

XANTHOQUINODINS, NEW ANTICOCIDIAL AGENTS
PRODUCED BY *Humicola* sp.
PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL AND
BIOLOGICAL PROPERTIES

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Humicola sp. FO-888, a soil isolate, was found to produce a series of new anticoccidial compounds. Five active compounds, designated xanthoquinodins A1, A2, A3, B1 and B2 were isolated from the fermentation broth of the producing strain by solvent extraction and HPLC. Xanthoquinodins inhibited the growth of *Eimeria tenella* in an *in vitro* assay system using BHK-21 cells as a host. No schizont in the cells was observed at concentrations of 0.02~0.2 µg/ml for xanthoquinodins A1, A3, B1 and B2 and at 0.02 µg/ml for xanthoquinodin A2.

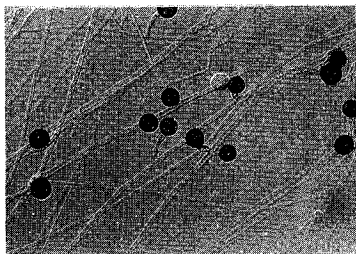
Coccidiosis in chickens, turkeys, geese or ducks is a widespread disease caused by a group of parasitic protozoa. Most of the pathogens in the poultry belong to the genus *Eimeria*. Polyether ionophores such as monensin¹, lasalocid², salinomycin³, narasin⁴ and maduramicin⁵ have been used successfully as anticoccidial agents and as growth promotants. However, polyether-resistant *Eimeria* spp. are emerging with increasing frequency⁶. Therefore, the search for new anticoccidial agents against polyether-resistant *Eimeria* spp. is being intensified.

ONAGA and ISHII⁷ reported an *in vitro* assay system for the evaluation of anticoccidial activity using cultured animal cells. In this system it was expected to observe the life cycle of a coccidium from sporozoites to shizonts. Accordingly, we have established a similar assay system using BHK-21 cells as a host and monensin-resistant *E. tenella* as a parasite to screen new anticoccidial agents of microbial origin. During the screening course, a fungal strain FO-888 was found to produce a series of new anticoccidial agents. Eventually, five active compounds, termed xanthoquinodins A1, A2, A3, B1 and B2 were isolated. The physico-chemical properties of xanthoquinodin A1 are similar to those of M-4126, originally isolated as an antibacterial antibiotic by K. MIZUNO *et al.*⁸). In this paper, the taxonomy of the producing strain, fermentation, isolation, and physico-chemical and biological properties of xanthoquinodins are described.

Characteristics of the Producing Strain

The strain FO-888 was isolated from a soil sample collected at Hachinohe, Aomori, Japan. On potato-glucose agar, corn meal agar, malt extract agar and yeast extract-soluble starch (YpSs) agar, this strain grew rapidly to form white to gray colonies with diameter of 55~80 mm after incubation for 14 days at 25°C. At 37°C, growth is nil. Conidia were abundantly born on the colony surface. Conidia were solitary, and were produced directly on the vegetative hyphae or on short branches (Fig. 1). They were

Fig. 1. Micrograph of conidial structures of *Humicola* sp. FO-888 on corn meal agar. (scale: 10 μ m).



smooth, dark brown, unicellular, thick-walled, globose to subglobose, and 14~17 μ m in size. Cultural characteristics on cellulose agar were nearly the same as those on the previous agar media. The above characteristics indicate that strain FO-888 belongs to the genus *Humicola*⁹⁾. The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Humicola* sp. FO-888 with the accession number FERM-P 13160.

Fermentation

A slant culture of strain FO-888 grown on YpSs agar was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of 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 was 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%, $CaCO_3$ 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. 2. The production of xanthoquinodins A1, A2, A3, B1 and B2 was measured by HPLC under the following conditions: YMC packed column R-ODS-5 (4.6 \times 200 mm), 60% CH_3CN in 0.05% H_3PO_4 , UV detection at 340 nm, flow rate 0.7 ml/minute. Under these conditions, xanthoquinodin B1 was eluted first with a retention time at 34.0 minutes, followed by xanthoquinodins A3, B2, A1 and A2 at 43.0, 53.0, 56.0 and 62.0 minutes, respectively. The concentration of xanthoquinodin A1 reached a maximum at 168 hours.

Isolation

Six-day cultured broth (300 ml) was centrifuged at 3,000 rpm for 5 minutes to separate the supernatant and mycelia. To the supernatant was added H_3PO_4 (600 μ l) and the acidified supernatant was extracted with 300 ml of ethyl acetate. The extracts were evaporated *in vacuo* to yield a dark yellow material (101 mg). To the mycelia were added 70% aq acetone (300 ml) and H_3PO_4 (600 μ l). After centrifugation of this suspension at 3,000 rpm for 5 minutes, the supernatant was concentrated to remove acetone. The aqueous solution was extracted with 300 ml of ethyl acetate. The extracts were evaporated *in vacuo* to yield a dark yellow material (188 mg). Xanthoquinodins A1, A2, A3, B1 and B2 were finally purified by HPLC (YMC pack D-ODS-5, 20 \times 250 mm, 70% CH_3CN in 0.05% H_3PO_4 , UV detection at 340 nm, 6.0 ml/minute).

Fig. 2. A typical time course of xanthoquinodins production.

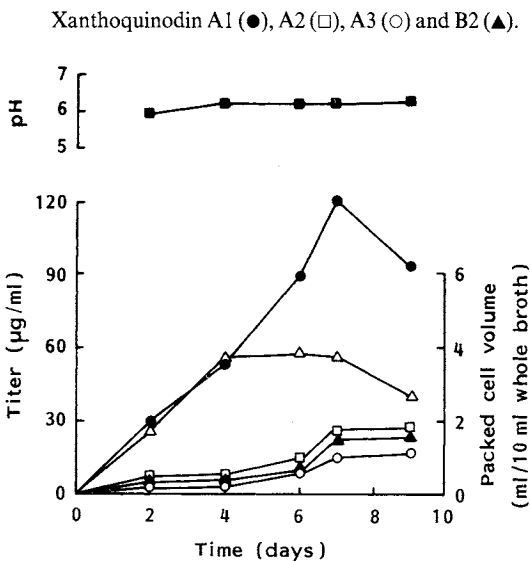
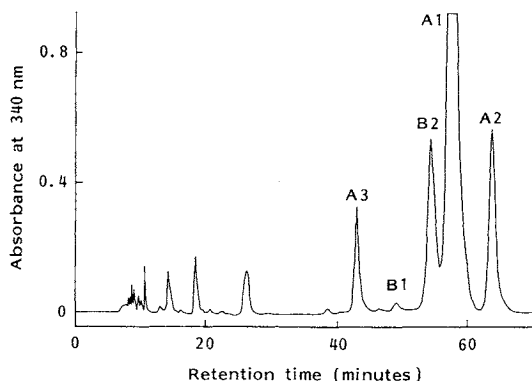


Fig. 3. A chromatographic profile of xanthoquinodins separated by preparative HPLC.



Column: YMC-Packed column ODS-5 (20 × 250 mm); mobile phase: CH₃CN - 0.05% aq H₃PO₄ (7 : 3); flow rate: 6.0 ml/minute; detection: 340 nm.

Fig. 4. UV spectra of xanthoquinodins (MeOH).

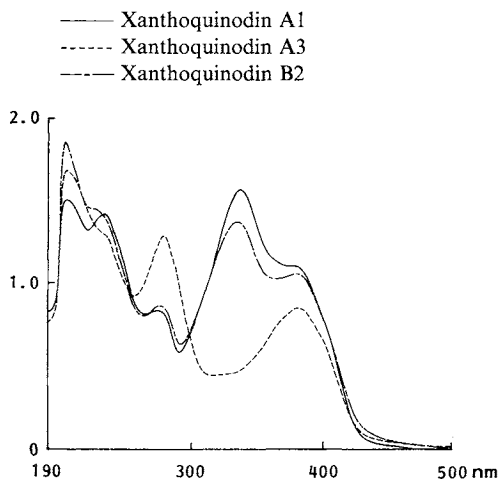


Table 1. Physico-chemical properties of xanthoquinodins.

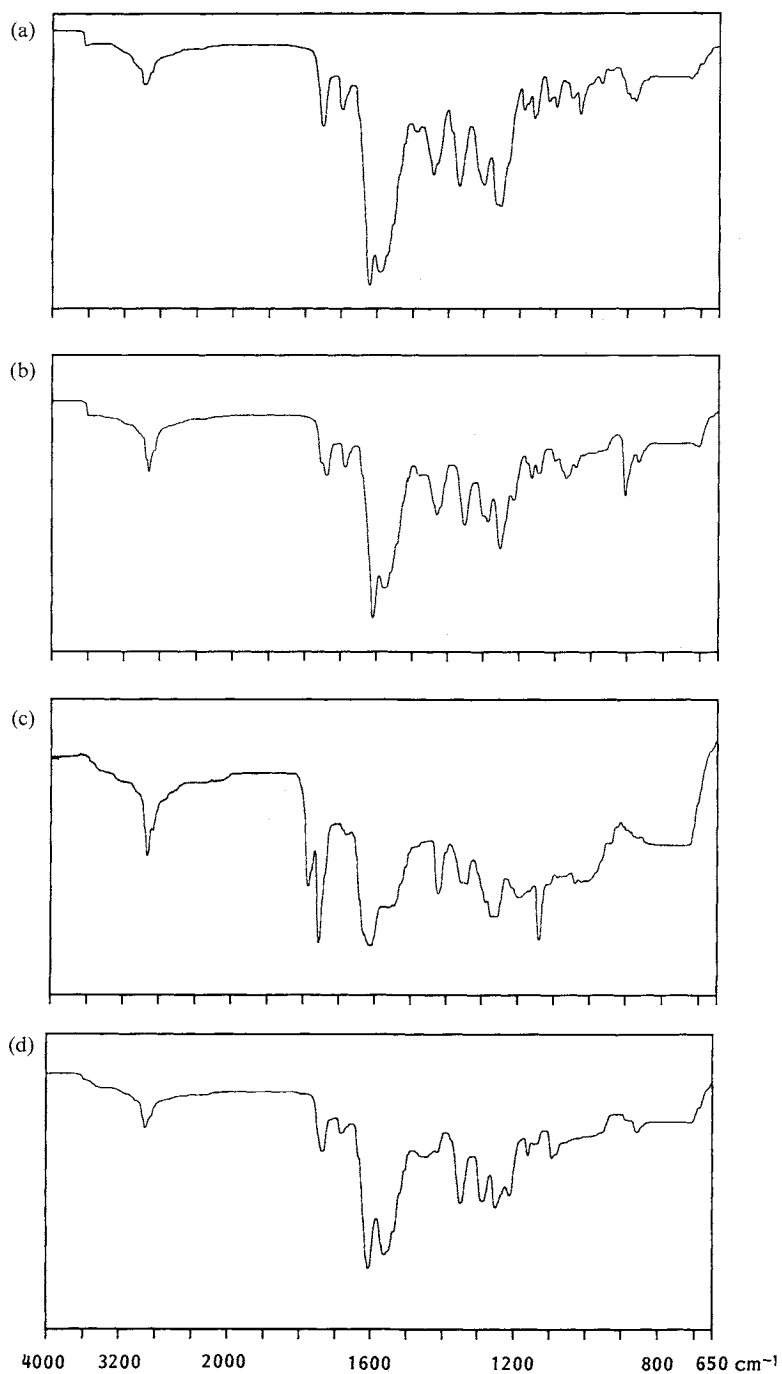
	Xanthoquinodin A1	Xanthoquinodin A2	Xanthoquinodin A3	Xanthoquinodin B1	Xanthoquinodin B2
Appearance	Yellow powder	Yellow powder	Yellow powder	Yellow powder	Yellow powder
$[\alpha]_D^{25}$ (c 0.1, MeOH)	+404°	+224°	+20°	+258°	+488°
Molecular formula	C ₃₁ H ₂₄ O ₁₁	C ₃₁ H ₂₄ O ₁₁	C ₃₁ H ₂₄ O ₁₁	C ₃₁ H ₂₄ O ₁₁	C ₃₁ H ₂₄ O ₁₁
HREI-MS (m/z)					
Calcd:	572.1317	572.1317	572.1317	572.1317	572.1317
Found:	572.1316	572.1319	572.1324	572.1301	572.1317
UV	230, 273, 335	230, 273, 335	232, 279, 378	226, 274, 334	226, 273, 333
IR	1735, 1680, 1600, 1570	1735, 1685, 1605, 1570	1783, 1752, 1605	1735, 1680, 1605, 1565	1735, 1680, 1605, 1565
Solubility					
Soluble:	MeOH, CHCl ₃	MeOH, CHCl ₃	MeOH, CHCl ₃	MeOH, CHCl ₃	MeOH, CHCl ₃
Insoluble:	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O

Total extracts from the supernatant and mycelium (289 mg) were dissolved in methanol (2.89 ml) and each 100 μ l was injected (Fig. 3). Active fractions corresponding to xanthoquinodins A1, A2, A3, B1 and B2 were combined and concentrated to aqueous solutions, which were extracted with ethyl acetate to give yellow powders (73, 16, 11, 2 and 17 mg, respectively).

Physico-chemical Properties

The physico-chemical properties of xanthoquinodins A1, A2, A3, B1 and B2 are summarized in Table 1. The molecular formulas of xanthoquinodins A1, A2, A3, B1 and B2 were determined to be all C₃₁H₂₄O₁₁ on the basis of HREI-MS. UV spectra of xanthoquinodins A1, A3 and B2 are shown in Fig. 4. IR spectra of xanthoquinodins A1, A2, A3 and B2 are shown in Fig. 5. The physico-chemical properties of xanthoquinodins A1 and A2, and of xanthoquinodins B1 and B2 were very similar. Xanthoquinodin A3 was different from the other xanthoquinodins.

These data indicated that xanthoquinodins A2, A3, B1 and B2 are new compounds, but xanthoquinodin A1 appears similar to M-4126, which was originally isolated as an antibiotic active against Gram-positive

Fig. 5. IR spectra of xanthoquinodins A1 (a), A2 (b), A3 (c) and B2 (d) (CHCl_3).

bacteria⁸⁾. Their structures will be described elsewhere.

Biological Properties

Effect on Anticoccidial Activity in BHK-21 Cells System

BHK-21 cells (an established cell line from kidney cells of baby hamster obtained from Dainippon

Pharmaceutical Co.) were used as host cells. Cultures were routinely maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C in a 25-cm² flask (Corning Co.) containing 5 ml of EAGLE's minimum essential medium (MEM) supplemented with benzylpenicillin (50 U/ml), streptomycin (50 µg/ml) and fetal bovine serum (FBS, Flow, 7%, v/v).

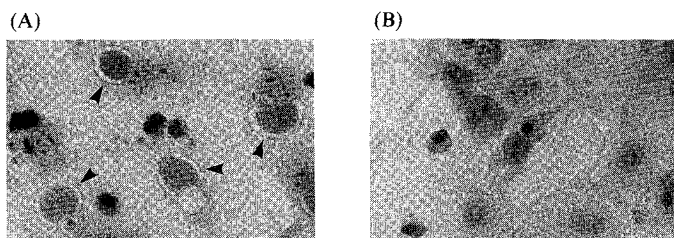
BHK-21 cells, cultured for three days, were dispersed using 0.125% (w/v) trypsin with 0.01% (w/v) EDTA and the cells were suspended in medium A (MEM supplemented with 5% FBS, benzylpenicillin (50 U/ml) and streptomycin (50 µg/ml)) at a density of 4×10^4 cells/ml. This cell suspension (100 µl) was used to inoculate each well of 96-flat-bottom-well microtiter plates (Corning Co.). The cells confluent after a 2-day incubation (37°C, 5% CO₂/95% air) were used for assay.

Eimeria tenella oocysts used are resistant to monensin. The oocysts were recovered from ceca of chickens (white leghorn) fed with a diet containing 120 ppm monensin and oocysts. The oocysts can usually be stored at 4°C as suspension in chromic acid (2.5%). Before assay, the oocysts, collected by centrifugation (800 rpm, 2 minutes), were washed twice with distilled water. The oocysts were resuspended in 20 ml of Purelox (Oyalox Co., sodium hypochlorite 6%) to sterilize them. After Purelox was removed by centrifugation (2,000 rpm, 2 minutes), the oocysts were washed three times with sterile phosphate buffered saline (PBS). A 5-ml suspension of pure oocysts ($2 \sim 4 \times 10^6$ /ml PBS) was ground with a tissue homogenizer at a speed of 600 rpm for 20 minutes at 4°C to give sporocysts. A mixed solution (3 ml of trypsin solution 2.5%, w/v, 2 ml of chicken bile acid and 20 ml of PBS) was added to the sporocyst suspension. Digestion of sporocysts was carried out at 41°C for 2 hours to yield sporozoites. After centrifugation of the suspension (3,000 rpm, 2 minutes), the precipitates were washed twice with PBS. Medium B (20~30 ml, MEM supplemented with benzylpenicillin (50 U/ml), streptomycin (50 µg/ml) and yeast extract (Difco Lab. 1%)) was added to the precipitates and the suspension was allowed to stand for 30 minutes. Unbroken oocysts and undigested sporocysts went down to the bottom. The supernatant with sporozoites was transferred carefully. The sporozoites were diluted to 3×10^5 /ml with medium B. After the medium of the 2-day cultured BHK-21 cells in each well was changed to 100 µl of medium B, the freshly prepared sporozoite suspension in medium B (100 µl) was further added to each well of the microtiter plates. The yeast extract is considered to promote the growth of *E. tenella*. Then samples dissolved in 4 µl of 50% ethanol, 100% ethanol or 100% methanol, were added to each well. The total volume per well was 204 µl. These plates were incubated at 41°C in a humidified atmosphere of 2% CO₂/98% air. After a three-day incubation the cells were rinsed with PBS, fixed with absolute methanol and stained with hematoxylin solution [hematoxylin (Wako Pure Chemical Industries, Ltd.) 0.1%, sodium iodate 0.02%, aluminium potassium sulfate 5.0%, citric acid 0.1%, chloral hydrate (Kanto Chemical Co.) 5.0%, w/v] for 10~15 minutes.

Anticoccidial activity was judged by microscopic observation of the formation of mature shizonts in

Fig. 6. Shizont formation in BHK-21 cells.

A; monensin (0.01 µg/ml), B; xanthoquinodin A1 (0.1 µg/ml). The arrow shows the mature shizont.



the cells. Since monensin-resistant *E. tenella* was used in the assay, mature schizonts were formed in the BHK-21 cells in the presence of monensin at 0.01 $\mu\text{g/ml}$ (Fig. 6A). Higher concentrations of monensin caused severe cytotoxicity to the host cells. On the other hand, no mature schizonts were observed in the presence of xanthoquinodin A1 at 0.1 $\mu\text{g/ml}$ (Fig. 6B). Thus, all xanthoquinodins showed anticoccidial activity at concentrations higher than 0.02 $\mu\text{g/ml}$ (Fig. 6, Table 2), indicating a similar anticoccidial potency. However, cytotoxicity was observed at concentrations higher than 2.0 $\mu\text{g/ml}$ of xanthoquinodins A1, A3, B1 and B2 and higher than 0.2 $\mu\text{g/ml}$ of xanthoquinodin A2. This indicated that xanthoquinodin A2 was the most cytotoxic agent.

Other Biological Activities

Antimicrobial activity of xanthoquinodins was tested at 1 mg/ml by paper disc (6 mm, Toyo Roshi) method. Xanthoquinodins A1, A2, A3, B1 and B2 showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 (diameter of inhibition zone: 12, 11, 8, 8, 12 mm), *Micrococcus luteus* ATCC 9341 (8, 9, 0, 0, 10 mm), *Staphylococcus aureus* FDA 209P (9, 9, 6, 6, 10 mm), *Acholeplasma ladlawli* PG8 (10, 7, 0, 0, 0 mm) and *Bacteroides fragilis* ATCC 23745 (8, 8, 0, 0, 7 mm), respectively.

Discussion

To screen new anticoccidial agents active against monensin-resistant *E. tenella* a cell assay was introduced with BHK-21 cells as a host and monensin-resistant *E. tenella* as a parasite. In this assay, in fact, monensin showed no anticoccidial activity (Fig. 6 and Table 2). But xanthoquinodins were found to inhibit the growth of the parasite in the cells (Fig. 6). Since *E. tenella* can be propagated from sporozoites to shizonts in BHK-21 cells, the inhibition step of xanthoquinodins might lie within the parts of the life cycle. A preliminary *in vivo* test showed that the growth of monensin-resistant *E. tenella* in ceca was completely inhibited when xanthoquinodin A1 was added to the diet at 35 ppm, but increment of body weight in host chickens was low.

Acknowledgment

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Table 2. Anticoccidial activity of xanthoquinodins A1, A2, A3, B1 and B2 in an *in vitro* assay.

Compounds	Minimum effective concentration ($\mu\text{g/ml}$)	
	Anticoccidial activity ^a	Cytotoxicity ^b
Xanthoquinodin A1	0.02	2.0
Xanthoquinodin A2	0.02	0.2
Xanthoquinodin A3	0.02	2.0
Xanthoquinodin B1	0.02	2.0
Xanthoquinodin B2	0.02	2.0
Monensin	— ^c	0.02

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 (Fig. 6).

^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.

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