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Quinolactacins A, B and C: Novel Quinolone Compounds from *Penicillium* sp. EPF-6

I. Taxonomy, Production, Isolation and Biological Properties

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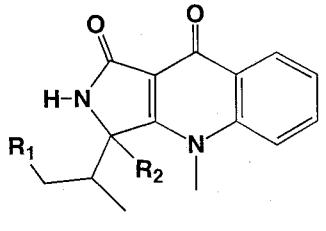
Quinolactacins A (**1**), B (**2**) and C (**3**), novel quinolone antibiotics have been found from the cultured broth of a fungal strain isolated from the larvae of the mulberry pyralid (*Margaronia* pyloalis Welker). The fungal strain, EPF-6 was identified as *Penicillium* sp. from its morphological characteristics. Quinolactacins were obtained from the culture medium by solvent extraction and chromatographic purification. Compound **1** showed inhibitory activity against tumor necrosis factor (TNF) production induced by murine peritoneal macrophages and macrophage-like J774.1 cells stimulated with lipopolysaccharide (LPS).

Since entomopathogenic fungi possess a parasitic function, the secondary metabolites from the fungi seem to be interesting as a source of screening for new insecticides[1] and immunosuppressants[2,3]. We have conducted a search for new compounds from entomopathogenic fungi by chemical screening using an HPLC analytical program. Recently, we reported two novel pyridone compounds, pyridovericin and pyridomacrolidin, which were isolated from the entomopathogenic fungus, *Beauveria bassiana* EPF-5, as a novel protein kinase inhibitor[4,5]. During our further screening for bioactive compounds from fungi, we isolated three novel quinolone compounds, quinolactacins A (**1**), B (**2**) and C (**3**), from the cultured broth of *Penicillium* sp. EPF-6, which was isolated from the larvae of mulberry pyralid (*Margaronia* pyloalis Welker), as shown in Fig. 1. The compound **1** showed inhibitory activity of TNF production by murine macrophages and macrophage-like J774.1 cells stimulated with LPS[6,7]. In this paper, we describe the taxonomy of the producing strain, fermentation, isolation and biological properties of **1**, **2** and **3**. The physico-chemical properties and structure

Fig. 1. Structures of quinolactacins A (**1**), B (**2**) and C (**3**).



1: $R_1 = CH_3$, $R_2 = H$
2: $R_1 = H$, $R_2 = H$
3: $R_1 = CH_3$, $R_2 = OH$

elucidation are reported in an accompanying paper[8].

Materials and Methods

Taxonomic Studies

The quinolactacins (**1**~**3**) producing strain, EPF-6, was isolated from larvae of the mulberry pyralid (*Margaronia* pyloalis Welker). This strain has been deposited in the culture collection at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, as *Penicillium* sp. EPF-6 with an accession number of FERM P-17738. Taxonomic studies of the strain EPF-6 were conducted according to the method of PITT[9]. For the evaluation of culture characteristics, the strain was inoculated in potato dextrose agar (PDA, Eiken Chemical Co., Ltd.), malt extract agar (MEA, malt extract 2.0%, peptone 0.1%, glucose 2.0%, agar 2.0%), CZAPEK's agar ($NaNO_3$ 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, sucrose 3.0%, agar 2.0%) and SABOURAUD's dextrose agar (SDA, Difco) media, for 14 days at 24°C. Morphological observation was carried out using a light microscope (Nikon, OPTIPHOTO-2) and a scanning electron microscope (JEOL JSM-T220).

Chemical Screening

The search for novel compounds from fungal metabolites by HPLC analysis was performed under the following conditions: column: ODS column (Senshu Pak PEGASIL ODS, 4.6×250 mm); solvent: a linear gradient from 20% methanol to 80% methanol for 50 minutes; UV detection at 254 nm; flow rate of 0.7 ml/minute. The detection of fungal metabolites by silica gel TLC analysis was performed under the following conditions: silica gel TLC (Merck Kieselgel 60 F_{254} precoated glass plates, No. 5715, 0.25 mm); solvent: $CHCl_3$: MeOH = 10 : 1; color reaction: iodine vapor and Ehrlich reagent.

Assay for Antimicrobial Activity

The antimicrobial activity of quinolactacins (**1**~**3**) was evaluated by a conventional agar dilution method using Müller-Hinton medium for bacteria and SABOURAUD's dextrose medium for filamentous fungi and yeasts. The tested organisms were as follows: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* FDA 209P JC-1, *S. aureus* CAY 26101, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* NCTC 10490, *P. aeruginosa* CAY 10746, *Proteus mirabilis* IFM OM-9 SS-12, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 26108, *Schizosaccharomyces pombe* YFC 429, *Rhodotorula acuta* JCM 1602, *Cryptococcus* sp. YFC 75, *Aspergillus niger* ATCC 9642, *A. fumigatus* TIMM 1776, *Mucor hiemalis* IFO 9401, and *Trichophyton interdigitale* YFC 284.

Stimulation of TNF Production by Murine Macrophages

Female C3H/HeN mice (Nippon Clea Co.) were used at 10 to 15 weeks of age. Murine macrophages were isolated by peritoneal lavage 4 days after intraperitoneal injection of 1.5 ml of sterile 3% thioglycolate broth, as described previously[7]. Cells were washed with serum-free RPMI 1640 medium (ICN Biomedicals, Costa Mesa, Calif., U.S.A.), 4 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were plated at 2×10^5 cell/well in 96-well plates (Nunc, Roskilde, Denmark) for TNF induction. Cells were cultured for 2 hours at 37°C with 5% CO_2, then washed three times with serum-free RPMI 1640 to remove nonadherent cells. The remaining cells were stimulated with 10 ng/ml *Salmonella minnesota* LPS (Sigma Chemical Co.) in 200 μl of RPMI 1640 medium containing 2% FBS. Stimulation times were 3 hours for the TNF assay.

TNF Assay

TNF activity was determined by the fibroblast cytotoxic assay using the TNF sensitive cell line, L929, essentially as described previously[10]. L929 cells were plated in 96-well culture plates at 5×10^4 cell/well in 100 μl of RPMI 1640 medium supplemented with 5% FBS and incubated for 22 hours. The cells were then cultured for an additional 18 hours in the presence of serial dilutions of test supernatants and 1 μg/ml of actinomycin D (Merck Co.). The viability of cells was determined by a quantitative colorimetric staining assay using a tetrazolium salt (MTT, Sigma Chemical Co.). TNF activity is expressed as U/ml where one unit is the amount of TNF causing 50% lysis of L929 cells.

Results and Discussion

Taxonomy of the Producing Strain

Cultural characteristics of strain EPF-6 were as follows. Colonies on CZAPEK's agar at 24°C attained a diameter of 20~25 mm in 7 days and were velvetinous, and olive gray in color. Conidia formation was good. The reverse sides of the colonies were dark green. Exudate and soluble pigment were absent. Colonies on MEA and SDA at 24°C attained a diameter of 15~20 mm in 7 days and were felty to velvetinous, brownish white to pale orange, and olive gray at the center in color. Conidia formation was moderate. The

Fig. 2. Scanning electron micrograph of the strain EPF-6.

Bar represents 5 μm.

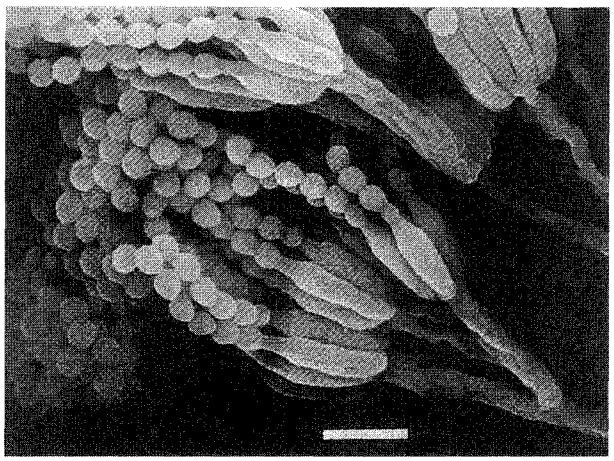
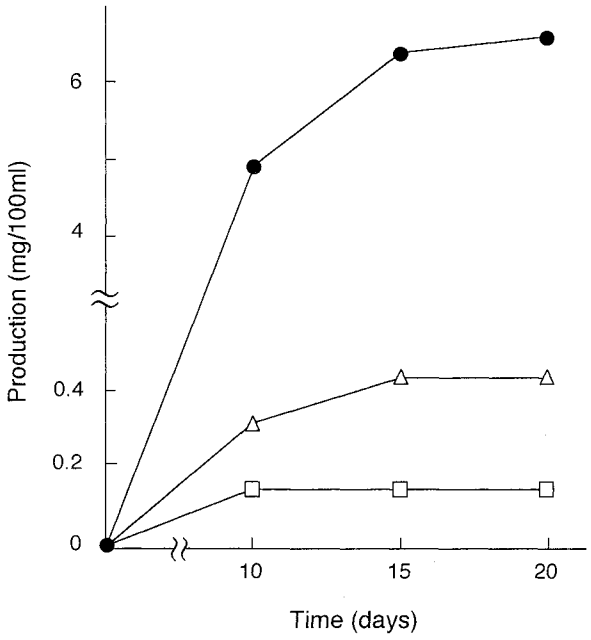


Fig. 3. Time course of quinolactacins A (**1**), B (**2**) and C (**3**) production by *Penicillium* sp. EPF-6.

1 (●), **2** (△), **3** (□).



reverse sides of the colonies were pale orange to pale brown. Exudate and soluble pigment were absent. The optimal temperature for growth of this strain was between 20~28°C. Morphological observation was conducted under a microscope. When strain EPF-6 was grown on PDA at 24°C for 14 days, the conidiophores were borne from the substrate hyphae, and the penicillia were biverticillate as shown in Fig. 2. The stipes were smooth, and 120~200×3~5.5 μm. The metulae were 10~16×3~4.5 μm. The phialides were 6~10×2~3 μm. The conidia were globose to oblate, smooth-walled, 2~2.5 μm in diameter. Based on the above-described characteristics, strain EPF-6 was identified as a member of the genus Penicillium[11] and named *Penicillium* sp. EPF-6.

Fermentation

A slant culture of the strain EPF-6 grown on potato dextrose agar (Difco) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.0%, potato starch 2.0%, polypeptone 0.5%, yeast extract 0.5%, and $CaCO_3$ 0.4%. The pH was adjusted to 7.0 before sterilization and incubated for 4 days at 28°C on a rotary shaker at 200 rpm. Three ml of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 50 ml of the production medium (SC-1) consisting of brown rice 30.0%, yeast extract 0.07%, sodium tartrate 0.03% and K_2HPO_4 0.03%. The fermentation was carried out for 21 days at 28°C under a stationary conditions. The time course of fermentation is shown in Fig. 3. The production of quinolactacins was measured by analytical HPLC under the following conditions: column, Supelcosil ABZ+plus® (Supelco Inc., 4.6×250 mm); mobile phase: acetonitrile-H_2O, 20:80; UV detection at 210 nm; flow rate of 0.7 ml/minute. Compounds **1**, **2** and **3** were eluted with retention times of 15.1, 9.3 and 17.1 minutes, respectively. The analytical HPLC elution profile of **1**, **2** and **3** is shown in Fig. 4. Cultivation conditions exemplified by the replacement of the production medium by P-5 (glucose 3.0%, glycerol 7.0%, meat extract 3.0%, $NaNO_3$ 0.2%, polypeptone 0.8%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 5.0), P-6 (glucose 2.0%, maltose 3.0%, Soybean meal 5.0%, cornsteep liquor 0.2%, polypeptone 0.3%, NaCl 0.3%, pH 6.0), and P-9 (glycerol 5.0%, potato starch 5.0%, malt extract 0.5%, yeast extract 0.5%, pH 6.0) media, resulted in extremely low production of all quinolactacins (data not shown).

Isolation

The procedure for the isolation of **1**, **2** and **3** is shown in Scheme 1. The 21-day old whole broth (5 liters) was added to acetone (20 liters). After the acetone extracts were

Fig. 4. Analytical HPLC chromatogram of mycelial extract from *Penicillium* sp. EPF-6.

Quinolactacins A (**1**), B (**2**) and C (**3**).

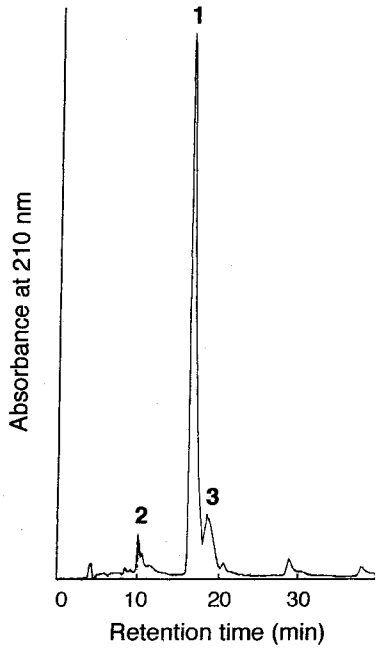
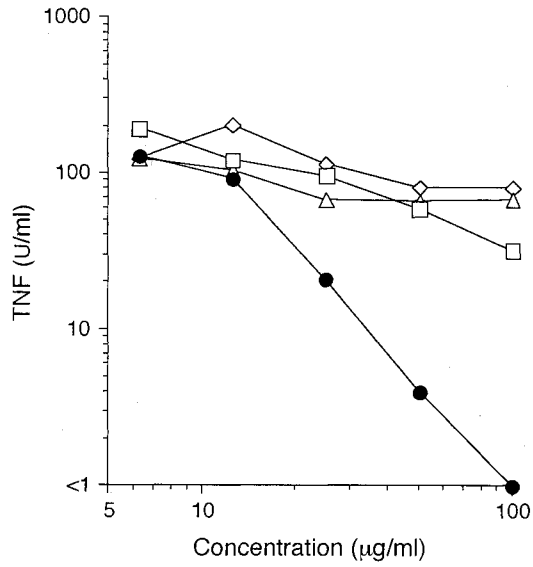


Fig. 5. Quinolactacins inhibition of LPS-induced TNF production by murine macrophages.

1 (●), **2** (△), **3** (□), Control (◇).



Scheme 1. Isolation procedure of quinolactacins A (**1**), B (**2**) and C (**3**).

Whole broth of *Penicillium* sp. EPF-6 (5 liters)
- extracted with acetone
- concentrated *in vacuo*

Aqueous solution (3 liters)
- Diaion HP-20 column chromatography
  washed with 20% acetone
  eluted with 40% acetone
- concentrated *in vacuo*
- extracted with $CHCl_3$
- concentrated *in vacuo*

$CHCl_3$ extract (1.5 g)
- Silica gel column chromatography
  eluted with $CHCl_3$ - MeOH (100 : 1 ~ 5 : 1)
- Sephadex LH-20 column chromatography
  eluted with MeOH

Crude powder (390 mg)
- Prep. HPLC (Supelcosil ABZ+plus)
  eluted with 30% acetonitrile

1 (138 mg) **2** (12 mg) **3** (9 mg)

filtered and concentrated, the resulting aqueous solution (3 liters) was applied to a Diaion HP-20 column (Mitsubishi Chemical Co., 3×10 cm) and washed with 20% acetone (1 liter). The active fraction was eluted with 40% acetone (1 liter) and concentrated *in vacuo*. The aqueous solution was extracted with $CHCl_3$ (500 ml). The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brownish oil (1.50 g). The oily substance was subjected to silica gel column chromatography (Wakogel C-300, Wako Pure Chemical Industries, Ltd.) and eluted with a stepwise gradient of $CHCl_3$ - MeOH (100 : 1, 50 : 1, 20 : 1, 10 : 1 and 5 : 1, stepwise, 120 ml each). The fraction eluted with $CHCl_3$ - MeOH (5 : 1) was concentrated *in vacuo* to give 970 mg of crude powder, which was applied to a Sephadex LH-20 column (Amersham Pharmacia Biotech, 2×100 cm) and eluted with MeOH. The fractions containing quinolactacins were concentrated and further purified by a reversed phase preparative HPLC (column: Supelcosil ABZ+plus®, 21.2×250 mm, Supelco Inc.) developed with 30% acetonitrile (flow rate: 5.0 ml/minute, detection: UV 275 nm) to give **1** (138 mg, retention time: 15.0 minutes), **2** (12 mg, retention time: 20.0 minutes) and **3** (9 mg, retention time: 23.0 minutes) as a white powder, respectively. The compounds **1**, **2** and **3** possess a novel quinolone structure with molecular formula, $C_{16}H_{18}N_2O_2$ (MW 270), $C_{15}H_{16}N_2O_2$ (MW 256) and $C_{16}H_{18}N_2O_3$ (MW 286),

respectively on the basis of HRFAB-MS and NMR spectral analyses.

Biological Properties

Inhibitory Effect on TNF Production by Quinolactacins

Bacterial lipopolysaccharide (LPS) activates macrophages to produce and release variously active mediators, including tumor necrosis factor (TNF), interleukin-1 and nitric oxide (NO)[12]. The mediators, especially TNF, cause endotoxic shock syndrome[13]. As shown in Fig. 5, compound **1** inhibited LPS-induced TNF production by murine peritoneal macrophages in a dose-dependent manner with an IC_{50} of 12.2 μg/ml. The compound **1** also inhibited LPS-induced TNF production by macrophages-like J774.1 cells (data not shown). Compounds **2** and **3** did not show distinct inhibition of TNF production by murine macrophages.

Antimicrobial Activity

A wide variety of antimicrobial activity was expected for quinolactacins, since the compounds possess a novel quinolone skeleton. However, compounds **1**, **2** and **3** exhibited very weak antifungal activity only against *Aspergillus niger* ATCC 9642, and were inactive against a range of bacteria, filamentous fungi and yeasts.

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