Fudecalone, a New Anticoccidial Agent Produced by *Penicillium* sp. FO-2030

NORIKO TABATA, HIROSHI TOMODA, ROKURO MASUMA,
YUZURU IWAI and SATOSHI ŌMURA*

Research Center for Biological Function, The Kitasato Institute,
Minato-ku, Tokyo 108, Japan

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Penicillium sp. FO-2030, a soil isolate, was found to produce a new anticoccidial compound. The active compound, designated fudecalone, was isolated from the fermentation broth of the producing strain by solvent extraction, silica gel column chromatography and preparative HPLC. The structure of fudecalone was elucidated to be 3,3a,6,6a,7,8,9,10-octahydro-1-hydroxy-4,7,7-trimethyl-1*H*-naphtho[1,8a-c]furan-6-one mainly by spectroscopic studies including various NMR measurements. The anticoccidial activity using cell systems indicated that schizont formation of monensin-resistant *Eimeria tenella* was completely inhibited by fudecalone at concentrations more than 16 μM.

Recently, we have reported new anticoccidial agents of microbial origin, xanthoquinodins[1], diolmycins[2], hynapenes[3], arohynapenes[4] and cytosaminomycins[5]. From our continuous screening program[1] using BHK-21 cells as a host and monensin-resistant *Eimeria tenella* as a parasitic protozoan, the fungal strain FO-2030 was found to produce a new anticoccidial agent named fudecalone (Fig. 1). In this paper, the taxonomy of the producing strain, fermentation, isolation, physicochemical properties, structure elucidation and biological characteristics of fudecalone are described.

Materials and Methods

General Experimental Procedures

Kieselgel 60 (E. Merck) was used for column chromatography. HPLC was carried out using Waters 600E system and an ODS packed column (YMC, D-ODS-5, 20 × 250 mm). UV spectrum was recorded on a Shimadzu UV-240 spectrophotometer. IR spectrum was recorded on a Horiba FT-210 infrared spectrometer. Melting point was measured with a Yanaco micro melting point apparatus. Optical rotation was obtained with a JASCO DIP-370 digital polarimeter. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian XL-400 spectrometer.

Taxonomic Studies

For the identification of the fungus, potato dextrose agar (Difco), malt extract agar, CZAPEK's agar, corn meal agar (Difco) and YpSs agar (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 2.0%, pH 6.0) were used. Morphological observations were made under a microscope (Olympus Vanox-S A H-2) and a scanning electron microscope (Hitachi S-430).

In Vitro Anticoccidial Activity

Anticoccidial activity was assayed according to the established method[1] using BHK-21 cells as a host and monensin-resistant *Eimeria tenella* as a protozoan.

Antimicrobial Activity

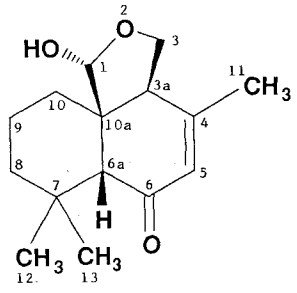
Antimicrobial activity was tested using paper disks (i.d. 6 mm, Toyo Roshi Co.). Bacteria were grown on Müeller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato dextrose agar. Antimicrobial activity was observed after 24-hour incubation at 37°C for bacteria and after 48-hour incubation at 27°C for fungi and yeasts.

Results

Taxonomy of the Producing Organism

The fungal strain FO-2030 was originally isolated from a soil sample collected at Numazu-shi, Shizuoka, Japan. This strain grew rapidly to form white to pale yellow colonies with a diameter of 50~90 mm after incubation for 14 days at 25°C. The colony surface was velvety to floccose. Conidia formation was moderate on potato dextrose agar, malt extract agar and YpSs agar.

Fig. 1. Structure of fudecalone.



Mass color of conidia was ivory to pale olive. In contrast conidia were poorly produced on CZAPEK's agar and corn meal agar. The colors of the reverse side of the colony and of soluble pigment were dark reddish brown to brownish black on potato dextrose agar. Exudate and sclerotia were not observed. When the strain FO-2030 was grown on potato dextrose agar at 25°C for 7 days, the conidiophores were borne from substrate hyphae, and the penicillia were monoverticillate as shown in Fig. 2. The phialides were $7.5 \sim 10 \times 2 \sim 3\,\mu m$. The conidia were globose to subglobose with a smooth surface and $2.5 \sim 3.0\,\mu m$ in diameter. From the above characteristics, the strain FO-2030 was identified as a member of the genus *Penicillium*[6,7] and named *Penicillium* sp. It was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as *Penicillium* sp. FO-2030 with an accession number of FERM P-14000.

Fermentation

A slant culture of the strain FO-2030 grown on YpSs agar was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flask was shaken on a rotary shaker for 3 days at 27°C. Two hundred ml of the seed culture were transferred to 20 liters of a production medium (soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast 0.3%, KCl 0.3%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $CaCO_3$ 0.2%, pH 6.5) in a 30-liter jar fermentor. The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. The production of fudecalone was measured by HPLC under the following conditions: column, YMC pack D-ODS-5 (20×250 mm); a linear gradient from 40% CH_3CN in 0.05% H_3PO_4 to 70% CH_3CN for 40 minutes; UV detection at 215 nm; flow rate at 6.0 ml/minute. Under these conditions, fudecalone was eluted with a retention time of 30.5 minutes (Fig. 4). The concentration of fudecalone reached a maximum at 96 hours.

Fig. 2. Scanning electron micrograph of penicillia and conidia of strain sp. FO-2030 on potato dextrose agar (Hitachi S-430).

Bar represents 5 μm.

Fig. 3. A typical time course of fudecalone production.

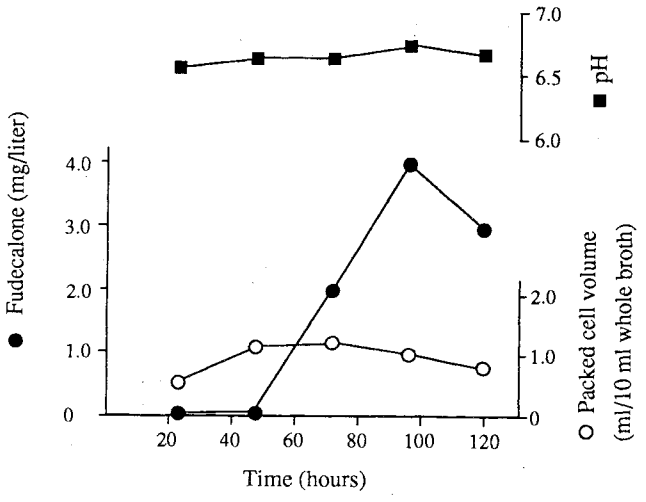
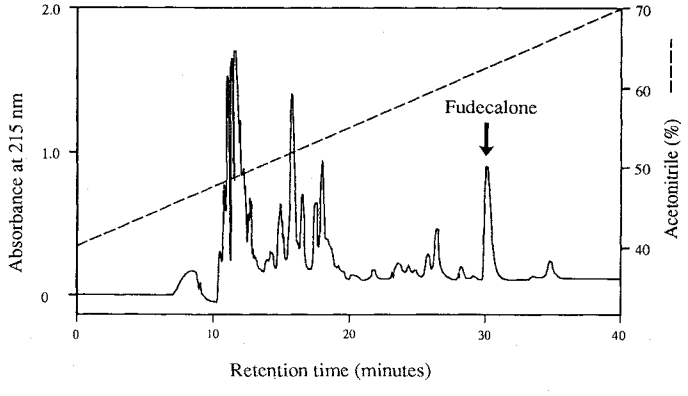


Fig. 4. A chromatographic profile of fudecalone separated by preparative HPLC.

Column, YMC-packed column D-ODS-5 (20×250 mm); solvent, a linear gradient 40% CH_3CN H_3PO_4 to 70% CH_3CN in 0.05% (0~40 minutes); UV at 215 nm; 6.0 ml/minute.



Isolation

The 96-hour old fermented broth (20 liters) was extracted with 20 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brown material (23.4 g). The material was applied on a silica gel column (Kieselgel 60, 1,200 ml), and the column was washed with 6.0 liters of chloroform. The active component was eluted with 6.0 liters of chloroform-methanol (99 : 1), and each 500 ml was successively collected. The 6th to 12th fractions containing fudecalone were combined and concentrated *in vacuo* to give a light yellow material (2.6 g). This material was dissolved in methanol (100 ml), and the solution was cooled at 4°C for 1 hour. Then, the precipitated white material was removed by paper filtration, and the supernatant was concentrated *in vacuo* to dryness to yield a colorless material (646 mg). Fudecalone was finally purified by preparative HPLC under the same conditions described above. The colorless material was dissolved in 6.46 ml of methanol and each 250 μl was injected. The active fraction was concentrated and extracted with ethyl acetate to give pure fudecalone (3.9 mg) as white powder.

Physico-chemical Properties

The physico-chemical properties of fudecalone are summarized in Table 1. It is soluble in methanol, ethanol, acetonitrile, acetone, ethyl acetate and chloroform, slightly soluble in benzene, but insoluble in water. The UV spectrum showed maxima at 203 (ε 10,300) and 228 nm (ε 12,700) (Fig. 5). The IR spectrum (KBr) showed the absorption at 1676 cm^{-1}, suggesting the presence of $\alpha\beta$-unsaturated ketone[8] (Fig. 6).

Structure Elucidation of Fudecalone

The molecular formula of fudecalone was determined to be $C_{15}H_{22}O_3$ (*m/z* found; 250.1572, calcd 250.1570) by HREI-MS analysis. ^{13}C and ^{1}H NMR spectra ($CDCl_3$) showed 15 carbon and 21 proton signals, respectively. The DEPT spectra indicated the presence of three $-CH_3$, three $-CH_2-$, one $-O-CH_2-$, two $-CH-$, one acetal, one $=CH-$ and four quaternary carbons. To fulfill the molecular formula of fudecalone, the presence of one hydroxyl group was suggested. It was supported by the fragment ion peak of *m/z* 233 $(M-OH)^+$ in the

Table 1. Physico-chemical properties of fudecalone.

	Fudecalone
Appearance	White powder
$[\alpha]_D^{28}$ (*c* 0.1, CH_3OH)	+4.4°
Molecular formula	$C_{15}H_{22}O_3$
HREI-MS (*m/z*)	
Calcd:	250.1570
Found:	250.1572
UV $\lambda_{max}^{CH_3OH}$ (nm)	203 (10,300), 228 (12,700)
IR ν_{max}^{KBr} (cm^{-1})	1676, 1466, 1441
Melting point	250~252°C (dec)
Solubility	
Soluble:	CH_3OH, $CHCl_3$, CH_3CN, Acetone, C_2H_5OH, Ethyl acetate
Insoluble:	H_2O
Color reaction	
Positive:	50% H_2SO_4
Negative:	Ninhydrin reagent

Fig. 5. UV spectrum of fudecalone (10 μg/ml CH_3OH).

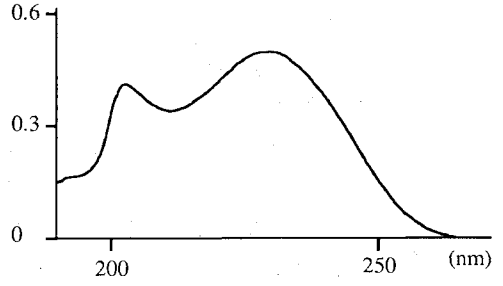


Fig. 6. IR spectrum of fudecalone (KBr).

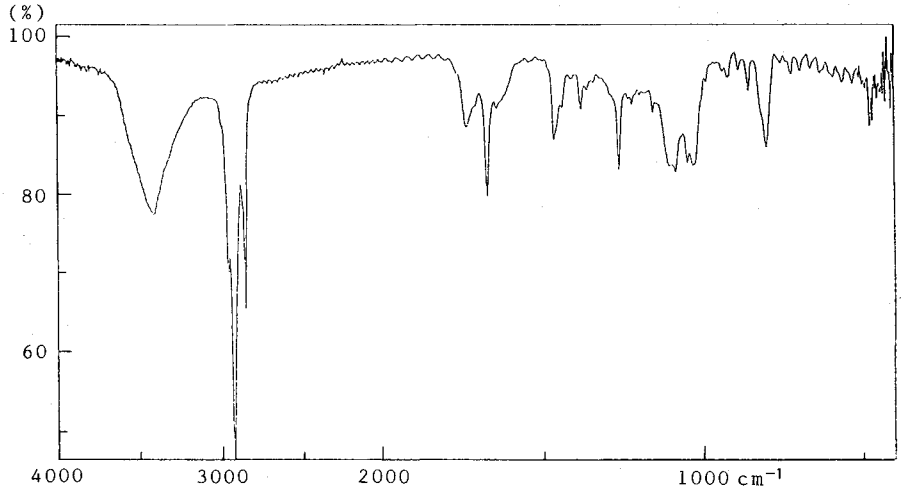


Table 2. ^{1}H and ^{13}C NMR chemical shifts of fudecalone.

Carbon No.	Fudecalone	
	^{13}C chemical shifts ppm[a]	^{1}H chemical shifts ppm[b]
C-1	100.87	5.47 (1H, s)
C-3	68.40	3.96 (1H, dd, J=8.8,4.0 Hz), 4.25 (1H, t, J=8.8 Hz)
C-3a	52.06	2.61 (1H, dd, J=8.8,4.0 Hz)
C-4	153.12	
C-5	128.60	5.82 (1H, t, J=1.2 Hz)
C-6	197.77	
C-6a	56.61	2.38 (1H,s)
C-7	32.50	
C-8	43.11	1.26 (1H, m), 1.44 (1H, m)
C-9	19.63	1.54 (1H, m), 1.62 (1H, m)
C-10	37.62	1.25 (1H, m), 1.86 (1H, m)
C-10a	51.10	
C-11	21.75	1.82 (3H, t, J=1.2 Hz)
C-12	32.25	1.24 (3H, s)
C-13	21.44	1.16 (3H, s)

[a] Each sample was dissolved in $CDCl_3$. Chemical shifts are shown with reference to $CDCl_3$ as 77.7 ppm.
[b] Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm.

FAB-MS spectrum. The connectivity of proton and carbon atoms was confirmed by the ^{13}C-^{1}H COSY spectrum (Table 2). As shown in Fig. 7, two partial structures I and II were proposed from further NMR experiments. Evidence supporting the structure I includes: 1) The ^{1}H-^{1}H COSY spectrum showed the cross peaks between H-3a (δ 2.61) and H_2-3 (δ 3.96 and 4.25), and 2) ^{13}C-^{1}H long range couplings of ^{2}J and ^{3}J observed in the heteronuclear multiple bond correlation (HMBC) spectrum showed the cross peaks from H_2-3 (δ 3.96 and 4.25) to C-4 (δ 153.12), from H-5 (δ 5.82) to C-3a (δ 52.06) and C-11 (δ 21.75) and from H_3-11 (δ 1.82) to C-3a (δ 52.06), C-4 (δ 153.12) and C-5 (δ 128.60). Evidence supporting the structure II includes: 1) Three methylenes, H_2-8 (δ 1.26 and 1.44), H_2-9 (δ 1.54 and 1.62) and H_2-10 (δ 1.25 and 1.86), were assigned to each geminal protons by the ^{13}C-^{1}H COSY spectrum (Table 2). The proton sequences were determined by the 1D homonuclear Hartmann-Hahn (HOHAHA) experiment. When the signal at H-10b (δ 1.86) was locked, positive signals were sequentially observed at increasing mixing times; H-10a (δ 1.25) signals at 0.02 seconds, H_2-9 (δ 1.54 and 1.62) signals at 0.04 seconds, and then H_2-8 (δ 1.26 and 1.44) signals at 0.06 seconds (Fig. 8). 2) The HMBC spectrum showed the cross peaks from H_2-8 (δ 1.26 and 1.44) to C-6a (δ 56.61), C-7 (δ

Fig. 7. Partial structures I and II of fudecalone.

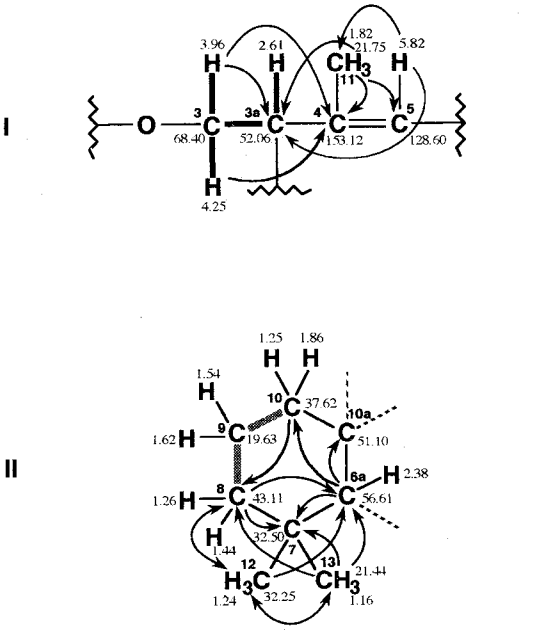


Fig. 8. HOHAHA experiments of fudecalone.

1) Normal ^{1}H NMR (1.0~2.0 ppm), 2) mixing times of 0.02 second, 3) 0.04 second, and 4) 0.06 second.

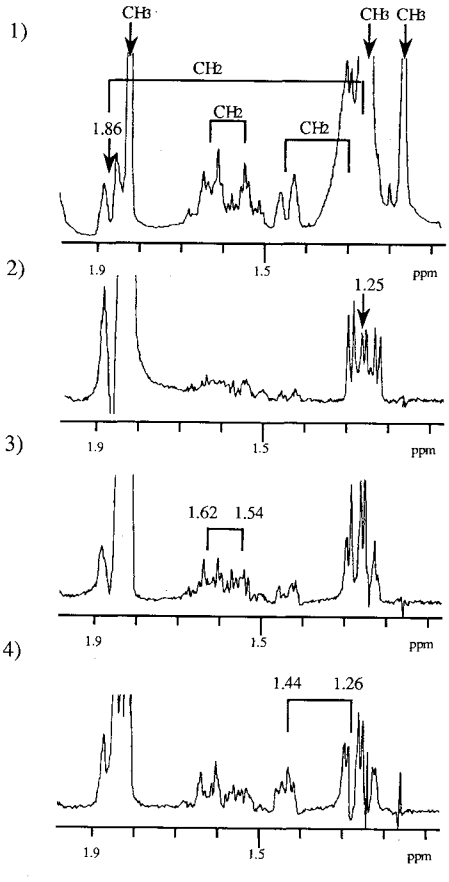
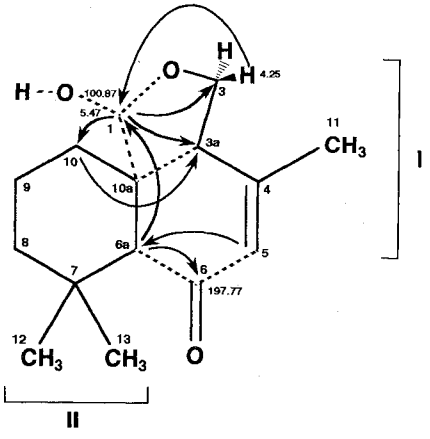


Fig. 9. HMBC analysis of fudecalone.



H ⟶ C ^{1}H-^{13}C long-range coupling (HMBC experiment)

32.50) and C-12 (δ 32.25), from H_3-12 (δ 1.24) to C-8 (δ 43.11), C-13 (δ 21.44) and C-6a (δ 56.61), from H_3-13 (δ 1.16) to C-8 (δ 43.11), C-12 (δ 32.25), C-7 (δ 32.50) and C-6a (δ 56.61), and from H-6a (δ 2.38) to C-7 (δ 32.50), indicating that the $-C^{7}(CH_3)_2-C^{6a}H-$ sequence follows the C-8 position of the three methylene sequence, and 3) cross peaks from H-6a (δ 2.38) to C-10a (δ 51.10) and C-10 (δ 37.62) revealed a 6-membered ring.

The two partial structures I and II were connected by HMBC experiments (Fig. 9). Cross peaks from H-5 (δ 5.82) to C-6a (δ 56.61) and from H-6a (δ 2.38) to C-6 (δ 197.77) indicated the connection of C-6a to C-5 *via* C-6 carbonyl carbon, and cross peaks from H_2-10 (δ 1.25 and 1.86) to C-3a (δ 52.06) indicated the attachment of C-10a quarternary carbon to C-3a to form a 6-membered ring in the decaline skeleton. The presence of a 5-membered ring in fudecalone was also suggested because of the observation of cross peaks from H-1 (δ 5.47) to C-3 (δ 68.40), C-3a (δ 52.06) and C-10 (δ 37.62) in the HMBC spectrum and the degree of unsaturation. Finally the remaining hydroxyl group should be attached to C-1 (δ 100.87) due to the comparable ^{13}C chemical shift with a hemiacetal carbon[8]. Taken together, the general structure of fudecalone was deduced to be 3,3a,6,6a,7,8,9,10-octahydro-1-hydroxy-4,7,7-trimethyl-1*H*-naphtho[1,8a-c]furan-6-one.

Stereochemistries of Fudecalone

Relative stereochemistries of fudecalone were studied by the NOE experiments. Fudecalone has the four chiral carbons, C-6a, C-3a, C-10a and C-1. Molecular modeling revealed that the geometry of the angular C-3a and C-10a carbons should be 3a*S**, 10a*S**. Concerning the

Fig. 10. Two possible stereochemistries for C-6a of fudecalone.

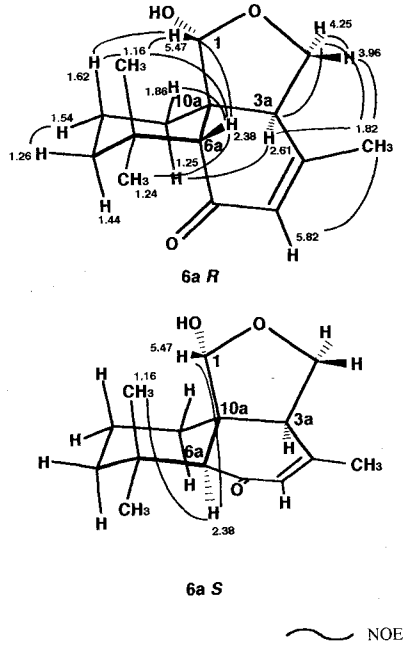


Table 3. Anticoccidial activity of fudecalone *in vitro*.

Compounds	Minimum effective concentration (μM)	
	Anticoccidial activity[a]	Cytotoxicity[b]
Fudecalone	16	160
Monensin	—[c]	0.03

BHK-21 cells stained with hematoxylin solution was microscopically observed. In control experiments (no drug) infected sporocysts grew in the cells to form mature shizonts.

[a] No mature shizonts observed in the cells when the drug was added to the culture medium at the indicated concentrations.

[b] No BHK-21 cells observed when the drug was added to the culture medium at the indicated concentrations.

[c] No anticoccidial acitivity.

geometry of C-1, NOE observation between H-1 (δ 5.47) and each of H_3-13 (δ 1.16), H-9a (δ 1.62) and H-6a (δ 2.38) indicated that C-1 has the *R** configuration. On the basis of these stereochemistries, two possible geometries for C-6a are illustrated in Fig. 10. NOE observation between H-6a (δ 2.38) and each of H_3-13 (δ 1.16) and H-1 (δ 5.47) indicated that the 6a*R** configuration is reasonable for the structure. Taken together, fudecalone has 1*R**, 3a*S**, 6a*R** and 10a*S** configurations (Fig. 1).

Biological Properties

Anticoccidial activity of fudecalone is shown in Table 3. Fudecalone exhibited anticoccidial activity at above 16 μM and cytotoxic activity against BHK-21 cells at above 160 μM.

Fudecalone showed no antimicrobial activity *in vitro* at a concentration of 1 mg/ml against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Bacteroides fragilis*, *Acholeplasma laidlawii*, *Candida albicans*, *Saccharomyces sake*, *Aspergillus niger*, *Pyricularia oryzae*, and *Mucor racemosus*.

Discussion

The structure of fudecalone was elucidated to have a unique naphtofuran ring (Fig. 1). The similar tricyclic ring systems were defined in the structures of corymobtins[9] and clerodane derivatives[10], both of which were isolated from plants. Since all of the plant-derived compounds consist of diterpenes, fudecalone appears to be an only sesquiterpene forming such a ring system.

Fudecalone is an unstable compound. After storage at 4°C for 2 months, about 60% of fudecalone was cleaved at the hydroxyfuran ring to form aldehyde and alcohol residues (data not shown). The cleaved derivative did not show anticoccidial activity.

The biological properties of corymobtins and clerodanes were not reported, and it might be interesting to test whether or not they show anticoccidial activity.

Acknowledgment

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