Herquiline B, a New Platelet Aggregation Inhibitor Produced by *Penicillium herquei* Fg-372

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A new herquiline analog, herquiline B was isolated from the culture broth of *Penicillium herquei* Fg-372. Herquiline B contains one piperazine and two cyclohexenones. The pyrrolidine ring of herquiline A was cleaved to yield herquiline B. The IC_{50} value of herquiline B against platelet aggregation induced by ADP and platelet-activating factor were 1.6 and 5.0 μM , respectively.

Herquiline (**1**, Fig. 1) was discovered as an alkaloid from microorganisms by our group and showed weak inhibitory activity against platelet aggregation induced by ADP.^{1,2)} In the course of screening for another alkaloid from the culture broth of herquiline producing strain, *Penicillium herquei* Fg-372, we found a new herquiline analog, herquiline B (**2**). Now we renamed **1** herquiline A. The structure elucidation revealed that the pyrrolidine ring of **1** was cleaved to yield **2**. Compound **2** inhibited platelet aggregation stronger than **1**.

Here, we report the production, isolation, physico-chemical properties, structure elucidation, and biological activities of **2**.

Materials and Methods

General

NMR spectra were obtained with a Valian Unity 400 spectrometer. 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 rotation was recorded on a JASCO model DIP-181 polarimeter. Melting point was measured with a Yanaco micro melting point apparatus MP-S3.

Media

The seed medium was composed of glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, K_2HPO_4 0.1%, agar 0.1% in distilled water. The pH was adjusted to 5.6~5.8. The production medium was composed of maltose 2.0%, glucose 0.1%, ammonium acetate 0.5%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.01%, yeast extract 0.2%, agar 0.1%. The pH was adjusted to 6.0 prior to sterilization.

Assay for Platelet Aggregation

The inhibitory activity against platelet aggregation was assayed using platelet rich plasma (PRP) from citrated-rabbit blood. PRP (90 μl) was placed in a 96-well plate and preincubated at 37°C for 5 minutes. Five μl of **1** or **2** was added to the PRP. After 3 minutes preincubation, the reaction was started by adding platelet-activating factor (PAF, 0.3 μM) or ADP (12.5 μM) as a platelet aggregating agent. The aggregation was measured, with frequently mixing, at 520 nm by Titertek Multiskan (Dainippon Pharmaceutical Co., Ltd., Osaka) at 1 minute intervals.

Antimicrobial Activity

Antimicrobial activity was tested for 14 species of microorganisms. An agar plate containing each microorganism put on 6-mm paper disk containing 50 μg of samples was incubated for 24~48 hours at 27°C or 37°C, on each appropriate condition. Then antimicrobial activity was estimated by measuring the diameter of inhibitory zone.

Fig. 1. Structures of herquelines A (**1**) and B (**2**).

Results and Discussion

Taxonomy of the Producing Organism Fg-372

Strain Fg-372 was originally isolated from a soil sample collected at Saitama Prefecture, Japan. For identification of fungus, CZAPEK's agar, malt extract agar and potato-dextrose agar were used. This strain grew restrictedly to form sage green to pea green colonies with diameter of 10~25 mm after incubation for 7 days at 27°C. Growth at 37°C was very poor in these media. The colony surface was velvety. The colors of the reverse side of the colony and soluble pigment were gold to dusty yellow. When the strain Fg-372 was grown on potato-dextrose agar at 27°C for 7 days, the conidiophores were borne from substrate hyphae, and the penicillia were biverticillate and usually symmetrical as shown in Fig. 2. The phialides were 9~10 × 2.5~3.5 μm, and its surface was smooth.

From the above characteristics, the strain Fg-372 was identified as the *Penicillium herquei*³⁾ and named *Penicillium herquei* Fg-372. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM P-14760.

Production and Isolation

The medium reported previously¹⁾ did not produce **1** by submerged culture. We examined various media for the production of **1** and a new production medium was found to produce **1** by submerged culture. Therefore minor analog could be obtained easier than before. Compound **2** was produced in the culture broth of both media. In the course of testing media, the production of **1** increased when tyrosine was added. It suggests that

tyrosine is a biosynthetic precursor of **1**.

The stock culture of the strain Fg-372 was inoculated into two 500-ml Erlenmeyer flasks containing 100 ml of the seed medium and the flasks were incubated on a rotary shaker at 27°C for 2 days. Then 200 ml of the seed culture was transferred into a 30-liter jar fermenter containing 20 liters of the production medium. The fermentation was carried out at 27°C for 7 days with aeration of 10 liters/minute and agitation of 250 rpm.

The isolation of herquelines was guided by Dragendorff reagent on TLC.

Acetone was added to whole broth (20 liters) and the mixture was stirred. After filtration, the acetone extract was evaporated to remove acetone. Residual solution was adjusted to pH 10 with NH₄OH and herquelines were extracted with CHCl₃. Then they were transferred into 0.1 N HCl aqueous solution. The water layer was adjusted to pH 10 again and they were extracted with CHCl₃. The CHCl₃ extract was concentrated to dryness to give a yellow oil (456 mg). The oil was dissolved in a small volume of CHCl₃ and applied on a column of NH₂-silica gel (Chromatorex NH-DM1020, Fuji Silysia Chemical, 20 g) prepared with hexane-ethyl acetate (95:5). The column was eluted stepwise with hexane-ethyl acetate (90:10, 80:20, and 0:100) and methanol. The eluate from hexane-ethyl acetate (80:20) was concentrated and further purified by silica gel column to give a white powder of **1** (108 mg). The fractions that were eluted with methanol and exhibited positive Dragendorff reagent in NH₂-silica gel were combined and concentrated to give 43.4 mg of yellow oil. It was dissolved in CHCl₃, applied on a silica gel column (2 g, Wakogel C-300, Wako Pure Chemical Ind.), and eluted with CHCl₃-methanol (20:1 and 10:1). The eluate from CHCl₃-methanol (10:1) was concentrated to give 11.1 mg of yellow oil. It was applied onto a silica gel TLC (Kieselgel 60F₂₅₄, Merck) and developed with CHCl₃-methanol (8:1). The zone that showed positive Dragendorff reagent was collected and

Fig. 2. Scanning electron micrograph of penicillia of strain Fg-372.

Bar represents 5 μm.

Table 1. Physico-chemical properties of **2**.

Appearance	White powder
MP (°C)	109~113
[α] _D ²⁵	-85.2 (c 0.3, MeOH)
Molecular formula	C ₁₉ H ₂₆ N ₂ O ₂
FD-MS (m/z)	315 (M+H) ⁺
HR FAB-MS: calcd	315.2074 (C ₁₉ H ₂₇ N ₂ O ₂)
found	315.2060 (M+H) ⁺
UV λ _{max} ^{MeOH} nm (ε)	203 (9100), 222 (sh, 1530), 285 (780)
IR ν _{max} (KBr) cm ⁻¹	3400, 2920, 1650, 1540, 1110
Solubility: soluble	MeOH, CHCl ₃
insoluble	H ₂ O, benzene, ethyl acetate

Table 2. The ^1H and ^{13}C NMR data of **1** and **2**.

Position	^{13}C	1 ^1H	^{13}C	2 ^1H
1	38.7 t	1.82 m (1H), 1.97 m (1H)	41.7 t	2.28 m (1H), 2.56 m (1H)
2	56.1 d	3.42 m (1H)	51.6 d	3.25 m (1H)
4	43.2 t	2.95 dd (1H, $J=7.5, 15.5$ Hz), 3.02 dd (1H, $J=2, 15.5$ Hz)	44.8 t	2.92 dd (1H, $J=5, 14$ Hz), 3.01 dd (1H, $J=3, 14$ Hz)
5	67.2 d	2.40 m (1H)	59.1 d	2.41 m (1H)
6	38.1 t	2.03 dd (1H, $J=3, 13.5$ Hz), 2.60 dd (1H, $J=2, 13.5$ Hz)	37.0 t	1.78 dd (1H, $J=4, 14.5$ Hz), 2.73 br d (1H, $J=14.5$ Hz)
7	141.4 s		135.7 s	
8	32.0 t	2.43 m (1H), 2.78 m (1H)	33.2 t	2.48 m (1H), 2.68 m (1H)
9	39.9 t	2.35 dd (1H, $J=2, 13$ Hz), 2.52 dd (1H, $J=6, 13$ Hz)	39.4 t	2.48 m (1H), 2.67 m (1H)
10	212.5 s		209.8 s	
11	51.7 d	3.95 m (1H)	50.9 d	3.95 m (1H)
11a	55.5 d	1.87 dd (1H, $J=2, 5$ Hz)	48.9 d	3.80 m (1H)
12	211.6 s		211.9 s	
13	39.3 t	2.45 m (2H)	39.7 t	2.48 m (1H), 2.68 m (1H)
14	25.5 t	1.57 m (1H), 1.86 m (1H)	30.8 t	2.32 m (1H), 2.61 m (1H)
14a	38.5 d	1.93 m (1H)	138.3 s	
14b	63.1 d	2.71 d (1H, $J=5$ Hz)	124.8 d	5.31 m (1H)
15	125.1 d	5.60 d (1H, $J=1.5$ Hz)	127.2 d	5.25 m (1H)
16-Me	43.4 q	2.17 s (3H)	42.9 q	2.26 s (3H)
17	61.6 t	2.22 dd (1H, $J=1, 12$ Hz), 2.25 dd (1H, $J=5.5, 12$ Hz)	53.2 t	2.57 m (2H)

The CDCl_3 signals (7.26 ppm of ^1H and 77.0 ppm of ^{13}C) were used as references. The coupling constants (Hz) are in parentheses.

extracted with methanol. The methanol extract was concentrated and subjected to reverse phase HPLC under the following conditions: column, Asahipak ODP-50 (i.d. 21.5×300 mm, Asahi Chemical Ind.); mobile phase, CH_3CN - 12.5 mM sodium borate (pH 10) buffer (40 : 60); flow rate, 3 ml/minute; detection, UV 220 nm. Under these conditions, the peak eluted at 15 minutes was collected to yield a white powder of **2** (5.2 mg).

Physico-chemical Properties

The compound **2** was isolated as a white powder. Its physical and chemical properties are summarized in Table 1. The molecular formula of **2** was deduced to be $\text{C}_{19}\text{H}_{26}\text{N}_2\text{O}_2$ by HR FAB-MS, which was same as that of **1**.

Structure Elucidation

The structure of **1** was elucidated on the basis of X-ray crystallographic analysis previously.²⁾ This time, we carried out 1D and 2D NMR experiments for **1** and accomplished the assignment of the ^1H and ^{13}C NMR signals (Table 2).

The structure of **2** was elucidated by the NMR spectral analysis. The DEPT and HMQC experiments of **2** revealed that **2** contains 1 methyl carbon, 8 methylene carbons, 6 methine carbons including 2 olefinic methines, 2 olefinic quaternary carbons, and 2 carbonyl carbons (Table 2). Comparing with the ^{13}C signals of **2** to **1**, two

Fig. 3. Structure of **2** elucidated by ^1H - ^1H COSY and HMBC.

new olefins appeared and two methines disappeared in that of **2**. The partial structures of **2** was established by the ^1H - ^1H COSY and they were pieced together into the final structure on the basis of the long-range coupling observed in the HMBC experiments (Fig. 3). The HMBC experiment indicated that the structure of **2** was same as that of **1**, except for N-3, C-14a, and C-14b. An olefinic proton at δ 5.31 (14b-H) was correlated with C-1 (δ 38.7), C-12 (δ 211.9), and C-14 (δ 25.5). C-14a (δ 138.3) was assigned by the correlation with 14- H_2 (δ 2.32, 2.61). Therefore, it was suggested that the bond between N-3 and C-14b of **1** was cleaved and a double bond was

formed between C-14a and C-14b. Thus, the structure of **2** containing one piperazine and two cyclohexenone rings was elucidated (Fig. 1).

Biological Properties

Inhibitory Activity of Platelet Aggregation

The preliminary data suggested that **1** showed a weak inhibition against blood platelet aggregation induced by ADP.¹⁾ Here we examined the inhibitory activity of herquines against platelet aggregation induced by PAF and ADP using PRP from rabbit blood. PAF and ADP caused a submaximal aggregation at 0.3 μ M and 12.5 μ M in the assay system, respectively. IC₅₀ values of herquines were shown in Table 3. Compound **2** inhibited platelet aggregation stronger than **1**.

A little change of the structure from **1** to **2** caused much inhibitory activity against platelet aggregation. Though the inhibitory mechanism is not clear, it may be due to the subcellular reaction because of its non-selective inhibitory mode for PAF- or ADP-induced aggregation. Further investigations are necessary to clarify the inhibitory mechanism of **2**.

Table 3. Inhibitory activity of **1** and **2** in platelet aggregation.

compound	IC ₅₀ (μ M)	
	PAF aggregation	ADP aggregation
1	240	180
2	5.0	1.6

Antimicrobial Activity

Compound **2** showed no antimicrobial activities at 50 μ g/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, *Piricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Candida albicans* KF 1, and *Saccharomyces sake* KF 26.

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