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Yaequinolones, New Insecticidal Antibiotics Produced by *Penicillium* sp. FKI-2140

I. Taxonomy, Fermentation, Isolation and Biological Activity

Ryuji Uchida, Rie Imasato, Yuichi Yamaguchi, Rokuro Masuma, Kazuro Shiomi, Hiroshi Tomoda, Satoshi Ōmura

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Abstract New nine insecticidal antibiotics designated yaequinolones were isolated from the culture broth of the fungal strain *Penicillium* sp. FKI-2140 by solvent extraction, centrifugal partition chromatography and HPLC. Yaequinolones showed growth inhibitory activity against brine shrimp (*Artemia salina*). Among them, yaequinolone F has the most potent activity with MIC value of 0.19 μg/ml.

Keywords yaequinolone, insecticidal, brine shrimp (*Artemia salina*), fungi, *Penicillium* sp.

Introduction

Our research group has focused on the discovery of biologically active compounds from microbial metabolites [1～8]. We have utilized brine shrimp (*Artemia salina*) as a test organism to screen for insecticidal agents and discovered new fungal metabolites with this assay system [1～3]. Further searches for insecticidal agents lead to the discovery of new compounds designated yaequinolones produced by fungal strain FKI-2140, a soil isolate of subtropical Okinawa in Japan. The strain, identified as *Penicillium* sp., was found to produce seven yaequinolones (**3** to **9**) along with nine known related compounds (**1**, **2** and **10** to **16**) [9～13], whose structures are shown in Fig. 1.

During the course of this study, compounds **1** and **2** were found to be identified as diastereomeric quinolinone alkaloids recently isolated from marine-derived fungus *Penicillium janczewskii* [9]. In this study, **1** and **2** were named yaequinolone A1 and A2, respectively. 4-Hydroxy-3,4-dihydro-3-methoxy-4-(4′-methoxyphenyl)-2(1*H*)-quinolinone (abbreviated quinolinone A in this study) (**10**) [10] and 4,5-dihydroxy-3,4-dihydro-3-methoxy-4-(4′-methoxyphenyl)-2(1*H*)-quinolinone (quinolinone B) (**11**) [10], were originally isolated as insecticidal antibiotics against *A. salina* from *Penicillium simplicissimum*. Peniprequinolone (**12**) [11] was isolated as a nematicidal antibiotic against root-lesion nematode *Pratylenchus penetras* from *Penicillium* cf. *simplicissimum*. Penigequinolones A and B (**13**, **14**) [11, 12], isolated as a mixture from *Penicillium* sp. NO. 410, were reported to show pollen-growth inhibitory activity and nematicidal activity against *P. penetrans*. 4′-Methoxycyclopeptin (**15**) [11] was isolated along with **12**. *trans*-Dehydro-4′-methoxycyclopeptine (**16**) [13] was previously reported as a synthetic intermediate of cyclopeptin. The structural elucidation of the yaequinolones is described in the accompanying paper [14] and elsewhere [3]. In this study, the taxonomy of the producing fungus, fermentation, isolation, and the biological activity of yaequinolones are described.

H. Tomoda (Corresponding author), **R. Uchida:** School of Pharmacy, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, E-mail: tomodah@pharm.kitasato-u.ac.jp

R. Imasato, Y. Yamaguchi, R. Masuma, S. Ōmura: Kitasato Institute for Life Sciences, & Graduate School of Infection Control Sciences, Kitasato University and The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

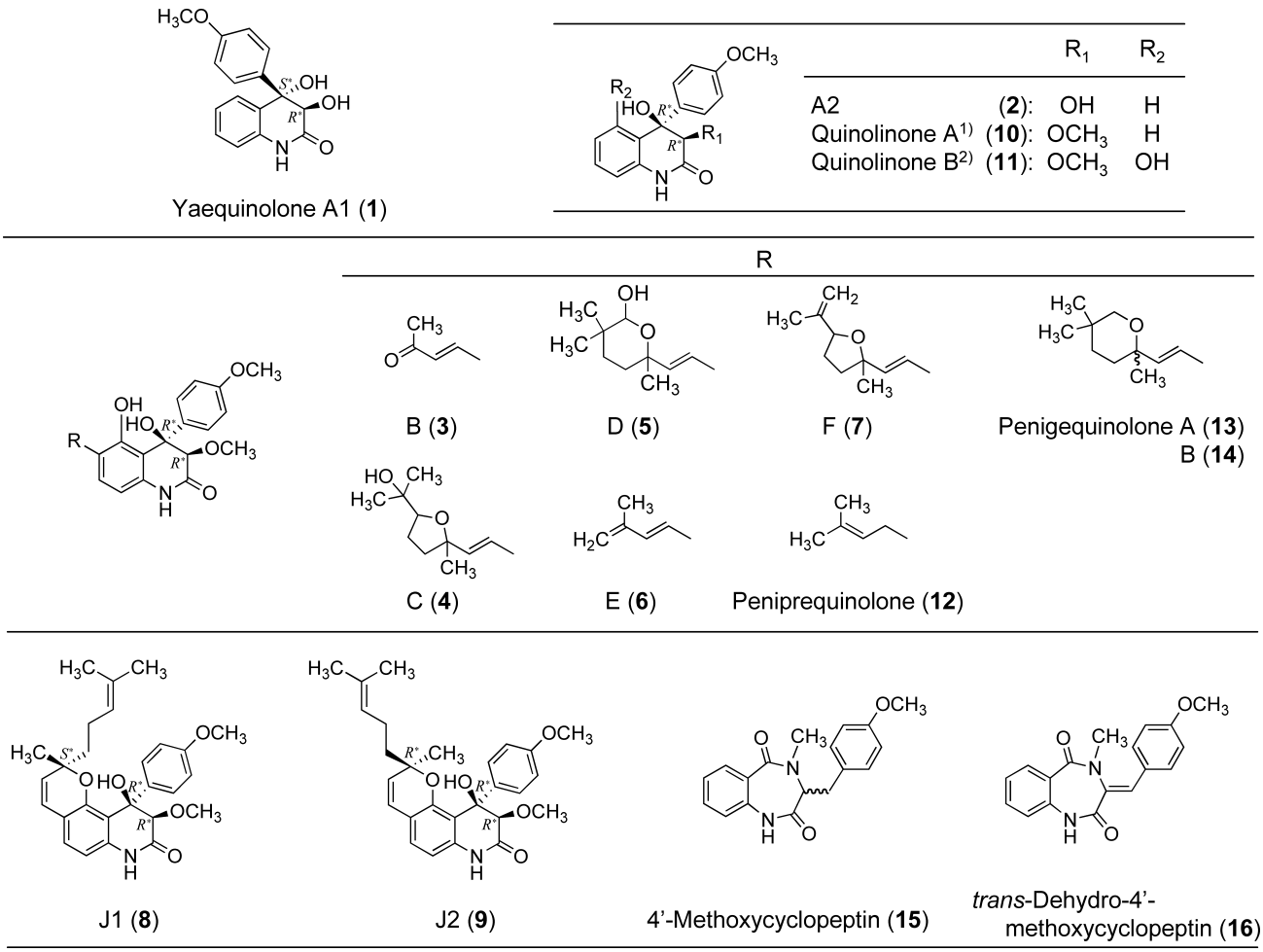

Fig. 1 Structures of yaequinolones (**1**～**9**) and related compounds (**10**～**16**).

1) Quinolinone A: 4-Hydroxy-3,4-dihydro-3-methoxy-4-(4′-methoxyphenyl)-2(1*H*)-quinolinone. 2) Quinolinone B: 4,5-Dihydroxy-3,4-dihydro-3-methoxy-4-(4′-methoxyphenyl)-2(1*H*)-quinolinone.

Materials and Methods

Taxonomic Studies

Strain FKI-2140 was isolated from a soil sample collected at Ishigakijima Island, Okinawa, Japan. Taxonomic studies and identification were conducted according to the procedures described by Pitt [15]. For the taxonomic studies of fungus, Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) were used. Morphological observations were done under a light microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (JEOL JSM-5600). Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago) was used for color names and hue numbers [16].

Fermentation Media

For production of yaequinolones, the seed medium contained 2.0% glucose, 0.2% yeast extract (Oriental Yeast Co.), 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% Polypeptone (Nihon Pharmaceutical CO., LTD.), 0.1% KH_2PO_4 and 0.1% agar. The pH was adjusted to 6.0 prior to sterilization. The production medium was composed of 1.0% glucose, 2.0% soluble starch, 2.0% soybean oil, 1.0% Pharmamedia, 0.5% meat extract, 0.1% $MgSO_4 \cdot 7H_2O$, 0.3% $CaCO_3$, 0.1% trace metals solution (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnCl_2 \cdot 4H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$, 0.1% $CuSO_4 \cdot 7H_2O$, 0.1% $CoCl_2 \cdot 6H_2O$) and 0.1% agar. The pH was adjusted to 6.0 prior to sterilization.

Fermentation

A stock culture of strain *Penicillium* sp. FKI-2140 was inoculated into a 500-ml Erlenmeyer flask with 100 ml of

the seed medium and incubated on a rotary shaker at 27°C for 3 days. The production culture was initiated by transferring 1 ml of the seed culture into each of sixty 500-ml Erlenmeyer flasks with 100 ml of the production medium and the fermentation was carried out at 27°C on a rotary shaker at 210 rpm. After 3 days, two flasks of the production culture were transferred into a 1 liter Roux flask and incubated for 11 days at 27°C under the stationary conditions.

Insecticidal Activity

Insecticidal activity was assayed by the microtiter-plate method using *A. salina* (Pfizer Consumer Inc) as a test organism [17]. Briefly, about 10 nuclei larvae hatched from eggs of *A. salina* in the culture medium (295 μl, 0.24% Tris, 2.57% NaCl, 0.47% $MgCl_2$, 0.07% KCl, 0.02% Na_2CO_3, 0.64% $MgSO_4$, and 0.11% $CaCl_2$, pH 7.0) were incubated with a sample (5 μl in DMSO solution) in a well of a 96-well microplate at 20°C. After 48 hours, the motility of *A. salina* was assessed visually in comparison with that of the control (no test sample).

Nematicidal Activity

Nematicidal activity was assayed by the microtiter-plate method using a free-living nematode, *Caenorhabditis elegans*, as reported previously [17]. Briefly, *C. elegans* was grown on an agar plate covered with *Escherichia coli* for 4 days at 20°C. About 5 organisms were incubated with a sample in a well of a 96-well microplate at 20°C. After 48 hours, the motility of the nematode was assessed visually under a microscope (×40, Olympus CK2) in comparison with that of the control (no test samples).

Antimicrobial Activity

Antimicrobial activity against the following 14 microorganisms was measured by a paper disk method [1]. *Bacteroides fragilis* ATCC23745, *Mycobacterium smegmatis* ATCC607, *Acholeplasma laidlawii* PG8, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* FDA209P, *Micrococcus luteus* PCI1001, *Escherichia coli* NIHJJ-2 IFO12734, *Pseudomonas aeruginosa* IFO3080, *Xanthomonas campestris* pv. *oryzae*, *Pyricularia oryzae* KF180, *Aspergillus niger* ATCC6275, *Mucor racemosus* IFO4581, *Candida albicans* and *Saccharomyces cerevisiae* were used for the assay.

Results

Taxonomy of the Producing Organism

Colonies on CYA were 27～30 mm diameter after 7 days at 25°C, floccose to velutinous, corrugate, and sage gray (24 ih) to mistletoe gray (24 1/2 ih) in color. The reverse was bamboo (2 gc) to cream (1 1/2 ca). Colonies on MEA were 28～30 mm diameter, velutinous, corrugate, dark olive green (24 1/2 nl) to mistletoe green (24 1/2 li) in color. The reverse was bamboo (2 gc) to covert tan (1 1/2 ca). Colonies on G25N were 11～13 mm diameter, floccose to velutinous, sulcate, and celadon gray (24 fe) to sage gray (24 ih) in color. The reverse was pearl pink (3 ca) to light yellow (2 ea). At 5°C and 37°C, no colonies were formed on CYA. Soluble pigment (light yellowish brown) was produced on CYA. Conidiophores on CYA were produced from subsurface or aerial hyphae, and were 30～75 μm long, smooth-walled, and bearing monoverticillate penicilli. Phialides were present at 3～10 per conidiophore, and were ampulliform, 8～12×2～3.5 μm in size and smooth-walled. Mature conidia after a 7-day incubation were spherical to subsperical, 2.8～3×2.7～3 μm, rough-walled, and present in divergent long chains. The conidia were produced sympodially, as shown by SEM observation (Fig. 2). From the above characteristics, strain FKI-2140 was considered to belong to the genus *Penicillium* [15, 19, 20].

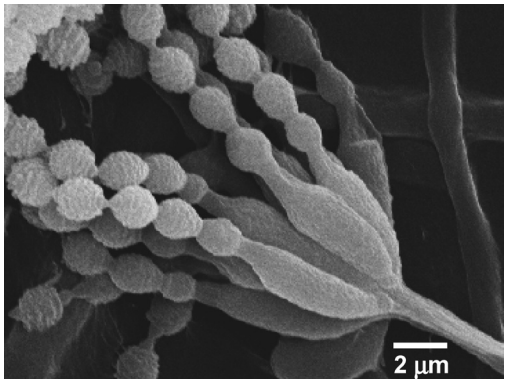

Fig. 2 SEM photomicrograph of conidia of FKI-2140. Bar represents 2 μm.

Isolation

The procedure for isolation of yaequinolones and related compounds is summarized in Fig. 3. The culture broth (6 liters) was treated with acetone (6 liters), and the mixture was centrifuged to obtain the supernatant, which was concentrated under reduced pressure. The resulting aqueous layer was extracted with an equal volume of ethyl acetate twice. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a dark brown oil. After treating with hexane - acetonitrile (1 : 1), the acetonitrile soluble fraction (1.75 g) was subjected to centrifugal partition chromatography (Sanki Engineering

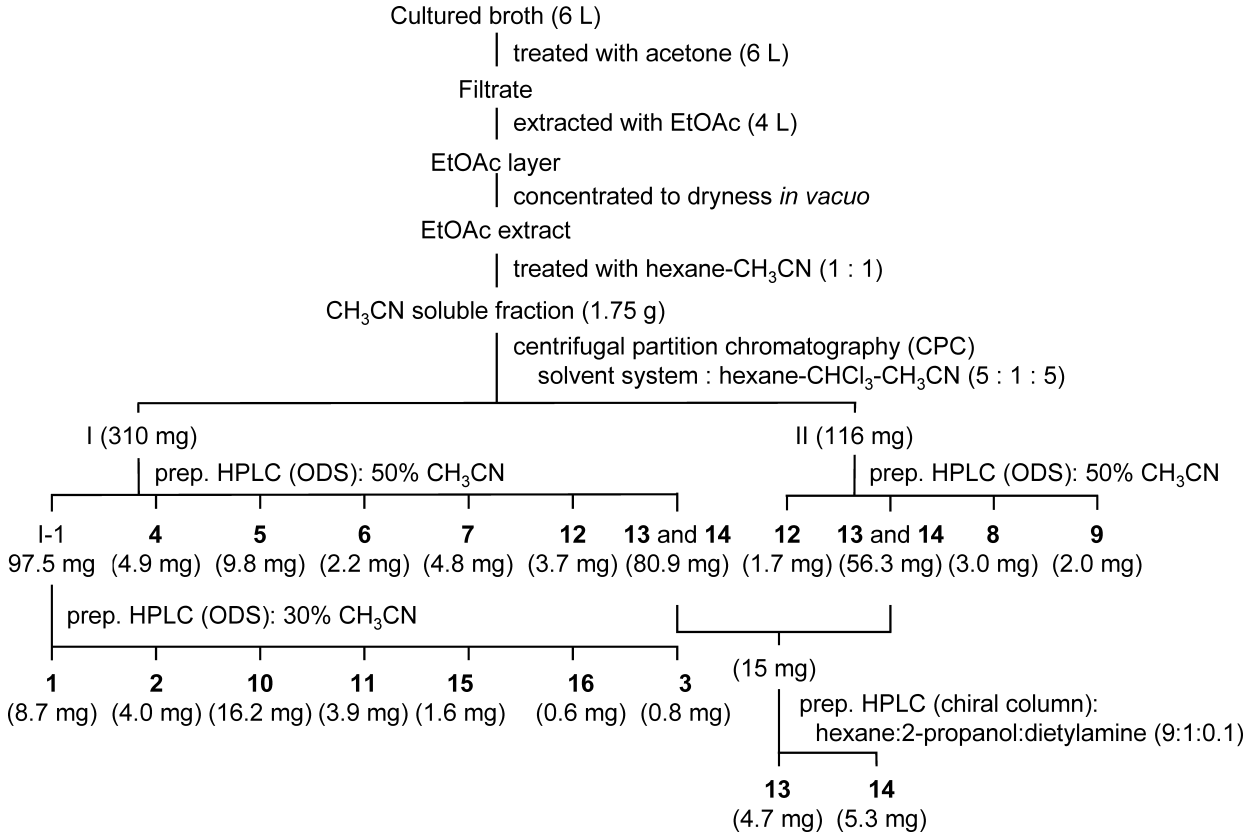

Fig. 3 Procedure for purification of yaequinolones and related compounds from the culture broth of *Penicillium* sp. FKI-2140.

Ltd.) under the following conditions: solvent system, the upper and lower layers hexane - chloroform - acetonitrile (5 : 1 : 5) as stationary and mobile phases, respectively; flow rate, 3 ml/minute; rotation speed, 1,200 rpm. The upper phase of the solvent was introduced by the ascending method (480 ml). The active compounds containing of yaequinolones were kept in the stationary phase and eluted after changing the eluent from the mobile phase to stationary phase. Then, two fractions (Fr. I and II) were collected and concentrated under reduced pressure.

Fr. I (310 mg) containing **1** to **7** and **10** to **16** was subjected to preparative HPLC under the following conditions: column, CAPCELL PAK C18 (Shiseido, i.d. 20×250 mm); mobile phase, 50% acetonitrile; flow rate, 8 ml/minute; detection, UV 210 nm. Seven fractions (Fr. I-1 to I-7) were collected; Fr. I-1 (containing **1** to **3**, **10**, **11**, **15** and **16**; retention time of 5 to 11 minutes), Fr. I-2 (**4**; 15 minutes), Fr. I-3 (**5**; 18 minutes), Fr. I-4 (**6**; 27 minutes), Fr. I-5 (**7**; 30 minutes), Fr. I-6 (**12**; 37 minutes) and Fr. I-7 (a mixture of **13** and **14**; 46 minutes). Each fraction was concentrated to dryness to give a mixture of **1** to **3**, **10**, **11**, **15** and **16** (97.5 mg), **4** (4.9 mg), **5** (9.8 mg), **6** (2.2 mg), **7** (4.8 mg), **12** (3.7 mg) and a mixture of **13** and **14** (80.9 mg) as a pale yellow powder. The mixture from Fr. I-1 (97.5 mg) was further subjected to preparative HPLC under the following conditions: column, CAPCELL PAK C18 (Shiseido, i.d. 20×250 mm); mobile phase, 30% acetonitrile; flow rate, 8 ml/minute; detection. UV 210 nm. Compounds **1** to **3**, **10**, **11**, **15** and **16** were eluted at retention times of 13, 17, 37, 23, 25, 39 and 30 minutes. Each fraction was collected and concentrated to dryness to give pure **1** (8.7 mg), **2** (4.0 mg), **3** (0.8 mg), **10** (16.2 mg), **11** (3.9 mg), **15** (1.6 mg) and **16** (0.6 mg) as a pale yellow powder. The mixture of **13** and **14** (15 mg) was purified by preparative HPLC under the following conditions: column, Chiral Pak IA (Daicel Chemical, i.d. 10×250 mm); mobile phase, hexane : 2-propanol : diethylamine (9 : 1 : 0.1); flow rate, 3 ml/minute; detection, UV 325 nm. Compounds **13** and **14** were eluted at retention times of 22 and 25 minutes, respectively. Each fraction was collected and concentrated to dryness to give pure **13** (4.7 mg) and **14** (5.3 mg) as a pale yellow powder.

Fr. II (116 mg) containing **8**, **9** and **12** to **14** was subjected to preparative HPLC under the following

Table 1 Insecticidal activities[a] of yaequinolones and related compounds

Compound		MIC (μg/ml)
Yaequinolone A1	**1**	>100
A2	**2**	100
B	**3**	50
C	**4**	12.5
D	**5**	6.25
E	**6**	6.25
F	**7**	0.19
J1	**8**	6.25
J2	**9**	6.25
Quinolinone A	**10**	>100
Quinolinone B	**11**	25
Peniprequinolone	**12**	0.78
Penigequinolone A	**13**	0.19
Penigequinolone B	**14**	0.19
4′-Methoxycyclopeptin	**15**	>100
trans-Dehydro-4′-methoxycyclopeptin	**16**	NT[b]

[a] Growth inhibition against *A. salina*. [b] NT: not tested.

conditions: column, CAPCELL PAK C18 (Shiseido, i.d. 20×250 mm); mobile phase, 50% acetonitrile; flow rate, 8 ml/minute; detection, UV 210 nm. Compounds **8**, **9**, **12** and a mixture of **13** and **14** were eluted at retention times of 41, 48, 26 and 37 minutes, respectively. Each fraction was collected and concentrated to dryness to give **8** (3.0 mg), **9** (2.0 mg), **12** (1.7 mg) and a mixture of **13** and **14** (56.3 mg) as a pale yellow powder.

Biological Activity

Insecticidal Activity

The minimum growth inhibitory concentrations (MIC) against the growth of *A. salina* are summarized in Table 1. Compounds **7**, **13** and **14** showed the most potent inhibition, with MIC values of 0.19 μg/ml, followed by **12**. Compounds **5**, **6**, **8** and **9** showed moderate activity, with MIC values of 6.25 μg/ml. However, **1**, **10** and **15** showed very weak inhibition at 100 μg/ml.

Other Activities

Compounds **1** to **16** showed almost no effect on the growth of *C. elegans* at 100 μg/ml, and no antimicrobial activity, at 10 μg/6 mm disk, against any of the 14 microorganisms tested.

Discussion

Penicillium sp. FKI-2140 produced nine yaequinolones (**1** to **9**) and seven structurally related known compounds (**10** to **16**). They showed insecticidal activity against *A. salina* at 0.19~100 μg/ml, no effect on *C. elegans* at 100 μg/ml, and no antimicrobial activity against 14 microorganisms. Compound **12** was previously reported to have weak nematicidal activity against *P. penetras* (82.4% toxicity at 1000 μg/ml), although it showed no inhibitory activity against the growth of *C. elegans* at 100 μg/ml. Compounds **13** and **14** were originally isolated as a mixture, but we were able to separate them by HPLC using a chiral column. They showed potent insecticidal activity against *A. salina*, although the mixture had been reported to be a moderate pollen-growth inhibitor (40% inhibition at 10 μg/ml). Regarding the insecticidal activity, as shown in Table 1, **7**, **13** and **14** had the most potent activity, followed by **12**. Compounds **5**, **6**, **8** and **9** showed moderate activity. However, **1**, **10** and **15** showed very weak inhibition even at 100 μg/ml. These results suggested that the presence of an isoprenyl-derived side chain in the structures is responsible for the anti-*A. salina* activity.

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