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

I. PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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

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

(Received for publication August 27, 1993)

Penicillium sp. FO-1611, a soil isolate, was found to produce a series of new anticoccidial compounds. Three active compounds, designated hynapenes A, B and C, were isolated from the fermetation broth of the producing strain by solvent extraction, silica gel column chromatography, gel filtration on Sephadex LH-20 and preparative HPLC. Hynapenes 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 123 μM, 34.7 μM and 34.7 μM for hynapenes A, B and C, respectively.

Recently, we have reported new anticoccidial agents of microbial origin, xanthoquinodins[1] and diolmycins[2]. From our continuous screening system using BHK-21 cells as a host and monensin-resistant *Eimeria tenella* as a parasitic protozoan, a fungal strain FO-1611 was found to produce a series of new anticoccidial agents. Three active compounds, termed hynapenes A, B and C (Fig. 1), were isolated. In this paper, the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological characteristics of hynapenes are described.

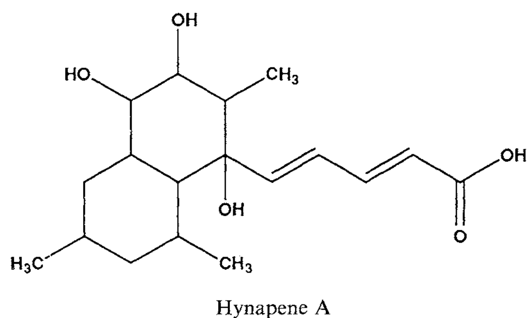
Characteristics of the Producing Strain

Strain FO-1611 was originally isolated from a soil sample. For the taxonomic test of the fungus, potato dextrose agar, malt extract agar, CZAPEK's agar and yeast extract - soluble starch (YpSs) agar were used. This strain grew rapidly to form pale green colonies with diameter of 30~40 mm after incubation for 14 days at 25°C. The colony surface was velvety. The conidial structures were abundantly produced on various agar media. The reverse color was pale gray to pale yellowish brown. When strain FO-1611 was grown on potato dextrose agar at 25°C for 7 days, the conidiophores borne from substrate hyphae, and penicillia were monoverticillate as shown in Fig. 2. The phialides were $7.5 \sim 10 \times 2 \sim 3\ \mu$m. The conidia were globe to subglobe and $2.0 \sim 2.5\ \mu$m in diameter, and its surface was smooth. From the above characteristics, the strain FO-1611 was identified as the genus *Penicillium*[3,4] and named *Penicillium* sp. FO-1611. This culture was deposited at the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology Japan, as FERM P-13399.

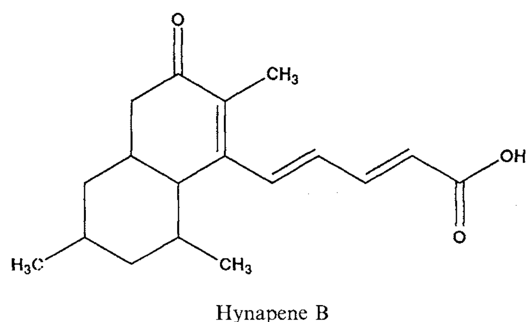
Fermentation

A slant culture of strain FO-1611 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 500-ml Erlenmeyer flasks containing 100 ml of seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%,

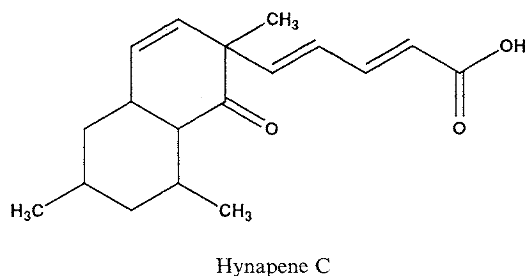
Fig. 1. Structures of hynapenes A, B and C.



Hynapene A



Hynapene B



Hynapene C

Fig. 2. Scanning electron micrograph of penicillia of strain FO-1611 on potato-dextrose agar (Hitachi, model S-430).

Scale: 5 μm.

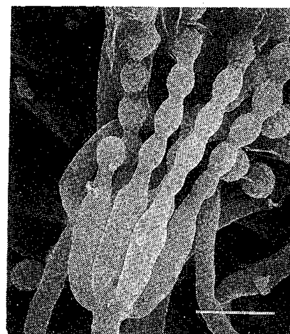
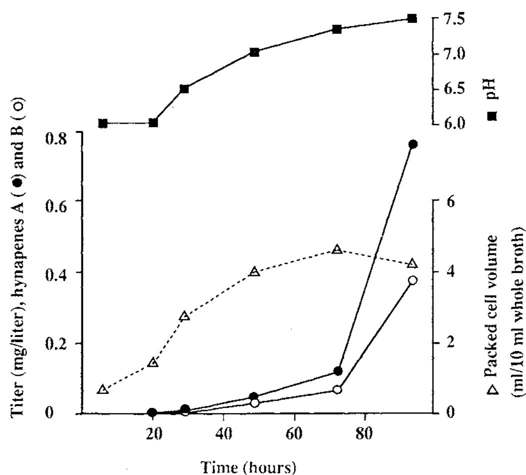


Fig. 3. A typical time course of hynapenes production.

Hynapenes A (●) and B (○).



Polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 3 days at 27°C. Two hundred ml of the seed culture was transferred into 20 liters 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 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 hynapenes A and B was measured by HPLC under the following conditions: column; YMC pack D-ODS-5 (20 × 250 mm), 60% CH_3CN in 0.05% H_3PO_4 (0 ~ 10 minutes) and a linear gradient from 60% CH_3CN in 0.05% H_3PO_4 to 100% CH_3CN (10 ~ 40 minutes), UV detection at 265 nm, flow rate 6.0 ml/minute. Under these conditions, hynapene A was eluted first with a retention time at 28.5 minutes, followed by hynapenes B and C at 29.5 and 35.5 minutes, respectively. The production of hynapenes A and B was observed after 30 hours and increased at least up to 93 hours.

Table 1. Physico-chemical properties of hynapenes.

	Hynapene A	Hynapene B	Hynapene C
Appearance	Yellow powder	Yellow powder	Yellow powder
$[\alpha]_D^{23}$ (*c* 0.1, MeOH)	−14°	+116°	+182°
Molecular formula	$C_{18}H_{28}O_5$	$C_{18}H_{24}O_3$	$C_{18}H_{24}O_3$
HREI-MS (*m/z*)			
Calcd:	324.1935	288.1724	288.1724
Found:	324.1926	288.1725	288.1724
UV λ_{max}^{MeOH} nm (ε)	263 (22,300)	238 (17,000), 297 (16,700)	202 (6,400), 252 (12,200), 261 (11,000)
IR (cm^{-1})	3402, 2920, 1686, 1637, 1543, 1458, 1406, 1383, 1302, 1248, 1151, 1011	3421, 2922, 1660, 1624, 1545, 1454, 1379, 1306, 1259, 1151, 1063, 1022	2924, 2389, 1718, 1691, 1633, 1456, 1416, 1375, 1302, 1265, 1003
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 93-hour cultured 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 (6.54 g). The material was applied on a silica gel column (E. Merck, Kieselgel 60, 300 ml). The column was washed with 1.5 liters of chloroform and 1.5 liters of chloroform - methanol (99 : 1). The active components were eluted with 1.5 liters of chloroform - methanol (98 : 2), and each 150 ml was successively collected. The 4th to 6th fractions enriched with hynapenes A, B and C were combined and concentrated *in vacuo* to give a brown material (252 mg). Further purification of hynapenes was carried out by gel filtration on Sephadex LH-20 (2 × 120 cm, MeOH, 0.2 ml/minutes). The active components were eluted with 500 ml of methanol, and each 2.5 ml was successively collected. The 81st to 91st fractions enriched with hynapenes A, B and C were evaporated *in vacuo* to give yellow materials (86 mg). Hynapenes A, B and C were finally purified by preparative HPLC (column; YMC pack D-ODS-5, 20 × 250 mm, solvent; 60% CH_3CN in 0.05% H_3PO_4 (0 ~ 10 minutes) and a linear gradient from 60% CH_3CN in 0.05% H_3PO_4 to 100% CH_3CN (10 ~ 40 minutes), UV at 265 nm, 6.0 ml/minute). The active fractions were concentrated and extracted with ethyl acetate to give pure hynapenes A (7.5 mg), B (3.8 mg) and C (5.9 mg) as yellow materials.

Table 2. Anticoccidial activity of hynapenes A, B and C in an *in vitro* assay.

Compounds	Minimum effective concentration (μM)	
	Anticoccidial activity[a]	Cytotoxicity[b]
Hynapene A	123	NT[d]
Hynapene B	34.7	139
Hynapene C	34.7	69.4
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 activity.

[d] NT: Not tested at higher concentrations than 123 μM.

Physico-chemical Properties

The physico-chemical properties of hynapenes A, B and C are summarized in Table 1. They are soluble in methanol, ethanol, acetonitrile, acetone, ethyl acetate, slightly soluble in chloroform and benzene and insoluble in water. The UV spectra show a maximum/maxima at 263 nm (ε 22,300) for hynapene A, at 238 (ε 17,000) and 297 nm (ε 16,700) for hynapene B and at 202 (ε 6,400), 252 (ε 12,200) and 261 nm (ε 11,000) for hynapene C. The IR spectra suggest the presence of a carbonyl group (carboxylic acid) in hynapenes (1686 cm^{-1} for hynapene A, 1660 cm^{-1} for hynapene B and 1691 cm^{-1} for hynapene C).

Biological Properties

Effect on Anticoccidial Activity in an *In Vitro* System

Anticoccidial activity *in vitro* was assayed as reported previously[1]. Anticoccidial activity of hynapenes is summarized in Table 2. Both hynapenes B and C showed anticoccidial activity at concentrations ranging above 34.7 μM. Hynapene A showed poor anticoccidial activity at concentration above 123 μM. When minimum effective concentrations were compared between anticoccidial activity (I) and cytotoxicity (II), the II/I ratio could be defined as specificity for anticoccidial activity. Hynapenes B and C showed the ratios of 4 and 2, respectively.

Other Biological Activities

Antimicrobial activity of hynapenes was tested at 1 mg/ml by paper disc (i.d. 6 mm, Toyo Roshi) method. Hynapenes A, B and C showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 (diameter of inhibition zone: 10, 12, 11 mm), *Mycobacterium smegmatis* ATCC 607 (0, 17, 0 mm) and *Pyricularia oryzae* (0, 9, 11 mm), respectively. Hynapenes A, B and C showed no antimicrobial activity against *Staphylococcus aureus* FDA 209P, *Micrococcus luteus* PCI 1001, *Escherichia coli* NIHJ, *Escherichia coli* NIHJ JC-2 IFO 12734, *Pseudomonas aeruginosa* P-3, *Xanthomonas oryzae*, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG8, *Candida albicans*, *Saccharomyces sake*, *Aspergillus niger* ATCC 6275, and *Mucor racemosus* IFO 4581.

Discussion

In the present investigation the hynapenes showed anticoccidial activity in an *in vitro* system. Hynapenes possess a common decalin core and a side chain of pentadienoic acid (Fig. 1). The structures will be reported in the following paper[5]. The order of anticoccidial activity was B=C>A (Table 1), suggesting the oxo group in the decalin core is important for potent anticoccidial activity. Furthermore, hynapene B showed the weakest cytotoxicity, resulting in the highest specificity among hynapenes. When production of hynapenes during fermentation was analyzed by HPLC, the peak corresponding to hynapene C (retention time of 35.5 minutes) was not detected. It might be that hynapene C is unstable after storage of the crude material at −20°C for two months.

Acknowledgment

We express our thanks to Mr. T. Kawakubo for characterization of the producing strain and to Mr. M. Kishi for preparing oocysts of *E. tenella*.

References

1) Tabata, N.; Y. Suzumura, H. Tomoda, R. Masuma, K. Haneda, M. Kishi, Y. Iwai & S. Ōmura: Xanthoquinodins, new anticoccidial agents produced by *Humicola* sp. Production, isolation and physico-chemical and biological properties. J. Antibiotics 46: 749~755, 1993

2) TABATA, N.; H. TOMODA, Y. TAKAHASHI, K. HANEDA, Y. IWAI, B. WOODRUFF & S. ŌMURA: Diolmycins, new anticoccidial agents produced by *Streptomyces* sp. I. Production, isolation and physico-chemical and biological properties. J. Antibiotics 46: 756～761, 1993

3) PITT, J. I. (*Ed.*): The Genus *Penicillium* and Its Teleomorphic States, *Eupenicillium* and *Talaromyces*. Academic Press, 1979

4) UDAGAWA, S.; K. TSUBAKI, G. HORIE, K. MIURA, K. MINOURA, M. YAMAZAKI, T. YOKOYAMA & S. WATANABE (*Ed.*): Classification and identification of genus *Penicillium*. *In* Kinrui Zukan. II. pp. 1076～1120, Kodansha, Tokyo, 1978

5) TABATA, N.; H. TOMODA, Y. IWAI & S. ŌMURA: Hynapenes A, B and C, new anticoccidial agents produced by *Penicillium* sp. II. Structure elucidation. J. Antibiotics 46: 1854～1858, 1993