Xanthoquinodin B3, a New Anticoccidial Agent Produced by *Humicola* sp. FO-888

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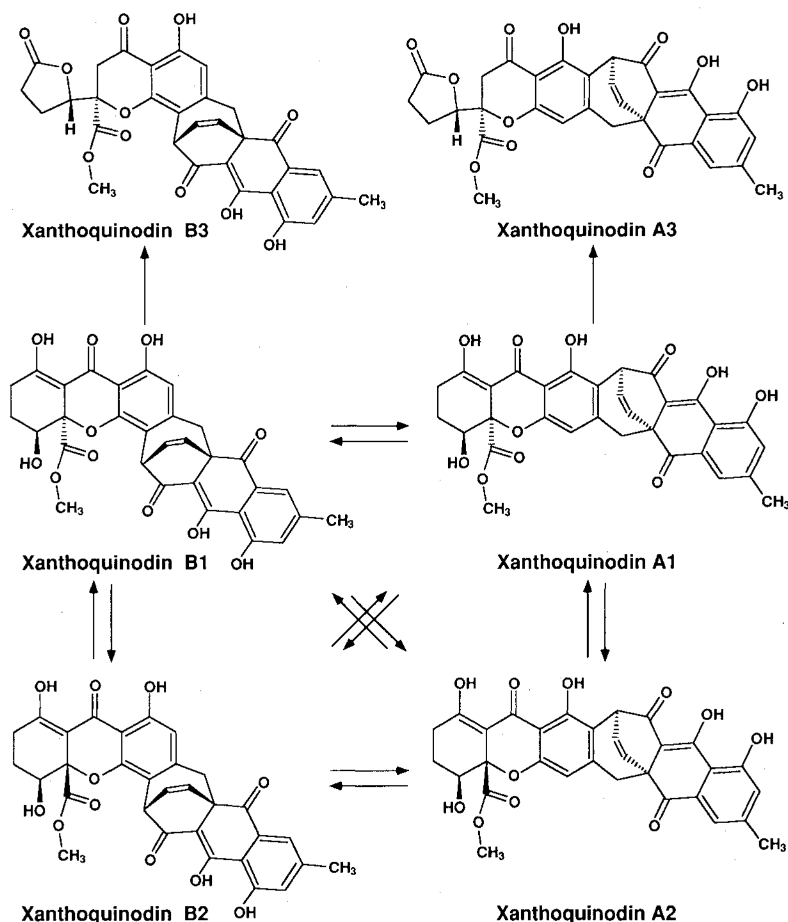
Xanthoquinodin B3, a new component of anticoccidial xanthoquinodins, which was detected in the culture broth of *Humicola* sp. FO-888, was isolated by heat treatment of the xanthoquinodins complex. Structural elucidation indicated that xanthoquinodin B3 has the same heterodimer of xanthone- and anthraquinone-derived monomers as other xanthoquinodins. Schizont formation of monensin-resistant *Eimeria tenella* in BHK-21 cells was inhibited by xanthoquinodin B3 at concentrations greater than 0.035 μM.

We have isolated xanthoquinodins A1, A2, A3, B1 and B2 as anticoccidial agents from the culture broth of *Humicola* sp. FO-888[1]. Xanthoquinodins are the first heterodimer of xanthone- and anthraquinone-derived monomers, both of which are biosynthesized *via* octaketide[2,3]. As reported previously, xanthoquinodins were interconvertible by heat treatment, but xanthoquinodin A3, a heat stable component, was produced only *via* xanthoquinodin A1. Existence of xanthoquinodin B3, which is heat stable and is produced *via* xanthoquinodin B1, has been deduced for B series of xanthoquinodins from the experiments. In fact, we have detected another

Fig. 1. Structures of xanthoquinodins A1, A2, A3, B1, B2 and B3.



component in the culture broth by HPLC, which showed the same UV spectrum as xanthoquinodin A3. We have obtained enough amount of the component by heat treatment of xanthoquinodins complex and identified with xanthoquinodin B3. In this paper, isolation, physico-chemical properties, structure elucidation and biological characteristics of xanthoquinodin B3 are described.

Materials and Methods

General Experimental Procedures

Humicola sp. FO-888 was used for the production of xanthoquinodins[1]. HPLC was carried out using the Waters 600E system.

Spectroscopic Studies

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a JASCO A-102 diffraction grating infrared spectrometer. Optical rotations were 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. The various NMR spectra were obtained on a Varian XL-400 spectrometer.

Interconversion of Xanthoquinodins by Heat Treatment

Each of the pure xanthoquinodins (0.3 mg), dissolved in 0.3 ml MeOH in a test tube (10 × 30 mm) sealed with a screw cap, was heated at 80°C for 85 minutes. The heat-treated xanthoquinodins were analyzed by HPLC using an ODS column (YMC, R-ODS-5, 4.6 × 200 mm) eluted with 70% acetonitrile in 0.05% H_3PO_4 at 0.7 ml/minute. Under the conditions xanthoquinodins A1, A2, A3, B1, B2 and B3 were eluted with retention times of 27.5, 30.5, 21.0, 23.5, 26.0 and 18.5 minutes, respectively. At time 0, each of the pure xanthoquinodins used in the reaction, showed only one peak by the HPLC analysis.

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 protozoan.

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Müeller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. 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 and Discussion

Isolation

The fermentation procedures were the same as reported previously[1]. Six-day old whole broth (100 ml) was extracted with 100 ml of ethyl acetate three times under the acidic conditions (added 200 μl of H_3PO_4). The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a yellow powder (82 mg). About half of the powder (40 mg) dissolved in 8.0 ml methanol in a test tube (20 × 50 mm) sealed with a screw cap was heated at 60°C for 3 days. Xanthoquinodins were purified by preparative HPLC (YMC pack D-ODS-5, 20 × 250 mm; 70% CH_3CN in 0.05% H_3PO_4; UV at 340 nm; 6.0 ml/minute). Xanthoquinodin B3 eluted first with a retention time of 38.0 minutes, followed by xanthoquinodins A3, B1, B2, A1 and A2 at 43.0, 47.0, 53.0, 56.0 and 62.0 minutes, respectively. After concentration, the aqueous fraction was extracted with ethyl acetate to yield pure xanthoquinodin B3 as a yellow powder (1.7 mg).

Physico-chemical Properties

The physico-chemical properties of xanthoquinodin B3 are summarized in Table 1. It is soluble in methanol, chloroform, acetonitrile, acetone, ethanol and ethyl acetate, and insoluble in water. The UV spectrum of xanthoquinodin B3 showed maxima at 232, 278 and 378 nm, which was similar to that of xanthoquinodin A3.

Structure of Xanthoquinodin B3

The molecular formula of xanthoquinodin B3 was determined to be $C_{31}H_{24}O_{11}$ on the basis of HREI-MS

Table 1. Physico-chemical properties of xanthoquinodin B3.

	Xanthoquinodin B3
Appearance	Yellow powder
Molecular weight	572
Molecular formula	$C_{31}H_{24}O_{11}$
FAB-MS(*m/z*)	
Positive	573 $[M+H]^+$ 595 $[M+Na]^+$
EI-MS(*m/z*)	572 $[M]^+$
HREI-MS(*m/z*)	
Found	572.1320
Calcd	572.1317
UV $\lambda_{max}^{CH_3OH}$ (nm)	232, 278, 378
IR $\nu_{max}^{CHCl_3}$ (cm^{-1})	1783, 1752, 1605
$[\alpha]_D^{25}$ (*c* 0.1, CH_3OH)	+ 40°
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

measurements (m/z found 572.1320, calcd 572.1317). The ^{1}H NMR spectrum ($CDCl_3$) of xanthoquinodin B3 displayed 24 proton signals and its ^{13}C NMR spectrum showed 31 resolved peaks (Table 2), supporting the molecular formula. The carbon atoms in the molecule were classified into one methyl, one oxy methyl, four methylene, one methine, one oxy methine, two quaternary, five sp^2 methine, eleven sp^2 quaternary and five carbonyl carbons by analysis of the DEPT spectra. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum (Table 2). From the ^{1}H-^{1}H COSY spectrum (Fig. 2), two proton sequences, $-C^5H_2-C^4H_2-C^3H-O-$ and $-C^{11'}H-C^{12'}H=C^{13'}H-$, were determined. ^{13}C-^{1}H long-range couplings of ^2J and ^3J observed in the HMBC spectrum (Fig. 2) gave the following evidences; 1) The cross peaks from H-3 (δ 4.82), H_2-4 (δ 2.35) and H_2-5 (δ 2.54, 2.61) to C-6 (δ 175.3), from H_2-7 (δ 2.91, 3.13) to C-2 (δ 84.0), C-3 (δ 80.7) and C-8 (δ 193.2), from H_2-4 to C-2 and C-3, and from H-3, H_2-7 and H_3-16 (δ 3.75) to C-15 (δ 169.8) indicated that the γ-lactone ring and methyl ester moiety were attached at C-2 as shown in partial structure I (Fig. 2). The absorption at 1783 cm^{-1} in the IR spectrum[4] and the fragment ion peak (m/z 513) of EI-MS also support the presence of a γ-lactone and a methyl ester moiety, respectively. 2) The cross peaks from 10-OH (δ 11.11) to C-9 (δ 105.8), C-10 (δ 160.3) and C-11 (δ 113.8), from H-11 (δ 6.17) to C-9, C-10 and C-13 (δ 114.5), from H_2-15' (δ 2.95, 3.04) to C-11, C-12 (δ 146.6), C-13, C-9'

Table 2. ^{1}H and ^{13}C NMR chemical shifts of xanthoquinodin B3.

Carbon No.	Xanthoquinodin B3	
	^{13}C chemical shifts ppm[a)]	^{1}H chemical shifts ppm[b)]
C-2	84.0	
C-3	80.7	4.82 (1H, t, J=7.0 Hz)
C-4	22.0	2.35 (2H, m)
C-5	27.6	2.54, 2.61 (2H, m)
C-6	175.3	
C-7	35.4	2.91 (1H, d, J=17.0 Hz) 3.13 (1H, d, J=17.0 Hz)
C-8	193.2	
C-9	105.8	
C-10	160.3	
C-10-OH		11.11 (1H, s)
C-11	113.8	6.17 (1H, s)
C-12	146.6	
C-13	114.5	
C-14	154.0	
C-15	169.8	
C-16	53.3	3.75 (3H, s)
C-1'	195.5	
C-2'	132.2	
C-3'	121.1	7.58 (1H, s)
C-4'	147.7	
C-5'	124.3	7.11 (1H, s)
C-6'	161.4	
C-6'-OH		11.50 (1H, s)
C-7'	115.1	
C-8'	182.0	
C-8'-OH		14.91 (1H, s)
C-9'	106.9	
C-10'	189.7	
C-11'	38.6	4.80 (1H, d, J=6.4 Hz)
C-12'	131.4	6.50 (1H, d, J=6.4 Hz)
C-13'	132.8	6.69 (1H, d, J=8.2 Hz)
C-14'	50.0	
C-15'	39.3	2.95 (1H, d, J=18.0 Hz) 3.04 (1H, d, J=18.0 Hz)
C-16'	22.2	2.45 (3H, s)

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. ^{1}H-^{1}H COSY, HMBC and HMQC experiments of xanthoquinodin B3.

^{1}H-^{1}H COSY: ▬, HMBC: H ⟶ C

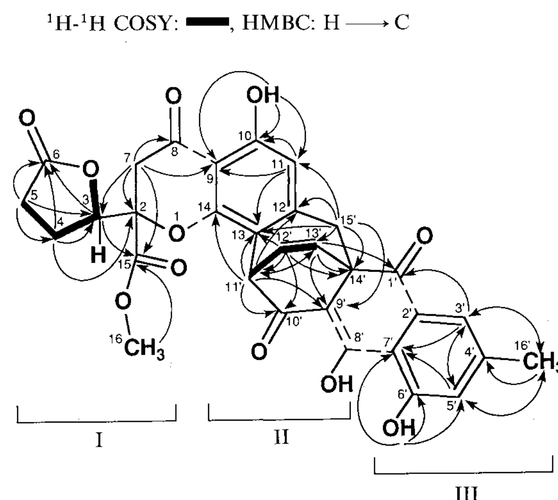
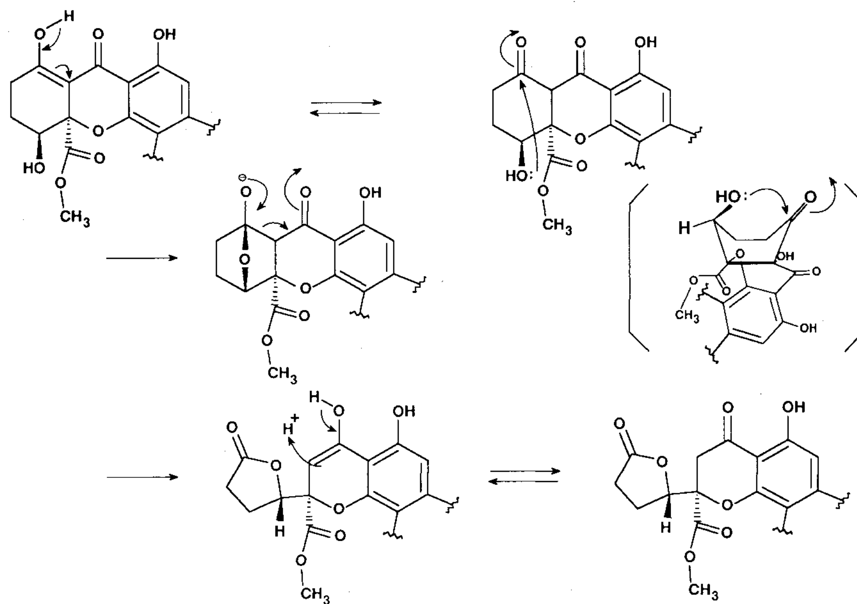


Table 3. Interconversion of xanthoquinodins by heat treatment.

Starting components	Products of xanthoquinodins (mol %)					
	A1	A2	A3	B1	B2	B3
Xanthoquinodin A1	11	14	34	9	32	0
Xanthoquinodin A2	10	55	0	8	27	0
Xanthoquinodin A3	0	0	100	0	0	0
Xanthoquinodin B1	4	12	0	68	10	6
Xanthoquinodin B2	10	21	0	13	56	0
Xanthoquinodin B3	0	0	0	0	0	100

Fig. 3. Conversion of xanthoquinodin B1 to xanthoquinodin B3.



(δ 106.9), C-13′ (δ 132.8) and C-14′ (δ 50.0), from H-11′ (δ 4.80) to C-12, C-13, C-14 (δ 154.0), C-9′, C-10′ (δ 189.7) and C-13′, from H-12′ (δ 6.50) to C-13, C-10′ and C-14′, and from H-13′ (δ 6.69) to C-9′ and C-14′ revealed the partial structure II, which was the same as the part of angularly conjugated system of xanthone and anthraquinone of the xanthoquinodins B1 and B2. 3) The long-range couplings from H_3-16′ (δ 2.45) to C-3′ (δ 121.1), C-4′ (δ 147.7) and C-5′ (δ 124.3), from 6′-OH (δ 11.50) to C-5′, C-6′ (δ 161.4) and C-7′ (δ 115.1), from H-5′ (δ 7.11) to C-3′ and C-7′, and from H-3′ (δ 7.58) to C-5′ and C-7′ showed the partial structure III, which was contained by all xanthoquinodins. 4) The long-range couplings were observed from H-13′ and H-3′ to C-1′ (δ 195.5), and the chemical shifts of C-2′ (δ 132.2), C-8′ (δ 182.0) and 8′-OH (δ 14.91) were comparable with those of other xanthoquinodins. Therefore, the connection of the partial structures II to III was suggested. 5) The cross peak from H_2-7 to C-9 suggested the connection of C-7 in I to C-9 in II *via* a carbonyl carbon C-8. Finally the presence of the cyclic ether was suggested because of the degree of unsaturation and the molecular formula. Taken together, the structure of xanthoquinodin B3 was elucidated as shown in Fig. 1.

Heat Treatment of Xanthoquinodins

The result of heat treatments is shown in Table 3. All xanthoquinodins except xanthoquinodins A3 and B3 thermodynamically interconverted to other components. Xanthoquinodin B3 was very stable towards heat treatment without any conversion to the other xanthoquinodins, and was produced only via xanthoquinodin B1 (Fig. 1). A possible synthetic sequence from xanthoquinodin B1 to B3 is shown in Fig. 3.

Table 4. Anticoccidial activity of xanthoquinodin B3 *in vitro*.

Compounds	Minimum effective concentration (μM)	
	Anticoccidial activity[a]	Cytotoxicity[b]
Xanthoquinodin B3	0.035	3.50
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.

Biological Properties

Anticoccidial Activities

The result of the *in vitro* anticoccidial activity[1] of xanthoquinodin B3 is shown in Table 4. The activity was as potent as those of xanthoquinodins A1, A3, B1 and B2.

Other Biological Activities

Xanthoquinodin B3 showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 (diameter of inhibition zone: 8.0 mm) and *Staphylococcus aureus* FDA 209P (6.5 mm). But no antimicrobial activity was observed against the following microorganisms; *Micrococcus luteus*, *Mycobacterium smegmatis*, *Pyricularia oryzae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, *Saccharomyces sake*, *Mucor racemosus* and *Aspergillus niger*.

Acknowledgments

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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, K. Matuzaki & S. Ōmura: Structure and biosynthesis of xanthoquinodins, anticoccidial antibiotics. J. Am. Chem. Soc. 115: 8558~8564, 1993
3) Matuzaki, K.; N. Tabata, H. Tomoda, Y. Iwai, H. Tanaka & S. Ōmura: The structure of xanthoquinodin A1, a novel anticoccidial antibiotic having a new xanthone-anthraquinone conjugate system. Tetrahedron Lett. 34: 8251~8254, 1993
4) Fresenius, W.; J. F. K. Huber, E. Pungor, G. A. Rechnitz, W. Simon & Th. S. West: *In* Tables of spectral data for structure determination of organic compounds, 2nd English ed. I135~140, Springer-Verlag, Berlin Heidelberg, 1989