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Xanthoepocin, a New Antibiotic from *Penicillium simplicissimum* IFO5762

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A new antifungal antibiotic xanthoepocin was isolated from the culture broth of *Penicillium simplicissimum* IFO5762. Xanthoepocin was obtained from the culture fluid by solvent extraction and chromatographic purification. It showed antibiotic activity against Gram-positive bacteria and yeasts.

In the course of screening of new antibiotics from microbial metabolites, we found xanthoepocin in the culture broth of *Penicillium simplicissimum* IFO5762. Xanthoepocin was produced both in the fermentation broth and mycelia as a major yellow pigment of the strain.

In this paper, we describe the fermentation, isolation, structure determination and biological property of xanthoepocin.

Materials and Methods

Microorganism

The xanthoepocin-producing strain, IFO5762, was purchased from Institute for Fermentation, Osaka (Osaka, Japan). This strain was maintained on a slant consisting of yeast extract 0.2%, starch 1.0%, $CaCl_2 \cdot 2H_2O$ 0.02% and agar 1.5% at 25°C.

Fermentation

A loopful of a mature slant culture of *P. simplicissimum* IFO5762 was inoculated into fifty 500 ml K-1 flasks containing 100 ml of the production medium consisting of soluble starch 2.0%, glucose 1.0%, yeast extract (Difco Laboratories) 0.3%, NZ-amine 0.3% and $CaCO_3$ 0.1%. The pH of the medium was adjusted to 7.0 before sterilization. Fermentation was carried out for 7 days at 30°C on a rotary shaker (200 rpm).

Isolation

To the fermented whole broth (5 liters) was added 2.5 liters of acetone and the mixture was vigorously stirred for 1 hour. Then the mixture was filtered through Celite and the filter cake was washed with 0.5 liters of acetone. The combined filtrates were evaporated to remove acetone and the resultant aqueous solution was extracted with 5 liters of ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a crude yellow extract (3.33 g). 770 mg of the crude extract was dissolved in 120 ml of methanol and defatted by partitioning with 180 ml of *n*-hexane four times. The residual methanol layer was concentrated *in vacuo* to give semi-pure xanthoepocin as a yellow powder (330 mg). Sephadex LH-20 gel filtration chromatography was employed to remove the remaining fatty acids in the active material. 20 mg of the semi-pure extract was dissolved in 0.3 ml of CH_2Cl_2, applied on a column of LH-20 (500×15 mm i.d.) and eluted with CH_2Cl_2-MeOH (2 : 1). Fractions containing pure material were collected and evaporated *in vacuo* to afford 12 mg of xanthoepocin sodium salt.

Feeding of ^{13}C-Labelled Acetate

Sodium [1,2-$^{13}C_2$]acetate (1 mg/1 ml) was added to the production medium 24 hours after the inoculation.

Table 1. Physico-chemical properties of xanthoepocin sodium salt.

Appearance	Yellow powder
MP	>180°C (dec)
$[\alpha]_D^{28}$	-1363.8 (c=0.1, pyridine)
HRFAB-MS	
Found:	605.0923 $(M-H)^-$
Calcd:	605.0931 (for $C_{30}H_{21}O_{14}$)
Molecular formula	$C_{30}H_{22}O_{14}$
UV λ_{max} nm (log ε)	
in MeOH	244 (4.74), 282 (4.25), 427 (4.07)
in 0.001N HCl-MeOH	246 (4.76), 269 (4.52), 282 (sh), 306 (sh), 393 (4.02)
in 0.001N NaOH-MeOH	245 (4.76), 283 (4.28), 427 (4.10)
IR ν_{max} (cm^{-1})	3430, 1685, 1650
Solubility	
soluble in	DMSO, pyridine, THF
slightly soluble in	MeOH, EtOAc, $CHCl_3$
TLC (Rf)[a]	0.34
HPLC (Rt)[b]	9.5 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=20:1)

[b] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm i.d.), mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (60:40), flow rate: 0.7 ml/min, detection: UV-254 nm.

Fermentation was continued for 6 days at 30°C on a rotary shaker (200 rpm). According to the purification protocol described above, 163 mg of ^{13}C-enriched xanthoepocin was obtained from 1 liter of the cultured broth.

Biological Assay

Antibiotic acitivity in fermentation broths and purification samples were evaluated by the conventional paper disc assay using *Bacillus subtilis* as an indicator strain. MIC values were determined by the conventional serial two-fold dilution method against laboratory strains.

Results and Discussion

Physico-chemical Properties

The physico-chemical properties of xanthoepocin (**1**, sodium salt) are summarized in Table 1. **1** was obtained as a yellow powder with the decomposition point at 180°C. It was readily soluble in DMSO, pyridine and THF and slightly soluble in MeOH, ethyl acetate and $CHCl_3$. It was optically active with an $[\alpha]_D^{28}$ value of −1363.8° (c=0.1, pyridine). The UV-visible spectrum showed maxima at 244, 282 and 427 nm in MeOH. The shift of absorption maximum at 427 nm to 393 nm in acidic methanol suggested that **1** was obtained as a phenolate. The IR spectrum showed the presence of hydroxyl group (3430 cm^{-1}), carbonyl group (1685 cm^{-1}) and conjugated olefin group (1650 cm^{-1}). Molecular ion $[M-H]^-$ was detected at *m/z* 605 by FAB-MS. The molecular formula was determined to be $C_{30}H_{22}O_{14}$ ($[M-H]^-$, *m/z*, calcd: 605.0931, found: 605.0923), based on HRFAB-MS and NMR spectra.

Structure Determination

From a combination of HMQC, HMBC, NOESY and INADEQUATE experiments and derivatizations, the structure shown in Fig. 1 was deduced. NMR data of **1** are summarized in Table 2. ^1H NMR of **1** showed five singlet signals due to two aromatic or olefinic protons, one methine proton, and two methyl groups. In ^{13}C NMR spectrum of **1**, fifteen signals were observed: ten sp^2 carbons, three oxygenated sp^2 carbons and two sp^3 carbons due to a *C*-methyl and an *O*-methyl group. Taking into account the

molecular formula, the number of the observed carbon signals suggested that the molecule is a symmertic dimer. The C–H connectivity and C–H long-range couplings were analyzed by HMQC and HMBC experiments. An aromatic proton H-5 at 6.88 ppm, bonded to C-5 (106.44 ppm), was long-range coupled with C-4, C-6, C-9a and C-10a. HMBC correlations from an olefinic proton H-4 (6.07 ppm) to C-3, C-5 and C-10a confirmed the connection of C-4 at C-4a. Long-range couplings from the methyl group at 1.98 ppm to C-3 and C-4 indicated its substitution at C-3. The chemical shift of C-3 (152.85 ppm) suggested its bonding to an oxygen atom. HMBC correlations from H-5 to the carbonyl carbon C-6 (191.36 ppm) and H-9 to C-5a and C-9a established the connections of C-6 at C-5a and C-9 at C-9a. In addition, the observed NOEs between H-4 and H-5 and between H-4 and 3-Me proposed the presence of a

Fig. 1. Structure of xanthoepocin.

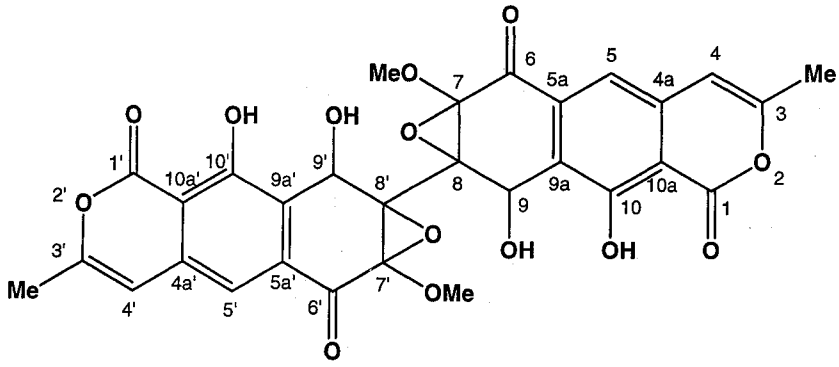
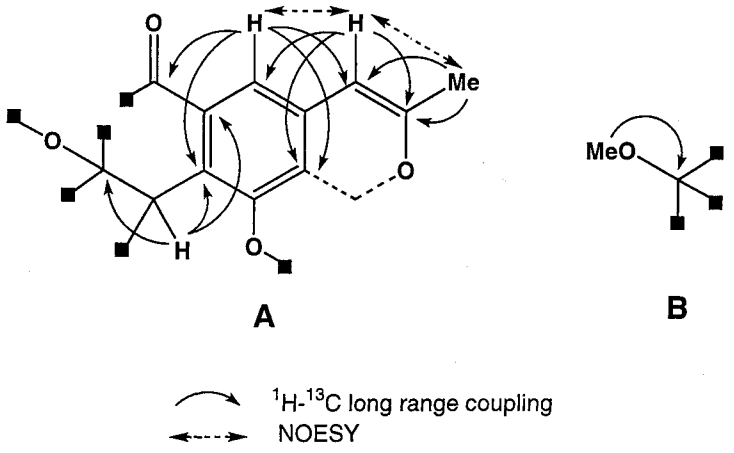


Table 2. ^{1}H and ^{13}C NMR data for xanthoepocin sodium salt (**1**) and derivatives A (**2**) and B (**3**).

Position	**1** (pyridine-d_5) ^{13}C	^{1}H	**2** ($CDCl_3$) ^{13}C	^{1}H	**3** (DMSO-d_6) ^{13}C	^{1}H
1	167.66		166.48		166.03	
3	152.85		154.68		154.66	
4	106.09	6.07 (s)	104.18	6.25 (s)	104.54	6.74 (s)
4a	134.76		137.36		137.90	
5	106.44	6.88 (s)	116.23	6.82 (s)	110.40	6.82 (s)
5a	138.72		139.88		146.97	
6	191.36		65.31	6.69 (s)	104.61	
7	83.32		84.67		100.89	
8	66.92		65.31		84.47	
9	67.14	5.74 (s)	63.74	5.46 (s)	68.81	4.31 (s)
9a	128.50		120.93		123.06	
10	172.71		160.56		158.74	
10a	111.03		106.06		105.32	
3-Me	18.56	1.98 (s)	19.36	2.29 (s)	18.87	2.27 (s)
6-$OCOCH_3$			169.84			
6-$OCOCH_3$			20.75	2.04 (s)		
6-OH						7.77 (s)
7-OMe	55.82	3.11 (s)	53.25	3.55 (s)	49.61	3.04 (s)
					51.64	3.72 (s)
10-OH				11.86 (s)		11.14 (s)

^{1}H and ^{13}C NMR spectra were measured at 400 MHz and 100 MHz respectively.

Fig. 2. Partial structures of **1** elucidated by HMBC and NOESY.



naphtalenoid substructure as shown in Fig. 2 (partial structure A). A long-range coupling from 7-OMe to a quarternary sp^3 carbon C-7 revealed the presence of partial structure B. Connectivities of the remaining atoms could not be determined by HMBC.

In order to elucidate the carbon framework, ^{13}C-enriched **1** was prepared for the INADEQUATE experiment by feeding sodium [1,2-$^{13}C_2$]acetate in the fermentation media because **1** was considered to be an aromatic polyketide. As shown in Fig. 3, the 2D-INADEQUATE revealed the presence of seven sets of C–C unit derived from the acetate: 3-Me/C-3, C-4/C-4a, C-5/C-5a, C-6/C-7, C-8/C-9, C-10/C-10a and C-10a/C-1. In addition, this result revealed the sites of connection between partial structures A and B and the connection of the carbonyl carbon C-1 at C-10a that led to the confirmation of the presence of the isocoumarin chromophore. The attachments of the oxygen functionality at C-8, C-9 and C-10 and the connectivities between C-7 and C-8 and between C-10 and C-10a were speculated.

Derivatization of **1** was carried out to elucidate the full structure. **1** was reduced by sodium borohydride and subsequently acetylated by acetic anhydride to give a mixture of three compounds. After the ODS chromatography, two unsymmetric and one symmetric products were obtained. The symmetric one, derivative A (**2**) was subjected to the NMR study due to the simplicity of its spectra. Important HMBC correlations of **2** are shown in Fig. 4. Detection of the long-range couplings from H-6 to C-5, C-5a, C-7, C-8 and the carbonyl carbon of the acetyl group allowed to confirm the site of the reduction and acetylation and also the connection between C-7 and C-8. HMBC correlations from a phenolic proton at 11.86 ppm to C-9a, C-10 and C-10a established the presence of a hydroxyl group at C-10 and the connection between C-10 and C-10a. Taking into account the chemical shifts at C-7 (84.67 ppm) and C-8 (65.31 ppm) and the molecular formula, **2** was expected to have an epoxy ring at C-7 and C-8 and dimerize at C-8. Thus, the structure as shown in Fig. 1 was proposed for **1**.

To confirm the proposed structure, **1** was converted to another derivative. **1** was treated with methanol in the presence of HCl to give a sole product, derivative B (**3**). In the ^{13}C NMR spectrum of **3**, the signals at C-7 and C-8 were shifted downfield from 83.32 ppm to 100.89 ppm and from 66.92 ppm to 84.47 ppm, respectively, as a result of the opening of the epoxy ring at C-7. Long-range couplings were observed from two methoxy groups at 3.04 and 3.72 ppm to C-7 and from H-9 to C-7 and C-8. The carbonyl signal at C-6 was shifted upfield from 191.36 ppm to 104.61 ppm. In the ^{1}H NMR, a hydroxyl proton appeared at 11.14 ppm, which had the HMBC correlations to C-5a, C-6 and C-7. These results indicated that the carbonyl group at C-6 is hydrated as shown in Fig. 5. FABMS did not show the ion peak of the hydrate but the dehydrated ion peak $[M-H_2O-H]^-$ at *m/z* 669.

Finally, the gross structure of **1** was determined as shown in Fig. 1. Its stereochemistry is not determined yet. **1** belongs to the xanthomegnin[1] class of fungal pigments, some members of which are known to be carcinogens[2] or uncouplers of respiratory chain[3]. Among those pigments, **1** is structurally related to floccosin[4~6], a mycotoxin from the dermatophyte *Epidermophyton floccosum*.

Fig. 3. Partial structure of **1** elucidated by 2D-INADEQUATE.

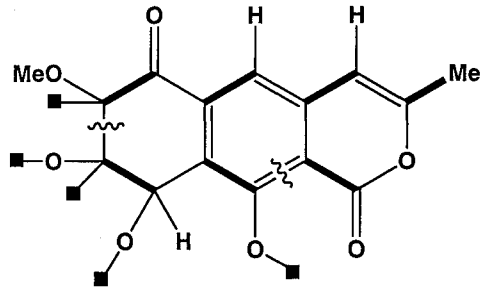


Fig. 4. Significant ^{1}H-^{13}C long-range couplings observed in the HMBC spectrum of **2**.

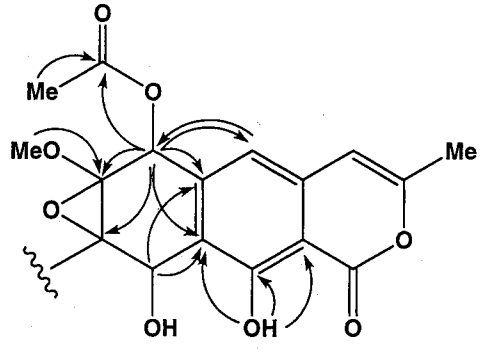
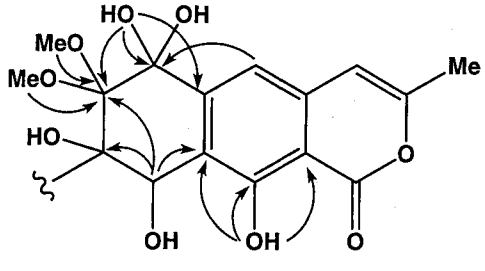


Fig. 5. Significant ^{1}H-^{13}C long-range couplings observed in the HMBC spectrum of **3**.



Biological Property

The antimicrobial activity of xanthoepocin (**1**) is summarized in Table 3. **1** inhibited the growth of MRSA and *Bacillus subtilis* with the MICs of 0.78~1.56 μg/ml. In addition, **1** exhibited activity against yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* with the MICs of 3.13~25 μg/ml. **1** was weakly cytotoxic to U937 cells with the IC_{50} value of 35 μg/ml.

Table 3. *In vitro* antibacterial activitiy of xanthoepocin.

Organisms	MIC (μg/ml)
Bacillus subtilis ATCC 6633	0.78
Staphylococcus aureus 209P JC-1	1.56
Staphylococcus aureus F-597 MRSA	1.56
Escherichia coli NIHJ JC-2	>100
Pseudomonas aeruginosa A3	>100
Proteus mirabilis ATCC21100	>100
Proteus vulgaris IFO3851	>100
Saccharomyces cerevisiae S-100	3.13
Candida albicans A9540	25.0
Candida tropicalis IFO1400	>100
Cryptococcus neoformans ATCC90112	12.5
Torulopsis glabrata IFO0622	6.25
Aspergillus fumigatus IFO8866	>100

Experimental

General

Melting points were determined on a Yanagimoto apparatus and are uncorrected. NMR experiments were performed on JEOL JNM-LA400 NMR spectrometer in the solvents specified. The FABMS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were recorded on a BECKMAN DU 640 spectrophotometer. IR spectra were recorded on a SHIMADZU FT IR-300 spectrophotometer. Optical rotation was measured on a HORIBA SEPA-300 polarimeter.

Reduction and Acetylation of Xanthoepocin (**1**)

To a solution of **1** (100 mg, 0.17 mmol) in a mixture of methanol (4 ml) and tetrahydrofuran (4 ml) was portionwise added sodium borohydride (200 mg, 5.3 mmol) with ice-cooling. After stirring for 3 hours, the mixture was concentrated *in vacuo*. The residue was dissolved in pyridine (3 ml) and acetic anhydride (3 ml) was added to the suspension. After stirring for 18 hours at room temperature, the reaction mixture was poured onto ice-water (100 ml) and extracted with ethyl acetate. The organic layer was

washed with water, saturated aqueous $CuSO_4$ solution and brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give a crude residue (78.3 mg). It was passed through a SiO_2 column (8 g) with the eluent of $CHCl_3$-MeOH (100 : 1) and the fractions were pooled and evaporated. The residue was applied on an ODS column (YMC ODS-AM, 200×45 mm i.d.) and eluted with 20~70% acetonitrile in 0.15% KH_2PO_4 buffer (pH 3.5). The fractions containing the derivative were evaporated to remove acetonitrile, extracted with EtOAc, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give 3.0 mg of derivative A (**2**) as a yellow amorphous: m.p. 155~157°C; UV λ_{max}^{MeOH} nm (log ε): 232 (4.62), 237 (4.62), 260 (4.43), 279 (sh), 348 (4.05), 390 (sh), $\lambda_{max}^{MeOH+HCl}$ 238 (4.64), 260 (4.47), 280 (sh), 347 (4.07), $\lambda_{max}^{MeOH+NaOH}$ 235 (4.49), 246 (sh), 258 (4.27), 369 (4.04); IR ν_{max} (KBr): 3450, 1750, 1685, 1650, 1625 cm^{-1}; HRFAB-MS *m/z* 693.1451 $[M-H]^-$ (calcd *m/z* 693.1456 for $C_{34}H_{29}O_{16}$).

Methanolysis of Xanthoepocin (**1**)

1 (10 mg, 17 μmol) was dissolved in 0.2 N HCl methanolic solution (50 ml) and stirred for 2 hours at 32°C. Then, water (400 ml) was added to the reaction mixture and extracted with EtOAc (350 ml). The organic layer was washed with water, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The methanol-adduct was precipitated by adding a small portion of acetonitrile to the residue. The precipitates were collected by filtration and washed with acetonitile to give 5.1 mg of derivative B (**3**) as a light yellow amorphous: m.p. 156~158°C; UV λ_{max}^{MeOH} nm (log ε): 238 (4.56), 258 (4.27), 278 (4.06), 349 (3.97), $\lambda_{max}^{MeOH+HCl}$ 238 (4.55), 258 (4.27), 278 (4.05), 349 (3.97), $\lambda_{max}^{MeOH+NaOH}$ 238 (4.52), 255 (sh), 278 (4.05), 349 (3.91), 393 (sh); IR ν_{max} (KBr): 3450, 1690, 1650, 1625 cm^{-1}; HRFAB-MS *m/z* 669.1448 $[M-H_2O-H]^-$ (calcd *m/z* 669.1456 for $C_{32}H_{29}O_{16}$).

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