245 (sh, 3.30), 309 (sh, 4.07), 325 (4.38), 340 (4.55), 359 (4.51) nm; IR ν_{max} (NaCl) 3730, 3593, 2922, 2856, 1797, 1743 cm^{-1}; HRFABMS *m*/*z* 466.2710 ($[M]^+$, 466.2719 calcd for $C_{29}H_{38}O_5$); ^1H and ^{13}C NMR, see Table 1.

4.5. Biological studies

4.5.1. Enzyme assays. NADH-fumarate reductase inhibitory activity was assayed using submitochondrial particles of *A. suum*. For the preparation of these particles containing NADH-fumarate reductase, muscle of *A. suum* (1 g) was homogenized in 3.5 mL of sodium phosphate buffer (120 mM, pH 7.0) and centrifuged at 1000×g for 10 min to remove cell debris. The supernatant was further centrifuged at 10,000×g for 30 min and the resultant mitochondrial precipitate was re-suspended in 3.5 mL of sodium phosphate buffer (120 mM, pH 7.0) before use.

NADH-fumarate reductase inhibitory activity was assayed by the following procedure; 80 μL of sodium phosphate buffer (120 mM, pH 7.0) containing 0.35 mM NADH and 7.2 mM disodium fumarate and 10 μL of DMSO/H_2O (1:1) solution of test compound were put into a 96-well plate and shaken for 3 min. The reaction was initiated by the addition of 10 μL of the submitochondrial fraction and the resultant mixture was incubated for 3 min at 37 °C. After incubation, absorbance at 340 nm was observed every 15 s for 10 min at 37 °C. The inhibitory activity was calculated as followed.

$$\text{Inhibition}(\%) = \left\{1 - \frac{\left(A_{slope}[\text{sample}] - A_{slope}[\text{no fumerate}]\right)}{\left(A_{slope}[\text{control}] - A_{slope}[\text{no fumerate}]\right)}\right\} \times 100$$

A: Absorbance at 340 nm.

NADH oxidase inhibitory activity and succinate-cytochrome *c* reductase inhibitory activity were assayed using submitochondrial particles of bovine heart. For preparation of submitochondrial particles from bovine heart muscles, chopped muscle (1 g) was homogenized in 5 mL of 120 mM sodium phosphate buffer (pH 7.0) and centrifuged at 1000×g for 10 min. The supernatant was further centrifuged at 10,000×g for 30 min and the resultant mitochondrial precipitate was re-suspended in 3.5 mL of sodium phosphate buffer (120 mM, pH 7.0). The suspension was centrifuged at 100,000×g for 30 min after ultrasonication and the submitochondrial particles were obtained as a precipitate. Before use, the precipitate was re-suspended in 3.5 mL of sodium phosphate buffer (120 mM, pH 7.0).

NADH oxidase inhibitory activity was assayed as follows: the phosphate buffer (80 μL) containing 0.70 mM NADH and 10 μL of DMSO/H_2O (1:1) solution of test compound were put into a 96-well plate and shaken for 3 min. The reaction was initiated by the addition of 10 μL of the submitochondrial fraction and the mixture was incubated for 3 min at 37 °C. After incubation, absorbance at 340 nm was observed every 15 s for 10 min at 37 °C. The inhibitory activity was calculated as followed.

$$\text{Inhibition}(\%) = \left\{1 - \frac{\left(A_{slope}[\text{sample}] - A_{slope}[\text{no enzyme}]\right)}{\left(A_{slope}[\text{control}] - A_{slope}[\text{no enzyme}]\right)}\right\} \times 100$$

A: Absorbance at 340 nm.

Succinate-cytochrome *c* reductase (SCRD) inhibitory activity was assayed by the following procedure: phosphate buffer (80 μL) containing 7.2 mM sodium succinate, 2.0 mg/mL of bovine cytochrome *c* and 1.3 mM potassium cyanide and 10 μL of DMSO/H_2O (1:1) solution of test compound were put into a 96-well plate and shaken for 3 min. The reaction was initiated by addition of 10 μL of the submitochondrial fraction and the mixture was incubated for 3 min at 37 °C. After incubation, absorbance at 550 nm was observed every 15 s for 10 min at 37 °C. The inhibitory activity was calculated as followed.

$$\text{Inhibition}(\%) = \left\{1 - \frac{\left(A_{slope}[\text{sample}] - A_{slope}[\text{no succinate}]\right)}{\left(A_{slope}[\text{control}] - A_{slope}[\text{no succinate}]\right)}\right\} \times 100$$

A: Absorbance at 550 nm.

Other enzyme activities were measured as described previously.[2a,14]

4.5.2. Cytotoxicity. Methanol solutions of test compound were put into a 96-well plate and dried completely. Jurkat cells were plated on the sample plate at a density of 2×10^4 cells/well with 100 μL of culture medium. After 3 days cultivation at 37 °C with 5% CO_2, cell density and morphological changes in the cells were observed under a microscope. After observation, 10 μL of MTT solution (5 mg/mL MTT in PBS) was added to the cells and the plate was incubated at 37 °C with 5% CO_2 for 2 h. Then, 50 μL of MTT solvent (0.7 M SDS, 50% DMF, 2.5% 1 M HCl and 2% acetic acid in H_2O) was added to the cells. After 1 h incubation at 37 °C, absorbance at 550 nm was measured.

4.5.3. Antimicrobial assay. Antimicrobial activity was measured using the agar dilution method, according to the method of the Japanese Society of Chemotherapy,[13] with the following strains: *Staphylococcus aureus* ATCC6538P, *Kocuria rhizophila* (*Micrococcus luteus*) ATCC9341, *Bacillus subtilis* ATCC6633, *Mycobacterium smegmatis* ATCC607, *Escherichia coli* NIHJ, *Salmonella typhimurium* KB20, *Pseudomonas aeruginosa* NBRC3080, *Xanthomonas oryzae* KB88, *Bacteroides fragillis* ATCC23745, *Morganella morganii* NBRC3168, *Proteus vulgaris* NBRC3167, *Acholeplasma laidlawii* Bm1 KB175, *Candida albicans* KF1, *Saccharomyces cerevisiae* ATCC9763, *Aspergillus niger* ATCC9642, *Pyricularia oryzae* KF180 and *Mucor racemosus* NBRC4581.

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