Andrastins A~C, New Protein Farnesyltransferase Inhibitors Produced by *Penicillium* sp. FO-3929

I. Producing Strain, Fermentation, Isolation, and Biological Activities

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New protein farnesyltransferase inhibitors, andrastins A~C, have been discovered in the cultured broth of *Penicillium* sp. FO-3929. Andrastins extracted from broth supernatant were purified by silica gel chromatography, ODS chromatography and HPLC. The IC_{50} of andrastins A, B, and C against protein farnesyltransferase were 24.9, 47.1, and 13.3 μM, respectively.

Ras proteins are subjected to posttranslational farnesylation at a cysteine the fourth residue from the C-terminus, in which protein farnesyltransferase (PFTase) is involved. Inhibition of PFTase would alter membrane localization and activation of Ras proteins[1]. In the course of screening for PFTase inhibitors of microbial origin, we have previously reported gliotoxins and pepticinnamins[2~4]. Additional new PFTase inhibitors, andrastins A, B, and C (**1**~**3**, Fig. 1), were found from the cultured broth of *Penicillium* sp. FO-3929[5].

In this paper, taxonomy of the producing strain and fermentation, isolation, physico-chemical properties, and biological activities of the andrastins are described.

Materials and Methods

General

Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively. Optical rotations were recorded on a JASCO model DIP-181 polarimeter. Melting points were measured with a Yanaco micro melting point apparatus MP-S3.

Materials

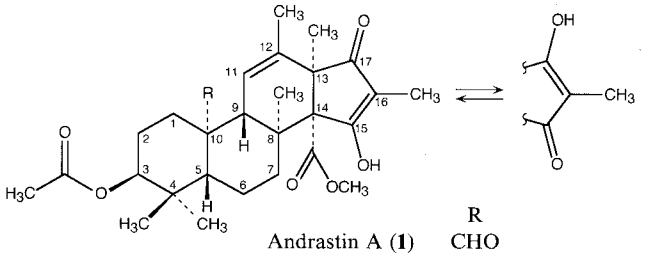
[^{3}H]-Farnesyl pyrophosphate (555 GBq/mmol) was purchased from Amersham. Partially purified PFTase was prepared as described previously[3].

For production of andrastins, the seed medium was composed of glucose 2.0%, yeast extract (Oriental Yeast Co.) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Daigo Nutritive Chemicals) 0.5%, KH_2PO_4 0.1%, and agar 0.1%. pH was adjusted to 6.0 prior to sterilizataion. The production medium was composed of soluble starch (Wako Pure Chemical Ind.) 1.5%, glycerol 0.5%, soybean meals 1.0%, fermipan (Gist-brocades) 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, and KH_2PO_4 0.05%. pH was adjusted to 6.5 prior to sterilization.

PFTase Assay

The assay method for PFTase was as described previously[3]. In brief, the standard reaction mixture contained in a final volume of 60 μl; 13 μg of partially purified PFTase from human cells THP-1, 1.3 μM of

Fig. 1. Structures of andrastins A, B, and C (**1**~**3**).



	R
Andrastin A (**1**)	CHO
Andrastin B (**2**)	CH_2OH
Andrastin C (**3**)	CH_3

recombinant p21 protein, 0.05 μM of [^{3}H]-farnesyl pyrophosphate, 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 5 mM DTT. PFTase was added and incubated for 30 minutes at 37°C. The reaction was stopped by addition of each 0.5 ml of 1% SDS in MeOH and 30% TCA. After vortexing and standing in ice for 60 minutes, the mixture was filtered on a Whatman GF/C filter and washed with 5 ml of 6% TCA. The dried filter was put into Ready Protein (Beckman) and finally counted in a liquid scintillation counter.

Antimicrobial Activity

Antimicrobial activity was measured against 14 species of microorganisms. Media for each microorganism are as follows: GAM agar (Nissui Seiyaku Co.) for *Bacteroides fragilis*; Bacto PPLO agar (Difco) supplemented with horse serum 10% and glucose 0.1% for *Acholeplasma laidlawii*; nutrient agar for the other bacteria; a medium composed of glucose 1.0%, yeast extract 0.5%, and agar 0.8% for fungi and yeasts. A paper disc (i.d. 8 mm) containing 50 μg of sample was placed on an agar plate seeded with a test microorganism. Bacteria were incubated for 24 hours at 37°C except *Xanthomonas oryzae*. Yeasts and *Xanthomonas oryzae* for 24 hours at 27°C. Fungi were incubated for 48 hours at 27°C. Antimicrobial activity was expressed as diameter of inhibitory zone.

Results and Discussion

Taxonomy of Producing Strain FO-3929

Strain FO-3929 was originally isolated from a soil sample collected at Shirokane, Minato-ku, Tokyo, Japan. From the characteristics described below, the fungus was identified as a strain of *Penicillium* sp. For the identification of the fungus, Czapek yeast extract agar (CYA), malt extract agar, 25% glycerol nitrate agar, and potato-dextrose agar were used.

Colonies on CYA grew rapidly, attaining a diameter of 50~55 mm with light olive gray in color after incubation for 7 days at 25°C. The reverse side of colonies was dull yellowish orange in color. No soluble pigment was produced.

Morphological observation was carried out under a microscope (Olympus Vanox-S AH-2). When grown on CYA at 25°C for 7 days, the conidiophores were born from substrate hyphae. Penicillia were mostly biverticillate with irregular monoverticillate occasionally as shown in Fig. 2. The phialides were 7.5~10.0 × 2~3 μm in size. The conidia were globe to subglobe and 2.5~3.0 μm in diameter and with smooth surface.

From the above characteristics, strain FO-3929 was identified as belonging to the *Penicillium* sp.[6], and named *Penicillium* sp. FO-3929. This strain has been deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan, as FERM P-14001.

Production and Isolation

A stock culture of strain FO-3929 was inoculated into six 500-ml Erlenmeyer flasks containing 100 ml of a seed medium and incubated on a rotary shaker at 27°C for 2 days. Then 600 ml of the seed culture thus obtained was transferred into a 50-liter jar fermenter containing 30 liters of a production medium. The fermentation was carried out at 27°C for 112 hours with agitation of 250 rpm and aeration of 15 liters/minute. A typical time course of the production of **1** is shown in Fig. 3.

The cultured broth (30 liters) was centrifuged and the supernatant was adjusted to pH 3 with 6N HCl. An equal volume of ethyl acetate was added and stirred for 30 minutes. The organic layer separated by centrifugation was concentrated under reduced pressure to give a brown oil (8.5 g). The oil was dissolved in a small volume of a $CHCl_3$-methanol (1 : 1), applied on a silica gel column (430 g, Silica gel 60, 40~63 μm, Merck) prepared with $CHCl_3$, and eluted with $CHCl_3$ and

Fig. 2. Photo micrograph of penicillia of strain FO-3929 on Czapek yeast extract agar (scale: 20 μm).

Fig. 3. A typical time course of the production of andrastin A.

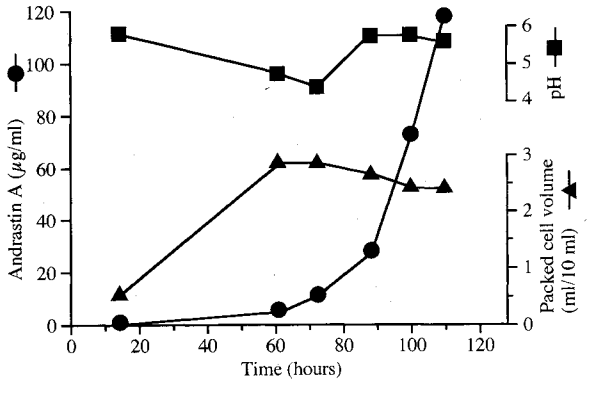


Table 1. Physico-chemical properties of **1**~**3**.

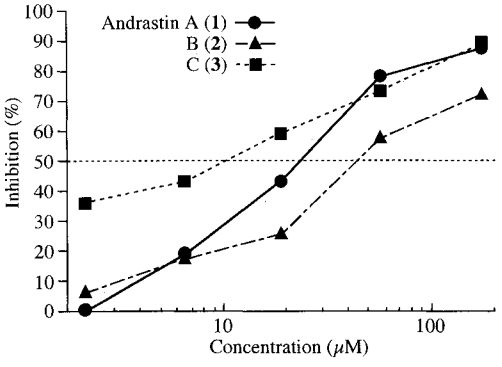
	1	2	3
Appearance	White powder	White powder	White powder
$[\alpha]_D^{26}$ (MeOH)	−46.4° (*c* 0.6)	−27.9° (*c* 1.0)	−30.3° (*c* 1.1)
mp (°C)	131~135	136~139	115~120
Molecular formula	$C_{28}H_{38}O_7$	$C_{28}H_{40}O_7$	$C_{28}H_{40}O_6$
Molecular weight	486	488	472
HR FAB-MS (*m/z*): calcd	487.2696 $(M+H)^+$	511.2671 $(M+Na)^+$	473.2903 $(M+H)^+$
found	487.2705 $(M+H)^+$	511.2694 $(M+Na)^+$	473.2907 $(M+H)^+$
UV λ_{max}^{MeOH} nm (ε)	211 (10580), 235 (sh, 5520), 250 (sh, 4010), 286 (10580)	211 (12220), 235 (sh, 6350), 250 (sh, 4760), 286 (11310)	211 (11820), 235 (sh, 5670), 250 (sh, 4540), 286 (11440)
UV $\lambda_{max}^{MeOH+HCl}$ nm (ε)	209 (9370), 235 (sh, 7470), 258 (7180), 300 (sh, 900)	207 (10260), 235 (8430), 258 (7740), 300 (sh, 1070)	207 (10630), 235 (7870), 258 (7920), 300 (sh, 950)
UV $\lambda_{max}^{MeOH+NaOH}$ nm (ε)	235 (sh, 6810), 250 (sh, 5230), 286 (14330)	235 (sh, 6110), 250 (sh, 4520), 286 (11970)	235 (sh, 6310), 250 (sh, 4890), 286 (13710)
IR ν_{max} (KBr) cm^{-1}	3440, 2950, 2879, 1737, 1716, 1625, 1452, 1434, 1376, 1326, 1247, 1214, 1054, 1031	3440, 2954, 2883, 1739, 1708, 1627, 1456, 1436, 1376, 1326, 1243, 1216, 1031, 1016	3440, 2950, 2877, 1737, 1716, 1625, 1454, 1434, 1380, 1324, 1245, 1221, 1054, 1031
Solubility: soluble	MeOH, EtOAc, $CHCl_3$	MeOH, EtOAc, $CHCl_3$	MeOH, EtOAc, $CHCl_3$
insoluble	H_2O, Hexane	H_2O, Hexane	H_2O, Hexane

$CHCl_3$-methanol. Andrastins were eluted with $CHCl_3$-methanol (99:1 and 96:4). The active fractions were concentrated under reduced pressure to give a yellow powder (4.5 g). The powder was dissolved in a small volume of methanol and applied on a ODS silica gel column (450 ml, YMC*GEL ODS-AQ-120-S50, YMC Co., Ltd.) prepared with CH_3CN-0.05% H_3PO_4 (3:2). Compounds **2** and **1** were eluted in this order with the same solution, and **3** with CH_3CN-0.05% H_3PO_4 (4:1). The fractions containing andrastins were concentrated separately under reduced pressure to give yellow powders of crude **1** (1.71 g), **2** (340 mg), and **3** (189 mg). They were further purified by HPLC under the following conditions: column, Senshu pak Pegasil ODS (i.d. 20 × 250 mm, Senshu Scientific Co., Ltd.); mobile phase, CH_3CN-0.05% H_3PO_4 (3:2) for **1**, CH_3CN-0.05% H_3PO_4 (1:1) for **2**, and CH_3CN-0.05% H_3PO_4 (4:1) for **3**; flow rate, 7 ml/minute; detection, UV 285 nm. Compounds **1**~**3** were eluded at 21, 14, and 20 minutes, respectively, under the above conditions. The active eluates of HPLC were concentrated to remove CH_3CN, extracted with ethyl acetate at pH 3, and concentrated to dryness to give white powders of **1** (1.48 g), **2** (330 mg), and **3** (36.6 mg).

Physico-chemical Properties

The physico-chemical properties of **1**~**3** are summarized in Table 1. The molecular formulae of **1**~**3** were revealed by HR-FAB-MS to be $C_{28}H_{38}O_7$, $C_{28}H_{40}O_7$ and $C_{28}H_{40}O_6$, respectively. The UV and IR spectra of **1**~**3** resembled one another, suggesting their structural similarity. The IR spectra exhibited characteristic absorptions at about 1740 cm^{-1}, suggesting the existence of ester groups. ^1H and ^{13}C NMR data as well as the details of the structure elucidations of **1**~**3** are described in the accompanying paper[7].

Fig. 4. PFTase inhibitory activities of andrastins.



Biological Properties

Inhibition of PFTase

As shown in Fig. 4, **1**~**3** inhibited PFTase in a dose-dependent manner. Among the three, compound **3** was the most potent with an IC_{50} value of 13.3 μM followed by **1** (24.9 μM) and **2** (47.1 μM). Recently KOSEMURA *et al.* reported a series of antifeedant and insecticidal compounds, the citreohybridones[8], whose structures are similar to andrastins[7]. Therefore the inhibitory activity of citreohybridones against PFTase was measured (Table 2). Among them, citreohybridone B showed the

Table 2. Inhibitory activity of andrastins and related compounds against PFTase.

	IC_{50} (μM)		IC_{50} (μM)
Andrastin A (**1**)	24.9	Isocitreohybridone A	46.0
Andrastin B (**2**)	47.1	Isocitreohybridone B	>167
Andrastin C (**3**)	13.3	Citreohybridonol	>167
Citreohybridone A	16.8	Citreohybriddione A	>167
Citreohybridone B	3.6	Citreohybriddione B	39.5

most potent inhibition with an IC_{50} value of 3.6 μM. The results suggest that the inhibitory activity is greater when methoxy or acetoxy residue are attached to the C-15 position than when attached to the C-17 position.

When **3** was preincubated with PFTase for 20 minutes before the enzyme assay, the inhibition did not change (data not shown), suggesting that **3** inhibits PFTase reversibly.

Antimicrobial Activity

Compounds **1**~**3** showed no antimicrobial activities at 50 μg/disk (paper disk method) against *Bacillus subtilis* PCI 219, *Staphylococcus aureus* ATCC 6538p, *Micrococcus luteus* ATCC 9341, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* PCI 602, *Xanthomonas oryzae* KB 88, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG 8, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Candida albicans* KF 1, and *Saccharomyces sake* KF 26.

Acknowledgments

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