Phenopyrrozin, a New Radical Scavenger Produced by *Penicillium* sp. FO-2047

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(Received for publication July 24, 1995)

A new radical scavenger, named phenopyrrozin, was isolated from the culture broth of *Penicillium* sp. FO-2047. Phenopyrrozin was purified from whole broth by solvent extraction, silica gel chromatography, and HPLC. The structure of phenopyrrozin was elucidated as 5,6,7,7a-tetrahydro-2-hydroxy-1-phenyl-3*H*-pyrrolizin-3-one. The IC_{50} of phenopyrrozin against lipid peroxidation induced by $Cr_2K_2O_7$ was 73 μg/ml. Phenopyrrozin also reduced chromosomal aberrations induced by paraquat.

An effective therapeutic agent for the treatment of free radical mediated diseases such as ischemia, trauma, and inflammation would be expected to decrease free radical levels. We have screened for new radical scavengers of microbial origin, and previously reported that isoflavonoids OH-1049 P, Q, and R possess free radical scavenger activity.[1] Other antioxidants from microbes were also reported by other investigators.[2~4] Our continuing screening efforts to find new radical scavengers culminated in the discovery of phenopyrrozin (**1**, Fig. 1) from the culture broth of *Penicillium* sp. FO-2047.

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

Materials and Methods

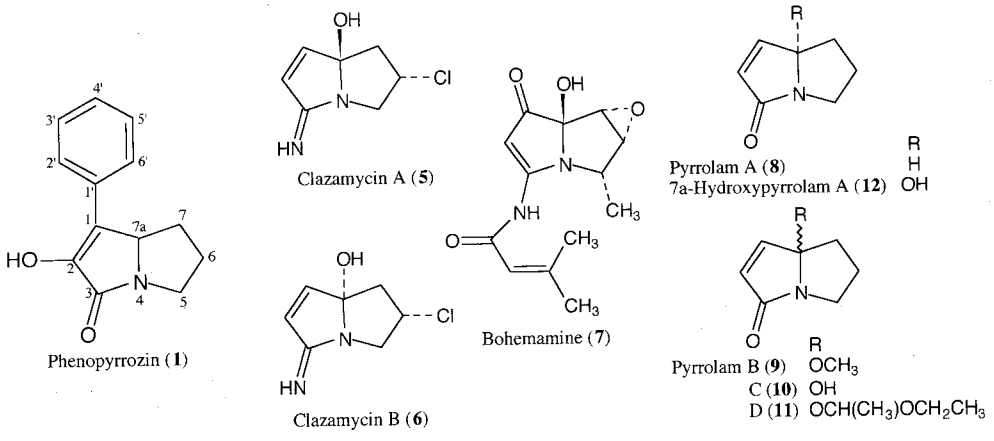
General

NMR spectra were obtained with Valian XL-400 and JEOL JNM-EX270 (for HMBC) spectrometers. Mass spectrometry was conducted on a JEOL JMS-DX300 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 (Oriental Yeast Co.) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Daigo Nutritive Chemicals, Ltd.) 0.5%, KH_2PO_4 0.1%, and agar 0.1%. The pH was adjusted

Fig. 1. Structures of phenopyrrozin (**1**) and pyrrolizidine compounds (**5**~**12**).



to 5.8 prior to sterilizataion. The production medium was composed of soluble starch 3.0%, glycerol 1.0%, soybean meals 2.0%, 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%. The pH was adjusted to 6.5 prior to sterilizataion.

Lipid Peroxidation Assay

Lipid peroxidation assay was done as reported previously.[5] In brief, microsomes from rat liver (2 mg protein/ml) were incubated with 1 mM $K_2Cr_2O_7$ and various concentrations of **1** in KCl-Tris buffer (pH 7.4) at 37°C for 1 hour. After the incubation period, thiobarbituric acid reacting substances (TBARS) were measured by BUEGE and AUST method.[6]

Assay of Chromosomal Aberrations Induced by Paraquat

Chinese hamster lung fibroblasts Don D-6 were maintained in EAGLE's minimal essential medium supplemented with 0.2 mM L-serine, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. The cells were grown as monolayer culture in plastic dishes under standard conditions of 5% CO_2 in air at 37°C. The cells were seeded at 2×10^4 cells/ml (2 ml per dish) in 8 cm^2 dishes, on the bottom of which were placed a suitable size of cover glass, and cultured for 48 hours. Paraquat (methyl viologen, Sigma) was dissolved in saline, and **1** and α-tocopherol were dissolved in DMSO. Each culture including control received the same volume (0.5%) of DMSO. Then cells were treated with paraquat and **1** or α-tocopherol for 3 hours, washed with Hanks' balanced salt solution, and reincubated with a fresh medium for further 19 hours, followed by arrest in metaphase with colcemid (0.1 μg/ml) for 2 hours. The cells were treated in hypotonic KCl (75 mM) for 30 minutes at 37°C, then fixed in MeOH - AcOH (3 : 1). Chromosomes were prepared on cover glass using an air-drying method and then stained in Giemsa (5% in phosphate buffer, pH 6.8 for 10 minutes). One hundred cells were analyzed per point. Only structural aberrations such as chromatid and chromosomal breaks, and exchanges were analyzed.

Antimicrobial Activity

Antimicrobial activity was tested for 14 species of microorganisms. An agar plate containing each microorganism put on 8-mm paper disk containing 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.

Glycosidase Assay

Inhibitory activities of α-glucosidase and α-mannosidase were measured as described previously.[7]

Catalytic hydrogenation of 1

A mixture of **1** (20 mg) and 10% palladium on activated carbon (40 mg) in MeOH (3 ml) was hydrogenated under an atmospheric pressure of hydrogen with stirring for 3 hours. The reaction mixture was filtered and concentrated under reduced pressure. It was purified by the HPLC under the condition described in the purification of **1** to give white powders of **3** (3.8 mg) and **4** (3.0 mg).

Compound **3**. FAB-MS *m/z* 218 $(M+H)^+$; $[\alpha]_D^{25}$ 65.9 (*c* 0.54, EtOH); UV λ_{max}^{EtOH} nm (ε) 207 (270), 255 (90), 290 (sh, 40); ^1H NMR ($CDCl_3$) δ 7.31 (2H, m, 3'-H and 5'-H), 7.28 (1H, m, 4'-H), 6.99 (2H, d, *J*=7.5 Hz, 2'-H and 6'-H), 4.90 (1H, d, *J*=6.5 Hz, 2-H), 4.12 (1H, ddd, *J*=6.5, 7.0, 8.3 Hz, 7a-H), 3.95 (1H, dd, *J*=6.5, 6.5 Hz, 1-H), 3.55 (1H, ddd, *J*=7.5, 7.5, 11.2 Hz, 5-H), 3.17 (1H, ddd, *J*=4.1, 8.2, 11.2 Hz, 5-H), 2.25 (1H, br s, 2-OH), 1.92 (1H, m, 6-H), 1.83 (1H, m, 6-H), 1.65 (1H, dddd, *J*=4.0, 7.0, 7.0, 12.5 Hz, 7-H), 1.05 (1H, ddd, *J*=8.3, 12.5, 17.2 Hz, 7-H); ^{13}C NMR ($CDCl_3$) δ 173.7 (C-3), 134.7 (C-1'), 129.3 (C-2' and C-6'), 128.7 (C-3' and C-5'), 127.6 (C-4'), 74.7 (C-2), 61.1 (C-7a), 51.7 (C-1), 41.7 (C-5), 25.8 (C-6), 25.6 (C-7).

Compound **4**. FAB-MS *m/z* 218 $(M+H)^+$; $[\alpha]_D^{25}$ 67.3 (*c* 0.43, EtOH); UV λ_{max}^{EtOH} nm (ε) 207 (340), 250 (120), 290 (90); ^1H NMR ($CDCl_3$) δ 7.38 (2H, m, 3'-H and 5'-H), 7.35 (2H, m, 2'-H and 6'-H), 7.32 (1H, m, 4'-H), 4.46 (1H, d, *J*=5.5 Hz, 2-H), 4.37 (1H, ddd, *J*=5.5, 8.0, 8.5 Hz, 7a-H), 3.59 (1H, ddd, *J*=7.7, 7.7, 12.0 Hz, 5-H), 3.24 (1H, ddd, *J*=4.0, 8.8, 12.0 Hz, 5-H), 3.15 (1H, dd, *J*=5.5, 8.5 Hz, 1-H), 2.90 (1H, br s, 2-OH), 2.2 (2H, m, 7-H), 2.19 (1H, m, 6-H), 1.47 (1H, m, 6-H); ^{13}C NMR ($CDCl_3$) δ 172.3 (C-3), 135.2 (C-1'), 128.9 (C-2' and C-6'), 128.6 (C-3' and C-5'), 127.6 (C-4'), 78.6 (C-2), 65.1 (C-7a), 54.2 (C-1), 40.1 (C-5), 30.7 (C-6), 27.2 (C-7).

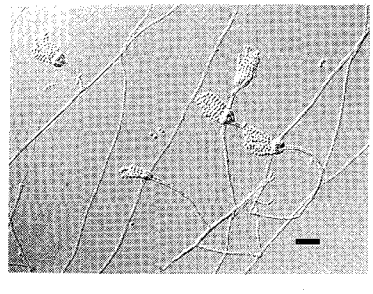
Results

Taxonomy of the Producing Organism FO-2047

Strain FO-2047 was originally isolated from a soil sample collected in Numazu, Shizuoka, Japan. For the taxonomic studies of the fungus, Czapek yeast extract agar, malt extract agar, 25% glycerol nitrate agar and potato-dextrose agar were used. This strain grew moderately to form pale green to grayish green colonies with diameter of 15 ~ 28 mm after incubation for 7 days

Fig. 2. Photo micrograph of penicillia of strain FO-2047 on Czapek yeast extract agar.

Bar represents 20 μm.



at 25°C. The colony surface was floccose to velvety. The conidial structures were moderately produced on various agar media. The reverse color was pale yellow to yellowish gray. When strain FO-2047 was grown on Czapek yeast extract agar at 25°C for 7 days, the conidiophores born from substrate hyphae, and penicillia were almost monoverticillate as shown in Fig. 2. The phialides were $5\sim10\times2\sim3\,\mu m$. The conidia were roughly globular, and $2\sim3\,\mu m$ in diameter, and its surface was smooth. The temperature permitting growth of the strain was 14~37°C, and optimum temperature for growth was 23~30°C.

From the above characteristics, the strain FO-2047 was identified to the genus *Penicillium*[8] and named *Penicillium* sp. FO-2047. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM P-14530. This strain also produced gliotoxin and acetylgliotoxin too as described previously.[9]

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

Appearance		White powder
MP (°C)		147~152
$[\alpha]_D^{25}$		−10.2 (*c* 0.6, MeOH)
Molecular formula		$C_{13}H_{13}NO_2$
Elemental analysis	calcd	C 72.54, H 6.09, N 6.51
	found	C 72.52, H 6.10, N 6.34
FD-MS (m/z)		215 (M^+)
HREI-MS:	calcd	215.0946 ($C_{13}H_{13}NO_2$)
	found	215.0938 (M^+)
UV λ_{max}^{EtOH} nm (ε)		206 (7,960), 215 (7,850), 227 (sh, 5,810), 240 (3,870), 295 (5,810), 340 (sh, 650)
UV $\lambda_{max}^{EtOH+HCl}$ nm (ε)		208 (sh, 5,380), 217 (6,450), 227 (sh, 5,160), 243 (3,440), 295 (5,590)
UV $\lambda_{max}^{EtOH+NaOH}$ nm (ε)		207 (16,660), 240 (sh, 4,840), 330 (5,590)
IR ν_{max} (KBr) cm^{-1}		3220, 1670, 1420, 1380, 1320, 1240, 1120, 850, 770
Solubility:	soluble	MeOH, EtOH, $CHCl_3$
	insoluble	H_2O

Production and Isolation

The stock culture of strain FO-2047 was inoculated into four 500-ml Erlenmeyer flasks containing 100 ml of a seed medium and incubated on a rotary shaker at 27°C for 3 days. Then 400 ml of the seed culture was transferred into a 30-liter jar fermenter containing 20 liters of a production medium. The jar fermenter was run at 27°C for 4 days with aeration and agitation.

The whole cultured broth (20 liters) was extracted with $CHCl_3$. The organic layer was concentrated under reduced pressure to give a brown oil (28.4 g). The oil was applied on a silica gel column (330 g, Silica gel 60, $40\sim63\,\mu m$, Merck) prepared with toluene, and eluted with toluene - ethyl acetate (4 : 1). The fractions containing **1** was concentrated under reduced pressure to give a crude material (1.27 g). It was dissolved in a small amount of methanol and the insoluble part was dried to give 487 mg of a powder. A part of this powder (50 mg) was dissolved in a small amount of $CHCl_3$ and applied on HPLC under the following conditions: column, Capcell pak C_{18} (i.d. 20 × 250 mm, Shiseido Co., Ltd.); mobile phase, 53% methanol; flow rate, 8 ml/minute; detection, UV 205 nm. Pure **1** eluted at 31 minutes was concentrated under reduced pressure to yield 23.6 mg of a white powder.

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was deduced by elemental analysis and HREI-MS as $C_{13}H_{13}NO_2$. Strong absorbance at 1670 cm^{-1} in the IR spectrum suggested an carbonyl residue. Chemical shifts in the ^1H and ^{13}C NMR of **1** are shown in Table 2. The ^{13}C-^1H COSY experiments revealed the connectivity of each proton and carbon.

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

Position	^{13}C	^1H
1	123.4 s	
2	143.6 s	
3	170.8 s	
5	41.8 t	3.22 ddd (1H, *J*=4.0, 7.2, 10.0 Hz)
		3.33 ddd (1H, *J*=10.0, 10.0, 10.0 Hz)
6	28.1 t	2.21 m (2H)
7	30.7 t	1.04 dddd (1H, *J*=10.0, 10.0, 10.0, 10.0 Hz), 2.38 m (1H)
7a	61.5 d	4.52 dd (1H, *J*=6.0, 10.0 Hz)
1'	133.0 s	
2' and 6'	126.3 d	7.73 d (2H, *J*=7.5 Hz)
3' and 5'	128.6 d	7.39 dd (2H, *J*=7.5, 7.5 Hz)
4'	127.1 d	7.25 dd (1H, *J*=7.5, 7.5 Hz)
2-OH		10.25 br s (1H)

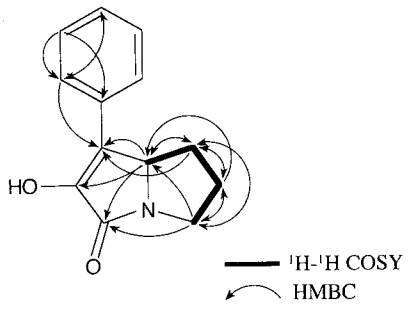
The DMSO-d_6 signals (2.50 ppm of ^1H and 39.5 ppm of ^{13}C) were used as references. The coupling constants (Hz) are in parentheses.

Structure Elucidation of Phenopyrrozin (1)

In the DEPT spectra, **1** showed 3 methylene, 6 methine, and 4 quaternary carbon signals. Five protons, δ 7.25 (1H), δ 7.39 (2H), and δ 7.73 (2H), suggested a monosubstituted benzene from their couplings in the ^{1}H NMR. A partial structure, $-CH_2-CH_2-CH_2-CH-$, was deduced by the ^{1}H-^{1}H COSY (Fig. 3). In the HMBC experiment, a long-range coupling between 3'-H (5'-H) and C-1' assigned C-1' to a quaternary carbon of the benzene ring (Fig. 3). Long-range couplings of 7-H_2/C-1, 7a-H/C-1 and 2'-H (6'-H)/C-1 proved the sequence of C-7a–C-1–C-1'. The acetylation of **1** afforded monoacetyl derivative **2** (FAB-MS, m/z 258 $(M+H)^+$), which confirmed the hydroxyl residue. The remaining hydroxyl (δ_H 10.25), olefine (δ_C 143.6), carbonyl (δ_C 170.8), and nitrogen were suggested to form γ-lactam with C-1 and C-7a by long range couplings of 5-H_2/C-3, 7a-H/C-2, and 7a-H/C-3.

To confirm the γ-lactam moiety, catalytic hydrogenation of **1** was carried out to give **3** and **4**. The ^{1}H-^{1}H COSY of **3** and **4** proved their sequences of 2-H–1-H–7a-H–7-H_2–6-H_2–5-H_2. So their structures were elucidated as diastereomers of hexahydro-2-hydroxy-1-phenyl-1*H*-pyrrolizin-3-one. Therefore olefinic C-1 and C-2 in **1** were suggested to be hydrogenated in **3** and **4**. Thus the structure of **1** was elucidated as 5,6,7,7a-tetrahydro-2-hydroxy-1-phenyl-3*H*-pyrrolizin-3-one (Fig. 1).

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



Biological Properties

Radical Scavenger Activitiy and Effect on Chromosomal Aberrations

The concentration of **1** that produced 50% inhibition of potassium dichromate-induced lipid peroxidation assessed by measuring TBARS was 73 μg/ml (Table 3).

The inhibitory effect of **1** on chromosomal aberrations induced by paraquat was examined *in vitro*. Treatment of Don D-6 cells with paraquat resulted in a dose dependent increase of chromosomal aberrations (data not shown). The chromosomal aberrations induced by paraquat were reduced by **1** dose dependently in the range of 35~70 μg/ml (Table 4). A potent antioxidant α-tocopherol showed similar effect at 17.5 μg/ml.

Antimicrobial Activity

Compound **1** showed antimicrobial activities at a high concentration (70 μg/disk, paper disk method) against *Micrococcus luteus* ATCC 9341 (inhibitory zone, i.d.

Table 3. The inhibition of dichromate-induced lipid peroxidation by **1**.

Concentration	Inhibition
100 μg/ml	59.6%
50	39.3
25	31.8
12.5	19.1
6.25	23.5
3.13	0

Table 4. Effect of **1** and α-tocopherol on chromosomal aberrations induced by paraquat on Don D-6 cells.

Compound	Concentration (μg/ml)	Number of cells observed	Cells with chromosomal aberrations (%)							
			Chromatid*			Chromosome*			Total	
			gaps	ctb	cte	csb	cse	oth	+gap	−gap
None		100	0	0	0	0	1	0	1	1
DMSO (control)		100	1	0	2	0	0	0	3	2
Paraquat	200	100	11	25	20	4	8	1	46	40
Paraquat + **1**	140	toxic								
	70	100	2	11	6	0	2	0	15	14
	35	100	9	14	14	0	9	0	26	24
	17.5	100	1	22	25	0	6	1	38	38
Paraquat	70	100	2	9	6	0	1	0	12	11
+ α-tocopherol	35	100	1	8	5	0	5	1	12	10
	17.5	100	1	4	11	0	1	0	13	12
	8.75	100	6	32	30	0	3	0	42	40

* ctb, chromatid breaks; cte, chromatid exchanges; csb, chromosome breaks; cse, chromosome exchanges; oth, multiple aberrations and others.

9.5 mm) and *Acholeplasma laidrawii* PG 8 (i.d. 18.0 mm). But it did not inhibit the growth of *Bacillus subtilis* PCI 219, *Staphylococcus aureus* ATCC 6538p, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* PCI 602, *Xanthomonas oryzae* KB 88, *Bacteroides fragilis* ATCC 23745, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Candida albicans* KF 1, and *Saccharomyces sake* KF 26 at the same concentration. Compounds **3** and **4** had no antimicrobial activities against the above 14 microorganisms at 50 μg/disk.

Inhibitory Activities of Glycosidases

Some pyrrolizidine alkaloids inhibit glucosidases[10] and known mannosidase inhibitor swainsonine has a indolizidine skeleton.[11] So inhibitory activities of **1** against glycosidases were tested. But **1** did not inhibit both yeast α-glucosidase and jack bean α-mannosidase at the concentration of 150 μM.

Discussion

Compound **1** has a pyrrolizidine skeleton. Many pyrrolizidine alkaloids have been purified from plant sources.[12] As for those of microbial origin, clazamycins A and B (**5**, **6**),[13] bohemamine (**7**),[14] pyrrolams A~D (**8**~**11**),[15] and 7a-hydroxypyrrolam A (**12**)[16] have been reported as far as we know. Though all of them were produced by Actinomycetes, **1** was obtained from fungi. Pyrrolizidine alkaloids show various biological activities; antitumor activity, inhibition of glucosidases, choline antagonist, mydriatic and antispasmodic activities, inhibition of cardiac contractility, *etc.*[10] Therefore biological activities of **1** are interested.

The toxicity of paraquat is mediated by superoxide anions generated by one electron transfer from paraquat to molecular oxygen.[17] NICOTERA[18] reported that BS lymphoblastoid cell line GM3403 demonstrated a near linear dose-dependent increase in total chromosomal aberrations when treated with increasing concentrations of paraquat, and that α-tocopherol, a potent inhibitor of lipid peroxidation, dose-dependently inhibited the exchange of sister chromatids of Don D-6 cells treated with paraquat. These findings, together with our present results, suggest that **1**, at least in part, acts as a suppressor of oxidative damage in DNA of Don D-6 cells.

Acknowledgments

We are grateful to Mr. TOMOYA KAWAKUBO and Ms. WAKAKO MOROI-OHTSUKA for characterization of the producing strain. We wish to thank Dr. HARUKI YAMADA and Ms. IZUMI OYA, Research Center for Biological Function, the Kitasato Institute, for glycosidases assay. We also wish to thank Ms. AKIKO HATANO, Ms. NORIKO SATO, Ms. AKIKO NAKAGAWA, and Ms. CHIKAKO SAKABE, School of Pharmaceutical Sciences, Kitasato University, for measurements of NMR and mass spectra. This work was supported in part by a grant from Japan Keirin Association.

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