NOTES

Arohynapene D, a New Anticoccidial Agent Produced by *Penicillium* sp. FO-2295

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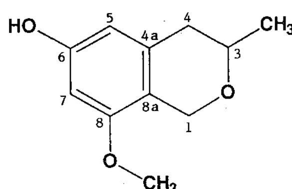
We have reported arohynapenes A and B and compound C as anticoccidial agents, which were produced by *Penicillium* sp. FO-2295[1]. Further isolation study from the strain led to the discovery of another active compound named arohynapene D (Fig. 1). In this paper, the isolation, physico-chemical properties, structure determination and biological characteristics of arohynapene D are described.

The seed and production media for arohynapene D production were the same as reported previously[1]. To obtain enough amount of arohynapene D, a jar fermentation was carried out at 27°C. The 96-hour old cultured broth (20 liters) was extracted with 20 liters of ethyl acetate. The extracts were dried with Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brown material (4.78 g). The material was applied on an ODS column (Senshu Sci., Co., ODS-SS-1020T, 100 ml). The first active fraction containing arohynapene D and compound C was eluted with 500 ml of 40% aq CH_3CN, and the second fraction containing arohynapene A and arohynapene B was eluted with 500 ml of 60% aq CH_3CN. For arohynapene D isolation, the brown material (37.3 mg), obtained by concentrating the 40% aq CH_3CN/fraction, was purified by preparative HPLC (column, YMC pack D-ODS-5, 20 × 250 mm; solvent, 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 from 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 D and compound C were eluted with retention times at 33.0 and 43.0 minutes, respectively. The fraction of arohynapene D was concentrated and extracted with ethyl acetate to give a white material (17.2 mg), which was further purified by preparative HPLC using an isocratic solvent (45% CH_3CN in 0.05% H_3PO_4). Arohynapene D was eluted with a retention time at 9.2 minutes. The active fraction was concentrated and extracted with ethyl acetate to give pure arohynapene D (4.1 mg) as white powder.

Fig. 1. Structure of arohynapene D.



The physico-chemical properties of arohynapene D are summarized in Table 1. It is soluble in methanol, ethanol, acetonitrile, acetone, ethyl acetate and chloroform, slightly soluble in benzene, and insoluble in water. The UV spectrum showed maxima at 210 (ε 10,400) and 282 nm (ε 2,250), which was very similar to that of compond C, suggesting that arohynapene D has the same chromophore as compound C.

The molecular formula of arohynapene D was

Table 1. Physico-chemical properties of arohynapene D.

Appearance	White powder
$[\alpha]_D^{28}$ (*c* 0.1, CH_3OH)	−12.0°
Molecular formula	$C_{11}H_{14}O_3$
HREI-MS (*m*/*z*)	
Calcd:	194.0943
Found:	194.0930
UV $\lambda_{max}^{CH_3OH}$ (nm)	210 (10,400), 282 (2,250)
IR ν_{max}^{KBr} (cm^{-1})	1606, 1497, 1447, 1380, 1315, 1260
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

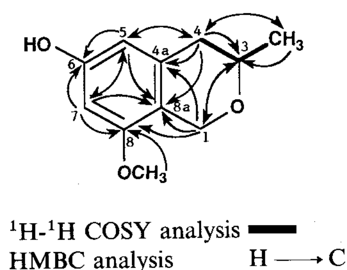
Table 2. ^1H and ^{13}C NMR chemical shifts of arohynapene D.

Carbon No.	Arohynapene D	
	^{13}C chemical shifts ppm[a]	^1H chemical shifts ppm[b]
C-1	64.41	4.57 (1H, dt, *J*=15.0, 2.0 Hz), 4.85 (1H, d, *J*=15.0 Hz)
C-3	70.21	3.73 (1H, m)
C-3-CH_3	21.46	1.33 (3H, d, *J*=6.0 Hz)
C-4	35.81	2.60 (2H, m)
C-4a	135.71	
C-5	106.53	6.16 (1H, d, *J*=2.0 Hz)
C-6	154.88	
C-7	96.28	6.23 (1H, d, *J*=2.0 Hz)
C-8	156.51	
C-8-O-CH_3	55.16	3.75 (3H, s)
C-8a	115.63	

[a] The 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.

Fig. 2. HMBC analysis of arohynapene D.



^1H-^1H COSY analysis ▬
HMBC analysis H ⟶ C

Table 3. Anticoccidial activity of arohynapene D *in vitro*.

Compound	Minimum effective concentration (μM)	
	Anticoccidial activity[a]	Cytotoxicity[b]
Arohynapene D	0.51	1.0
Monensin	—[c]	0.03

BHK-21 cells stained with hematoxylin solution was observed microscopically. In control experiments (no drug), infected sporozoites of monensin-resistant *Eimeria tenella* 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.

determined to be $C_{11}H_{14}O_3$ (*m/z* found 194.0930, calcd 194.0943) by HREI-MS analysis. ^{13}C and ^1H NMR spectra ($CDCl_3$) showed 11 carbon and 13 proton signals, respectively. The DEPT spectra indicated the presence of one $-CH_3$, one $-O-CH_3$, one $-CH_2-$, one $-O-CH_2-$, one $-O-CH-$, two $=CH-$ and four quaternary carbons. To fulfill the molecular formula of arohynapene D, the presence of one hydroxyl group was suggested. The connectivity of proton and carbon atoms was confirmed by the ^{13}C-^1H COSY spectrum as shown in Table 2. The ^1H-^1H COSY spectrum showed the $-CH_2-CH-CH_3-$ sequence (Fig. 2). ^{13}C-^1H long range couplings of ^2J and ^3J observed in the HMBC spectra are shown in Fig. 2, that is, 1) cross peaks from H_2-4 (δ 2.60) to C-4a (δ 135.71) and C-8a (δ 115.63) and from H_2-1 (δ 4.57 and 4.85) to C-3 (δ 70.21), C-4a (δ 135.71) and C-8a (δ 115.63) revealing a 6-membered ring, 2) cross peaks from H-5 (δ 6.16) to C-6 (δ 154.88), C-7 (δ 96.28) and C-8a (δ 115.63) and from H-7 (δ 6.23) to C-5 (δ 106.53), C-6 (δ 154.88), C-8 (δ 156.51) and C-8a (δ 115.63) also revealing another 6-membered ring, 3) cross peaks form H_2-1 (δ 4.57 and 4.85) to C-8 (δ 156.51), from H_2-4 (δ 2.60) to C-5 (δ 106.52) and from H-5 (δ 6.16) to C-4 (δ 35.81) indicating that the two 6-membered rings form an isochroman skeleton, and 4) cross peaks form $O-CH_3$ (δ 3.75) to C-8 (δ 156.51) indicating that the methoxy group is attached at the C-8 (δ 156.51) carbon. The remaining hydroxyl group should be attached to C-6 (δ 154.88) carbon because of the ^{13}C chemical shift[4]. Finally, the structure of arohynapene D was deduced to 6-hydroxy-8-methoxy-3-methylisochroman as shown in Fig. 1, which was C-5 demethyl compound C[1,3].

The *in vitro* anticoccidial activity[2] of arohynapene D is summarized in Table 3. Arohynapene D showed anticoccidial activity at a concentration 0.51 μM. Arohynapene D showed no antimicrobial activity *in vitro* (1 mg/ml).

Acknowledgment

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