NOVEL ANTINEMATODAL AND ANTIPARASITIC AGENTS FROM *PENICILLIUM CHARLESII*

I. FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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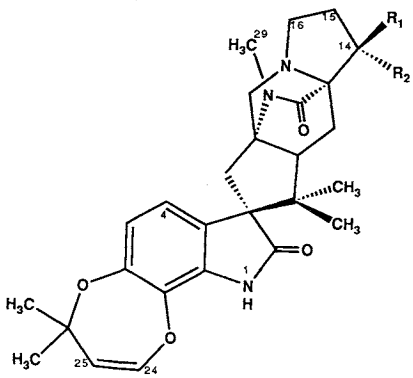
Paraherquamide and six novel analogs were isolated from the fermentation of *Penicillium charlesii* (ATCC 20841). All seven natural products displayed potent antinematodal activity against *Caenorhabditis elegans*. None of the novel analogs were more potent than paraherquamide.

Paraherquamide (**1**), a known fungal metabolite from *Penicillium paraherquei*[1,2], has been isolated from *Penicillium charlesii* (ATCC 20841) along with six structurally related novel analogs (paraherquamides B~G, **2**~**7**)[3] (Figs. 1 and 2). All seven metabolites as well as C-24, C-25 dihydroparaherquamide (**8**) possess antinematodal activity against *Caenorhabditis elegans*[4] (Table 1). Paraherquamide was also shown to have *in vivo* antiparasitic activity against *Trichostrongylus colubriformis* (nematoda) in gerbils[5]. The six novel metabolites (**2**~**7**) differ from paraherquamide (**1**) and the related marcfortines[6,7] at C-14 and in the dioxypino ring system. The fermentation, isolation

Table 1. Antinematodal activity of paraherquamide (**1**) and its analogs (**2**~**8**) against *Caenorhabditis elegans*.

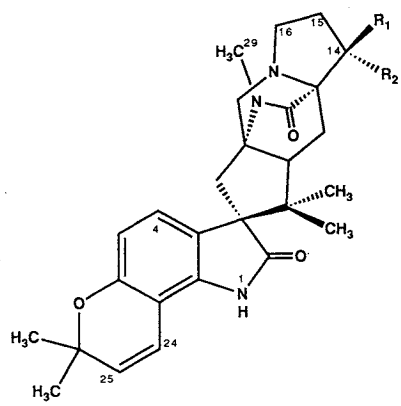
Compound	LD_{50} (μg/ml)
1	2.5
2	100
3	40
4	160
5	6
6	65
7	20
8	>200

Fig. 1. Paraherquamide and analogs **2**~**5**.



1 R_1=OH R_2=CH_3
2 R_1=H R_2=H
3 R_1, R_2=H_2C=
4 R_1, R_2=$-OCH_2-$
5 R_1=H R_2=CH_3

Fig. 2. Paraherquamide analogs **6** and **7**.



6 R_1=H R_2=CH_3
7 R_1=OH R_2=CH_3

and biological activity are described in this paper.

Materials and Methods

General Procedures

UV spectra, taken in MeOH, were recorded on a Beckman DU-8 spectrophotometer.

Mass spectra (EI and HR) were carried out on a Finnigan MAT 212 mass spectrometer and NMR (^1H and ^{13}C) on a Varian XL-300, which will be reported in the adjoining paper[8].

Fermentation

Fermentations were carried out on solid substrates in either shake flasks or large production trays.

Isolation

Isolation was accomplished by silica gel, Sephadex LH-20, and reverse phase HPLC chromatographies to provide paraherquamide (**1**) and six novel analogs (**2**~**7**). Reverse phase HPLC was used to monitor fermentation titers as well as to assess the purity of isolated compounds. Chromatography was carried out on a C-18 bonded phase column (4.6 mm × 25 cm, Whatman Partisil-10 ODS-3) eluted with 0.001 M H_3PO_4 - CH_3CN (70 : 30) at 1.5 ml/minute. Detection was by UV absorbance at 225 nm (Micromeritics-788). Capacity factors (K') for the seven compounds are indicated in Table 2.

Biological Assay

C. elegans N2 strain was cultivated on a NG agar plate covered with a lawn of *Escherichia coli*. Worms were rinsed off the agar plate with 5 mM Tris buffer (pH 7.35) at 22°C, washed twice by centrifugation at 1,000 × *g* for 2 minutes and then resuspended. Aliquots of the worm suspension (50 μl, approximately 100 worms) were placed into 13 × 100 mm glass test tubes. All eight compounds in DMSO were added to aliquoted samples of *C. elegans* in a final volume of 500 μl of 1% DMSO solution and incubated at 22°C for 16 hours. Motile worms were determined by examination using a low power dissecting microscope. Controls had >90% motile worm. LD_{50} values reported is the concentration at which 50% of the worms are immotile[9].

Results and Discussion

Fermentation

For the production of the active compounds, *P. charlesii* ATCC 20841 was cultivated in seed media and transferred to either flasks or large trays for production.

The producing strain, *P. charlesii*, as soil or vegetative growth in 10% glycerol, was cultivated in the following seed medium: corn steep liquor 5 g, tomato paste 40 g, oat flour 10 g, glucose 10 g, and trace

Table 2. Physico-chemical properties and chromatographic behavior of compounds **1**~**8**.

Compound	Molecular formula	MW	UV λ_{max}^{MeOH}	log ε	K'[a]	Rf[b]
1	$C_{28}H_{35}N_3O_5$	493	225	4.50	1.8	0.51
2	$C_{27}H_{33}N_3O_4$	463	225		3.1	0.42
3	$C_{28}H_{33}N_3O_4$	475	225		7.9	0.42
4	$C_{28}H_{33}N_3O_5$	491	225		7.1	0.51
5	$C_{28}H_{35}N_3O_4$	477	225	4.40	4.0	0.35
6	$C_{28}H_{35}N_3O_3$	461	245	4.46	4.8	0.30
7	$C_{28}H_{35}N_3O_4$	477	245	4.35	2.2	0.44
8	$C_{28}H_{37}N_3O_5$	495	225		3.2	0.51

[a] Whatman ODS-3, 10 μm particles, 1.5 ml/minute, MeOH - 0.01 M H_3PO_4 (3 : 7), 40°C. Rt's given as K'.
[b] Whatman KC18F reversed phase TLC: MeOH - H_2O (8 : 2).

elements mix 10 ml (containing $FeSO_4 \cdot 7H_2O$ 1 g, $MnSO_4 \cdot 4H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 0.025 g, $ZnSO_4 \cdot 7H_2O$ 0.2 g per liter of distilled water) in 1 liter of distilled water. The pH of the medium was adjusted to 6.8. Media in unbaffled flasks (50 ml in a 250-ml Erlenmeyer flask) were incubated with 1 ml of frozen culture at 28°C with rotary agitation at 212 rpm for 48 hours.

The production medium in 250-ml Erlenmeyer flasks consisted of the following ingredients: Cracked corn 10 g, yeast extract 0.5 g, sodium tartrate 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, L-cysteine 0.1 g, glycerol 0.5 ml, $CoCl_2 \cdot 6H_2O$ 0.002 g, and 15 ml distilled water. The flasks were autoclaved at 121°C for 20 minutes. After autoclaving, 10 ml of distilled water was added followed by a second autoclaving at 121°C for 20 minutes. After inoculation of the production medium with 2 ml of the seed medium the batch was incubated without agitation for 7 or 14 days at 25°C. Alternatively 10 ml of the seed medium was used to inoculate the contents of 2,000 ml flasks (containing eight times the amount of production medium) or 800 ml for the large trays (80 × 50 × 5 cm), containing three hundred times the amount of medium.

Isolation

Ethyl acetate extracts of fermentations were chromatographed on silica gel, Sephadex LH-20 (Pharmacia) and reverse phase HPLC to yield paraherquamide and six novel analogs. All chromatographies were followed for activity by bioassay.

Ethyl acetate extracts of small scale fermentations (nominally 640 ml broth) were dried over Na_2SO_4 and concentrated to an oil under vacuum. After trituration with MeOH and filtration, the MeOH soluble portion was chromatographed on Sephadex LH-20 (200 ml) in MeOH. The fractions of interest (0.55 to 0.75 column volume (c.v.)) were concentrated to an oil under vacuum and further fractionated on a Silica gel 60 (E. Merck) column (500 ml) with hexane - CH_2Cl_2 - MeOH (5 : 5 : 0.5). Paraherquamide eluted last (3.6 to 4.72 c.v.), while the earlier fractions (2.8 to 3.3 c.v.) contained 3 minor components. All were isolated by repeated preparative HPLC on a Magnum 20 column (Whatman ODS-3) eluted at 10 ml/minute of 68% aqueous methanol employing a Gilson 116 UV detector with an integrator (SP 4100). 1.6 mg of the desoxy compound **2**, 2.1 mg of the anhydro compound **3**, and 0.7 mg of the epoxide compound **4** were isolated as well as 204 mg of compound **1**, and were evaluated by bioassay.

One production tray (nominally 12 liters broth) of culture ATCC 20841 was extracted several times with EtOAc. The pooled extracts were concentrated to a volume of 300 ml to which was added 1 volume of MeOH. The resultant oily precipitate was removed, washed with MeOH and discarded. The MeOH solutions were evaporated to an oily solid which was redissolved in acetonitrile and extracted four times with *n*-hexane. The resulting acetonitrile solution was again taken to dryness, redissolved in CH_2Cl_2 and fractionated on silica gel (Grace, EtOAc, 4-liter). The fractions of interest (4.25 to 6 c.v.) were taken to dryness, dissolved in MeOH and chromatographed on Sephadex LH-20 (4 liters) in MeOH. The active fractions (0.75 to 0.85 c.v.) were combined, dried and washed with cold EtOAc. The EtOAc insolubles were dissolved in acetone and charged to a 1-liter Silica gel 60 (E. Merck) column in hexane - acetone (2 : 1). 3.5 g of compound **1**, 180 mg of compound **5** and 12 mg of compound **7** were isolated at 3.1 ~ 4.4, 1.5 ~ 2.5, and 2.65 ~ 2.9 c.v., respectively. The EtOAc filtrate from above was dried, dissolved in acetone and the same procedure followed above to afford 28 mg of compound **6** which eluted at 2.3 to 2.4 c.v. on a 120-ml silica gel column.

During the course of isolation and analyses of fermentation study samples, it was noticed that fermentation conditions had a profound effect on production of the analogs **2** ~ **7**. Indeed, while

paraherquamide was produced equally well in both shake flask and tray batches, compounds **2**~**4** were the only analogs detected in small fermentations; **5**~**7** clearly predominated in the larger batches.

Physico-chemical Properties

The physico-chemical properties of paraherquamides (**1**~**8**) are summarized in Table 2. All are soluble in methanol, ethyl acetate, acetone and dimethyl sulfoxide; but are practically insoluble in water. They give a positive response to iodine vapor and 50% H_2SO_4. The molecular formula and MW of each was assigned based on its mass spectrum.

Biological Activity

While all seven natural products displayed some degree of activity vs. *C. elegans*, paraherquamide (**1**) was the most potent as can be seen in Table 1. The C-24, C-25 dihydro derivative (**8**) had the weakest activty. Alkyl substitution at C-14 and/or unsaturation in the dioxypino ring appears to be critical for good activity. The deshydroxy analog compound **5** is the most potent of the analogs suggesting the C-14 hydroxyl is not crucial for activity.

Paraherquamide was less active against *C. elegans* versus avermectin B_{1a}; but more active than the benzimidazoles[10]. Paraherquamide was demonstrated to be an effective anthelmintic agent and was well tolerated at high dosage levels in gerbils. No anthelmintic data on any structurally related compounds have been reported to date. Semi-synthetic derivatives of paraherquamide will be reported separately[11,12].

Experimental

Hydrogenation of Paraherquamide (**1**)

5 mg of paraherquamide (0.01 mmol) was dissolved in 2 ml MeOH with 10% by weight of 10% Pd/C and hydrogenation carried out under 2.8 kg/cm^2 at 23.8°C for 2.5 hours. The reaction was complete as determined by HPLC analysis. Purification was carried out by HPLC (Whatman ODS-3, 40% aqueous MeOH) yielding 4 mg of C-24, C-25 dihydroparaherquamide (**8**). Under the same conditions, WILKINSON's catalyst afforded only 30% conversion at the end of 2.5 hours.

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