AROHYNAPENES A AND B, NEW ANTICOCCIDIAL AGENTS PRODUCED BY *Penicillium* sp.

TAXONOMY, FERMENTATION, AND STRUCTURE ELUCIDATION

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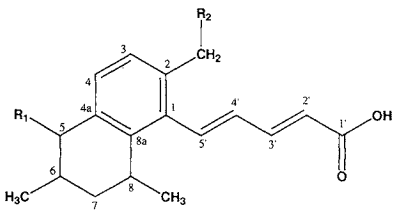
Penicillium sp. FO-2295, a water isolate, was found to produce a series of new anticoccidial compounds. Two active compounds, designated arohynapenes A and B, were isolated from the fermentation broth of the producing strain by solvent extraction and preparative HPLC. Arohynapene A was deduced to be (2*E*,4*E*)-5-(5-hydroxy-2,6,8-trimethyl-5,6,7,8-tetrahydronaphtalene)-2,4-pentadienoic acid, and arohynapene B was (2*E*,4*E*)-5-(2-hydroxymethyl-6,8-dimethyl-5,6,7,8-tetrahydronaphtalene)-2,4-pentadienoic acid. Arohynapenes inhibited the growth of *Eimeria tenella* in an *in vitro* assay using BHK-21 cells as a host. No schizont in the cells was observed at concentrations ranging above 35.0 μM and 7.0 μM for arohynapenes A and B, respectively.

Recently, we have reported the new anticoccidial agents of microbial origin, xanthoquinodins[1], diolmycins[2] and hynapenes[3]. From our continuous screening program using BHK-21 cells as a host and monensin-resistant *Eimeria tenella* as a parasitic protozoan, a fungal strain FO-2295 was found to produce a series of new anticoccidial agents. Two active compounds, named arohynapenes A and B (Fig. 1), were isolated. In this paper, the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure determination and biological characteristics of arohynapenes are described.

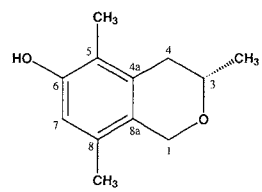
Taxonomy of the Producing Organism

The fungal strain FO-2295 was isolated from a water sample collected at a pond located in Tanegashima, Kagoshima, Japan. For identification of the fungus, potato-dextrose agar, malt extract agar, CZAPEK's agar and corn meal agar were used. This strain grew rapidly to form pale yellowish green to pale gray colonies with a diameter of 40~50 mm after incubation for 14 days at 25°C. The colony surface was abundantly produced on various agar media. The reverse color was dark green or pale yellow. Morphological observations were done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope

Fig. 1. Structures of arohynapenes A and B and (3*S*)-6-hydroxy-8-methoxy-3,5-dimethylisochroman (compound C).



Arohynapene A R_1=OH, R_2=H
Arohynapene B R_1=H, R_2=OH



Compound C

(Hitachi S-430). When the strain FO-2295 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, $2.0 \sim 2.5\,\mu m$ in diameter, and with a smooth. From the above characteristics, the strain FO-2295 was identified as a member of the genus *Penicillium*[4,5] and named *Penicillium* sp. FO-2295. It was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FEPM P-13400.

Fermentation

A slant culture of the strain FO-2295 grown on 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) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the 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. Ten ml of the seed culture were transferred to 100 ml of a production medium (sucrose 2.0%, glucose 1.0%, corn steep liquor 1.0%, meat extract 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, trace elements 200 ml (containing in g/liter: $FeSO_4 \cdot 7H_2O$ 1.0, $MnCl_2 \cdot 4H_2O$ 1.0, $ZnSO_4 \cdot 7H_2O$ 1.0, $CuSO_4 \cdot 5H_2O$ 1.0, $CoCl_2 \cdot 2H_2O$ 1.0), $CaCO_3$ 0.3% and agar 0.1%, pH 6.0) in a 500-ml Erlenmeyer flask. The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. The production of arohynapenes A and B was measured by HPLC under the following condi-

Fig. 2. Scanning electron micrograph of penicillia and conidia of *Penicillium* sp. FO-2295 on potato-dextrose agar (Hitachi S-430).

Bar represents 5 μm.

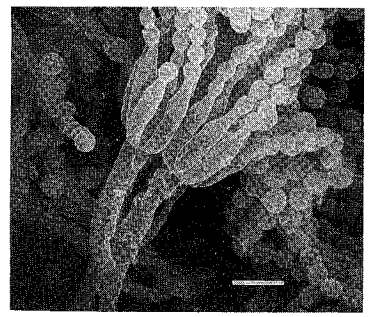
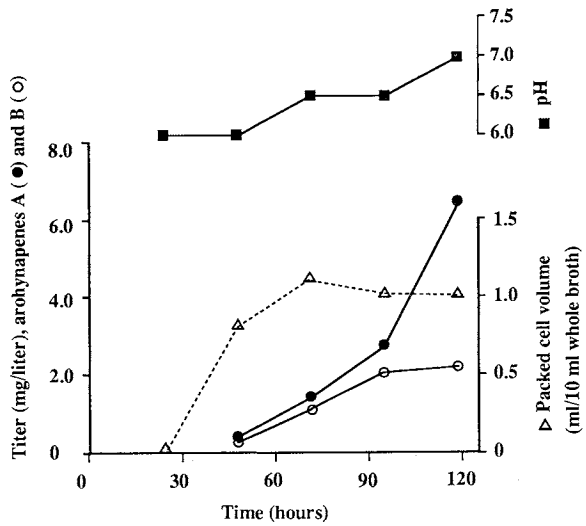


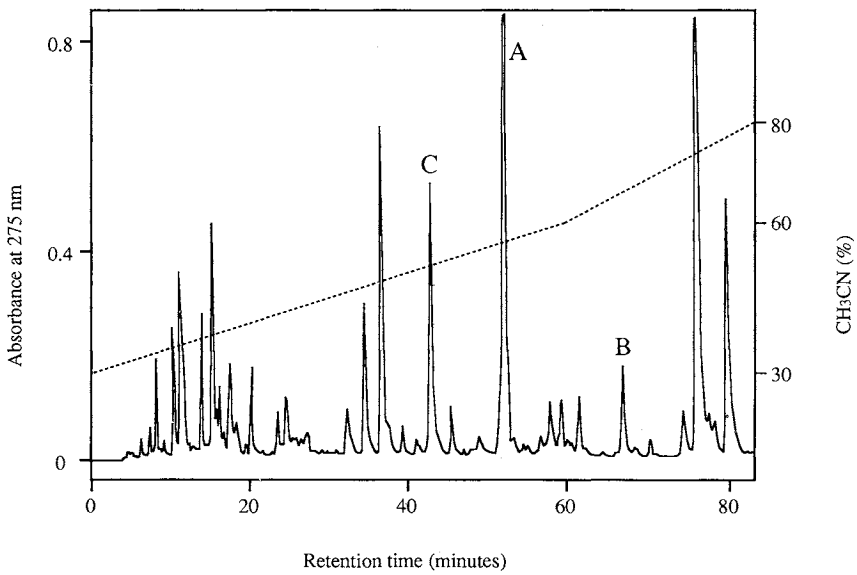
Fig. 3. A typical time course of arohynapenes production.

Arohynapenes A (●) and B (○).



tions: column, YMC pack D-ODS-5 (20×250 mm); a linear gradient from 30% CH_3CN in 0.05% H_3PO_4 to 60% CH_3CN in 0.05% H_3PO_4 (0~60 minutes) and a linear gradient 60% CH_3CN in 0.05% H_3PO_4 to 80% CH_3CN in 0.05% H_3PO_4 (60~80 minutes); UV detection at 275 nm; flow rate, 6.0 ml/minute. Under these conditions, arohynapene A was eluted first with a retention time at 52.5 minutes, followed by arohynapenes B at 67.0 minutes (Fig. 4). The production of arohynapenes A and B was observed after 48 hours and increased at least up to 120 hours.

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



Column, YMC-Packed column D-ODS-5 (20×250 mm); mobile phase, a linear gradient 30% CH_3CN in 0.05% H_3PO_4 to 60% CH_3CN in 0.05% (0~60 minutes) and a linear gradient 60% CH_3CN in 0.05% H_3PO_4 to 80% CH_3CN in 0.05% (60~80 minutes); flow rate, 6.0 ml/minute; detection, UV at 275 nm.

Table 1. Physico-chemical properties of arohynapenes.

	Arohynapene A	Arohynapene B	Compound C
Appearance	Yellow powder	Yellow powder	White powder
$[\alpha]_D^{23}$ (*c* 0.1, MeOH)	+38.4°	+74°	+102.2°
Molecular formula	$C_{18}H_{22}O_3$	$C_{18}H_{22}O_3$	$C_{12}H_{16}O_3$
HREI-MS (*m/z*)			
Calcd:	286.1568	286.1568	208.1099
Found:	286.1568	286.1576	208.1088
UV λ_{max}^{MeOH} nm (ε)	204 (11,400), 226 (9,400), 282 (9,000)	204 (11,400), 225 (9,400), 280 (9,000)	206 (27,500), 285 (2,100)
IR (cm^{-1}) (KBr)	2918, 1680, 1639, 1444, 1272, 1000	2918, 1675, 1633, 1509, 1053	1606, 1497, 1447, 1380, 1315, 1260
Solubility			
Soluble:	MeOH, $CHCl_3$, EtOH, EtOAc	MeOH, $CHCl_3$, EtOH, EtOAc	MeOH, $CHCl_3$, EtOH, EtOAc
Insoluble:	H_2O	H_2O	H_2O
Color reaction			
Positive:	50% H_2SO_4	50% H_2SO_4	50% H_2SO_4
Negative:	Ninhydrin reagent	Ninhydrin reagent	Ninhydrin reagent

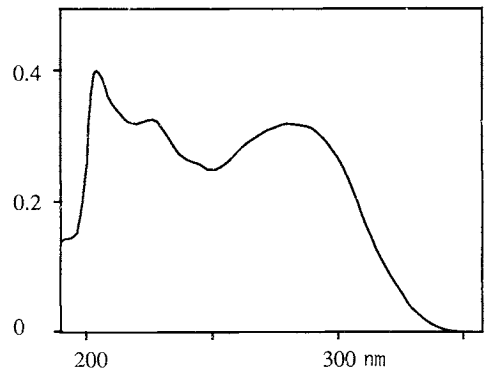
Isolation

The 120-hour fermented broth (2 liters) was extracted with 2 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brown material (452 mg). Arohynapenes A and B were directly purified by preparative HPLC, the conditions of which were the same as above. The crude material were dissolved in 4.52 ml of methanol and each 100 μl was injected. The active fractions (peaks A, B and C in Fig. 4) were concentrated and extracted with ethyl acetate to give pure arohynapenes A (9.4 mg), B (3.4 mg) and compound C (19.0 mg) as yellow materials. Compound C was identified as (3*S*)-6-hydroxy-8-methoxy-3,5-dimethylisochroman[6].

Physico-chemical Properties

The physico-chemical properties of arohynapenes A and B are summarized in Table 1. They are soluble in methanol, ethanol, acetonitrile, acetone, ethyl acetate, and chloroform; slightly soluble in benzene; and insoluble in water. The UV spectra show maxima at 204 (ε 11,400), 226 (ε 9,400) and 282 nm (ε 9,000) for arohynapene A (Fig. 5) and at 204 (ε 11,400), 225 (ε 9,400) and 280 nm (ε 9,000) for arohynapene B. The IR spectrum (KBr) of arohynapene A is shown in Fig. 6.

Fig. 5. UV spectrum of arohynapene A (10 μg/ml MeOH).



Structure Elucidation of Arohynapenes A and B

The molecular formulas of arohynapenes A and B were determined to be $C_{18}H_{22}O_3$ (*m/z* found arohynapene A; 286.1568, arohynapene B; 286.1576, calcd 286.1568) by HREI-MS analyses. ^{13}C NMR and ^1H NMR spectra ($CDCl_3$) showed 18 carbon signals and 20 proton signals, respectively. The DEPT spectra indicated the presence of three $-CH_3$, one $-CH_2-$, two $-CH-$, one $-O-CH-$, six $=CH-$ and

Fig. 6. IR spectrum of arohynapene A (KBr).

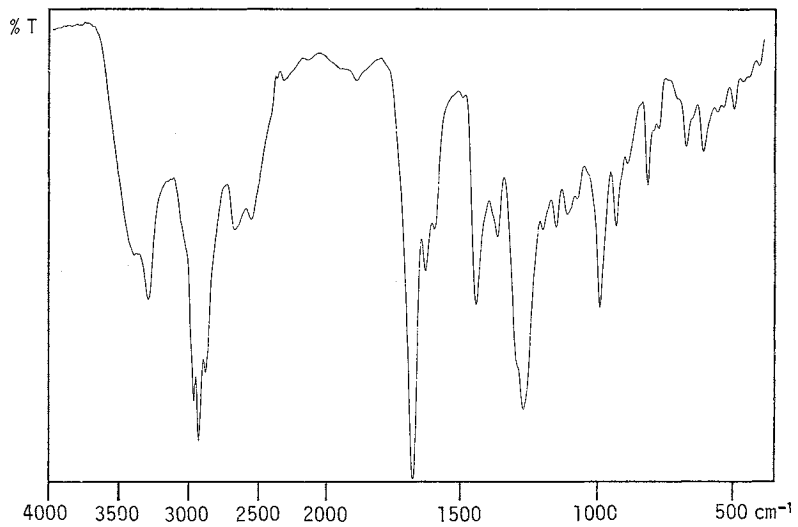


Table 2. ^{1}H and ^{13}C NMR chemical shifts of Arohynapenes A and B and compound C.

No.	Arohynapene A		Arohynapene B		(3*S*)-6-Hydroxy-8-methoxy-3,5-dimethylisochroman (Compound C)	
	^{13}C chemical shifts ppm[a]	^{1}H chemical shifts ppm[b]	^{13}C chemical shifts ppm[a]	^{1}H chemical shifts ppm[b]	^{13}C chemical shifts ppm[a]	^{1}H chemical shifts ppm[b]
C-1	135.8		135.1		64.6	4.57 (1H, d, J=15.2 Hz),
						4.89 (1H, d, J=15.2 Hz)
C-2	136.6		136.4			
C-2-CH_3	21.3	2.30 (3H, s)				
C-2-CH_2			63.7	4.62 (2H, s)		
C-3	128.1	7.06 (1H, d, J=8.0 Hz)	126.1	7.22 (1H, d, J=8.0 Hz)	70.6	3.74 (1H, m)
C-3-CH_3					21.7	1.38 (3H, d, J=6.3 Hz)
C-4	129.5	7.14 (1H, d, J=8.0 Hz)	128.9	7.05 (1H, d, J=8.0 Hz)	34.0	2.42 (1H, dd, J=16.5, 10.6 Hz),
						2.61 (1H, dd, J=16.5, 2.3 Hz)
C-4a	137.2		138.7		134.1	
C-5	73.2	4.49 (1H, m)	39.6	2.38 (1H, dd, J=15.0, 11.5 Hz),	112.9	
				2.65 (1H, dt, J=15.0, 3.0 Hz)		
C-5-CH_3					10.0	2.04 (3H, s)
C-6	33.3	1.76 (1H, m)	29.6	1.25 (1H, m)	152.7	
C-6-CH_3	17.7	1.11 (3H, d, J=6.5 Hz)	22.2	1.06 (3H, d, J=6.6 Hz)		
C-7	33.4	1.53 (1H, m),	41.4	1.11 (1H, m),	96.2	6.23 (1H, s)
		1.91 (1H, m)		2.16 (1H, m)		
C-8	30.4	3.18 (1H, m)	30.9	3.21 (1H, m)	153.9	
C-8-CH_3	24.5	1.19 (3H, d, J=6.5 Hz)	24.3	1.14 (3H, d, J=6.9 Hz)		
C-8-O-CH_3					55.0	3.68 (3H, s)
C-8a	141.0		141.2		115.2	
C-1′	171.3		170.8			
C-2′	112.0	5.93 (1H, d, J=15.5 Hz)	120.2	5.96 (1H, d, J=15.5 Hz)		
C-3′	146.8	7.57 (1H, dd, J=15.5, 11.0 Hz)	146.9	7.56 (1H, dd, J=15.5, 11.0 Hz)		
C-4′	131.1	6.42 (1H, dd, J=16.0, 11.0 Hz)	131.5	6.61 (1H, dd, J=15.5, 11.0 Hz)		
C-5′	141.3	7.10 (1H, d, J=16.0 Hz)	140.0	7.18 (1H, d, J=15.5 Hz)		

[a] Each sample were 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.

five quaternary carbons for arohynapene A and two $-CH_3$, two $-CH_2-$, one $-O-CH_2-$, two $-CH-$, six $=CH-$and five quaternary carbons for arohynapene B. To fulfill the molecular formulas of arohynapenes A and B, the presence of one hydroxyl and one carboxylic acid groups in both arohynapenes were suggested. In fact, it was supported by the following evidences; 1) The fragment ion peak of m/z 269 $[M+1-H_2O]^+$ in the FAB-MS spectra for arohynapenes A and B indicated the presence of a hydroxyl groups[7], 2) the chemical shifts of C-5′ (δ 73.2) for arohynapene A and C-2-CH_2 (δ 63.7) for arohynapene B in ^{13}C NMR suggested the presence of adjacent oxygen atoms, 3) the chemical shifts of C-1′ (δ 171.3) for arohynapene A and C-1′ (δ 170.8) for arohynapene B suggested a carbonyl carbon, and 4) the absorption at 1680 cm^{-1} for arohynapene A and at 1675 cm^{-1} for arohynapene B in IR spectra supported the presence of carboxylic acid groups. The connectivity of proton and carbon atoms was confirmed by the ^{13}C-^1H COSY spectra as shown in Table 2. The ^1H-^1H COSY spectra showed the three partial structures I, II and III for arohynapene A, and four partial structures I, II, III and IV for arohynapene B (Fig. 7). Furthermore, the proton sequences for arohynapene B were determined by differential selective proton decoupling spectra. Irradiation at H-6 (δ 1.25) showed the positive signals at H-6-CH_3 (δ 1.06) and H-7 (δ 1.11 and δ 2.16), suggesting the presence of the $-CH_2-CH-CH_3$ sequence. ^{13}C-^1H long range couplings of $2J$ and $3J$ observed in the heteronuclear multiple bond correlation (HMBC) spectra are shown in Fig. 8; 1) Cross peaks from H-5 (δ 4.49) to C-4 (δ 129.5), C-4a (δ 137.2), C-8a (δ 141.0) and C-6 (δ 33.3), from H-8 (δ 30.4) to C-1 (δ 135.8), C-4a (δ 137.2) and C-8a (δ 141.0), from H-3 (δ 7.06) to C-1 (δ 135.8) and C-4a (δ 137.2), and from H-4 (δ 7.14) to C-2 (δ 136.6) and C-8a (δ 141.0) revealed a decaline skeleton, 2) cross peaks from H-5′ (δ 7.10) to C-8a (δ 141.0) and from H-4′ (δ 6.42) to C-1 (δ 135.8) indicated that the olefine moiety is attached to the C-1 position of decaline core, and 3) cross peaks from H-2′ (δ 5.93) and H-3′ (δ 7.57) to C-1′ (δ 171.3) indicated the attachment of carbonyl carbon to the terminal olefine moiety. The respective coupling constants between H-2′ and H-3′ and between H-4′ and H-5′ were 15.5 and 15.5 Hz for arohynapene A and 15.5 and 16.0 Hz for arohynapene B, suggesting that the both olefins in the side

Fig. 7. Partial structures of arohynapenes A (a) and B (b).

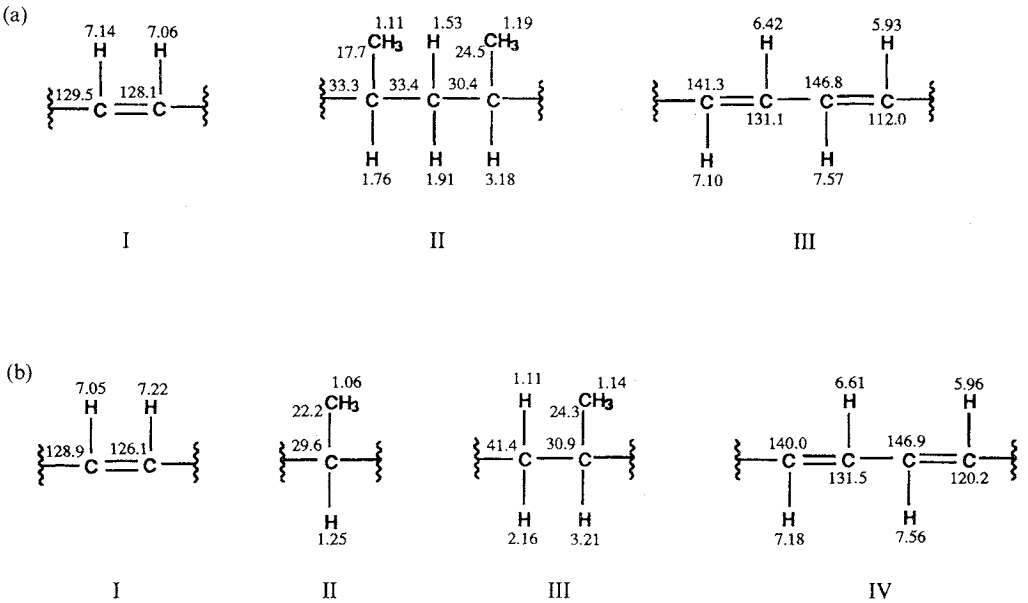
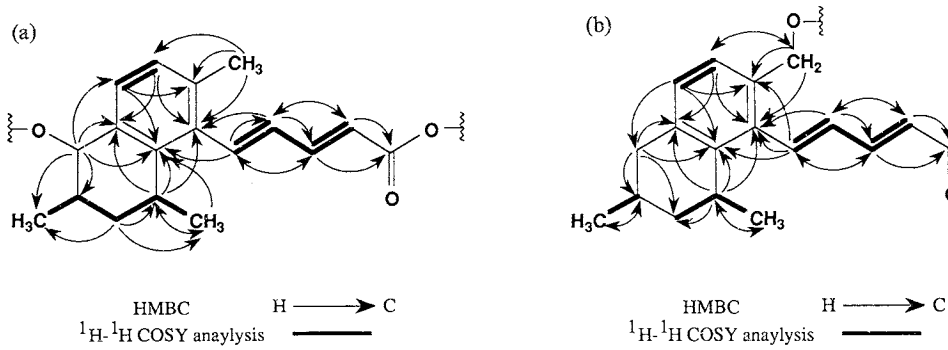


Fig. 8. HMBC analysis of arohynapene A (a) and B (b).



chain are *E* configurations[7]. On the basis of these results described above, the structures of arohynapenes A and B were deduced as shown in Fig. 1.

The physico-chemical and ^1H NMR spectral data of compound C (Tables 1 and 2) suggested that the structure including stereochemistry is identical to that of (3*S*)-6-hydroxy-8-methoxy-3,5-dimethylisochroman[6]. The ^{13}C chemical shifts assigned (Table 2) also supported the structure.

Biological Properties

Effect on Anticoccidial Activity in an *in vitro* System

Anticoccidial activity *in vitro* was assayed as reported previously[1]. Anticoccidial activity of arohynapenes is summarized in Table 3. Arohynapenes A and B and compound C showed anticoccidial activity at concentrations ranging above 35.0 μM, 7.0 μM and 67.0 μM, respectively.

Table 3. Anticoccidial activity of arohynapenes A and B and compound C *in vitro*.

Compounds	Minimum effective concentration (μM)	
	Anticoccidial activity[a]	Cytotoxicity[b]
Arohynapene A	35.0	140
Arohynapene B	7.0	140
Compound C	67.0	192
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 schizonts.

[a] No mature schizonts 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 activity.

Other Biological Activities

Arohynapenes A and B and compound C 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

Structures of arohynapenes are very similar to those of hynapenes[3]. All these compounds possess a trimethyldecaline core with a side chain of pentadienoic acid. The difference between arohynapene and hynapene is that one of the 6-membered rings in the decaline moiety is aromatized for arohynapenes but is saturated or monounsaturated for hynapenes. The order of anticoccidial potency in an *in vitro* system

is arohynapene B > hynapene B = hynapene C > arohynapene A > hynapene A, suggesting that aromatized ring in the decaline core is important for potent anticoccidial activity. Compound C was identified as (3*S*)-6-hydroxy-8-methoxy-3,5-dimethylisochroman, which was originally reported to be a new metabolite from a hybrid strain derived from *Penicillium citreo-viride*[6]. It is of interest that the compound was found to be produced inherently by *Penicillium* sp. FO-2295.

Experimental

Various NMR spectra were recorded on a Varian XL-400 (400 MHz) NMR spectrometer. Mass spectra were obtained on a JEOL model JMS-D 100 mass spectrometer. UV-visible spectra were measured on a Shimadzu UV-200S spectrometer. IR spectra were recorded on a Horiba FT-210 diffraction infrared spectrometer.

Acknowledgment

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