Pterulinic Acid and Pterulone, Two Novel Inhibitors of NADH : Ubiquinone Oxidoreductase (Complex I) Produced by a *Pterula* Species

I. Production, Isolation and Biological Activities

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Pterulinic acid (**1**) and pterulone (**2**), two novel halogenated antibiotics, were isolated from fermentations of *Pterula* sp. 82168. Both compounds exhibited significant antifungal and weak or no cytotoxic activities. **1** and **2** are effective inhibitors of eucaryotic respiration. The target of the antibiotics resides within the mitochondrial NADH : ubiquinone oxidoreductase (complex I).

In a previous investigation of antifungal metabolites produced by submerged cultures of the basidiomycete *Pterula* sp. 82168, strobilurin A[1], hydroxystrobilurin A[2] and oudemansin A[3] were isolated[2]. The strobilurins and oudemansins constitute a class of respiration inhibitors which inhibit the electron transfer in the bc_1 complex (complex III)[4,5]. In the course of further fermentations of the same *Pterula* strain, two new antifungal antibiotics were found. In this paper, we describe the production, isolation, and biological properties of pterulinic acid (**1**) and pterulone (**2**). The elucidation of their structures is subject of a second publication[6].

Materials and Methods

Pterula Species 82168

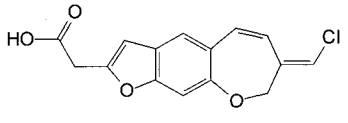
The strain and its maintenance have been described earlier[2].

General

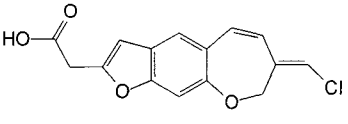
For analytical HPLC a Hewlett Packard 1090 series II instrument and for preparative HPLC a Gilson model 302 or Jasco PU-980 instrument were used.

Fermentation

Fermentations were carried out in 20 liters of YMG medium composed of (g/liter): yeast extract 10, maltose 4 and glucose 4, pH 5.5, in a Biolafitte C6 fermenter at 22°C with an aeration rate of 3.2 liters/minute and agitation (120 rpm). A well grown culture of *Pterula* sp. 82168 (250 ml) in the same medium was used as inoculum. During fermentation 100 ml samples were taken, the mycelia separated by filtration and the culture broth



(*E*)-1



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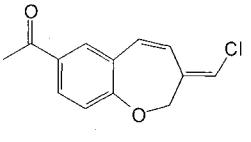

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† This article is dedicated to Prof. Dr. H.-G. KUBALL on occasion of his 65th birthday.

extracted with ethyl acetate. The residue obtained after evaporation of the organic solvent was taken up in 1 ml of methanol. The mycelia were extracted with methanol-acetone 1:1 and the crude extract taken up in 1 ml methanol. 10 μl of the concentrated solutions were assayed for antifungal activity in the agar plate diffusion assay with *Mucor miehei* as test organism. 10 μl were analyzed by analytical HPLC (Merck LiChrospher 100 RP-18, 5 μm; column 125 × 4 mm; flow: 1.5 ml/minute; gradient: H_2O-acetonitrile 0~50% in 15 minutes, 50~100% in 5 minutes; R_t pterulone = 16.8 minutes).

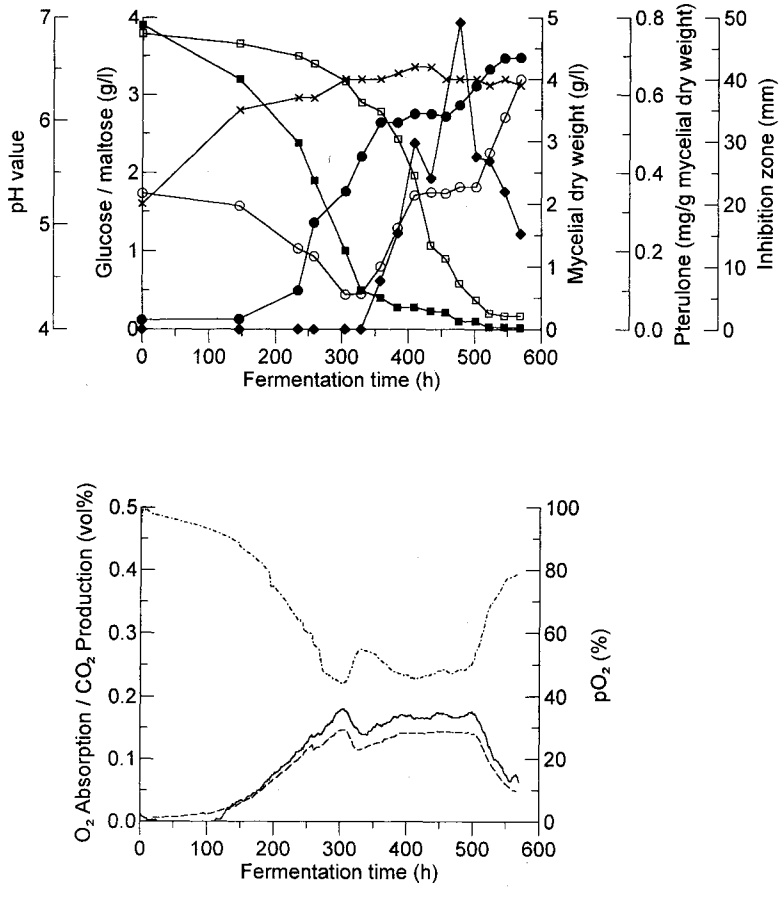
Isolation of Pterulinic Acid and Pterulone

After 20 days, when the content of pterulone in the mycelia was highest, the culture fluid (16 liters) was separated from the mycelia and passed through a column (30 × 6.5 cm) containing Mitsubishi Diaion HP 21 adsorber resin. The column was washed with water and the antifungal compounds (pterulinic acid, oudemansin, and strobilurins) eluted with two liters of acetone. The acetone eluate was concentrated and the crude product (900 mg) applied onto a column of silica gel (Merck 60; 0.063~0.2 mm; 30 × 2.9 cm). An enriched product (125 mg) was obtained by elution with cyclohexane-ethyl acetate 1:1. This was further purified by preparative HPLC on Merck LiChrosorb Diol [7 μm; column 250 × 25 mm; flow rate 5 ml/minute; gradient (% cyclohexane in *tert*-butyl methyl ether): 0~10 minutes, 30%; 10~40 minutes, 30~40%; 40~50 minutes, 40%; 50~80 minutes, 40~50%; 80~95 minutes, 50~100%; detection at λ = 210 nm, R_t pterulinic acid = 70 minutes]. Yield: 9.3 mg of pterulinic acid.

The lyophilized mycelia (65 g) were extracted twice with a total of 4 liters methanol-acetone 1:1. The crude extract (6.1 g) was applied onto a silica gel column (Merck 60, 0.063~0.2 mm; 30 × 6.5 cm). Upon elution with cyclohexane-ethyl acetate 7:3, 370 mg of an enriched extract were obtained. This was further purified by preparative HPLC on LiChrosorb Diol (7 μm; column 250 × 25 mm; flow rate 5 ml/minute; detection at λ = 210 nm) using a cyclohexane-*tert*-butyl methyl ether gradient: 0~20 minutes, 10%; 20~30 minutes, 10~20%; 30~80 minutes, 20%; 80~100 minutes, 20~100%. Pterulone (25 mg) was eluted after 40 minutes. Final purification of pterulone was achieved by preparative HPLC Merck LiChrospher RP-18 (10 μm; column 250 × 10 mm; flow rate 5 ml/minute), eluted isocratically with water-acetonitrile. Yield: 16 mg.

Fig. 1. Fermentation of *Pterula* sp. 82168 in 20 liters YMG medium.

○ pH; ■ glucose (g/liter); □ maltose (g/liter); ● mycelial dry weight (g/liter); ◆ pterulone concentration in mycelial extract (mg/g); × inhibition zone (mm) caused by 10 μl of culture filtrate extract, corresponding to 1 ml culture broth. ----- pO_2 (%); --- O_2 Absorption (vol%); —— CO_2 Production (vol%).



Test for Biological Activities

The assays for antimicrobial[7], phytotoxic[7], cytotoxic[8], nematicidial[9,10], and hemolytic[11] activities were carried out as described previously. The inhibition of respiration of *Penicillium notatum* was measured as described by WEBER *et al.*[12]. Mitochondria of bovine heart were prepared as reported[13,14]. The respiratory activities of isolated bovine heart mitochondria were measured with a Clarke-type oxygen electrode as described by BRANDT *et al.*[15].

Fig. 2. Oxygen uptake of germinated *Penicillium notatum* spores.

● Pterulinic acid; ◇ pterulone.

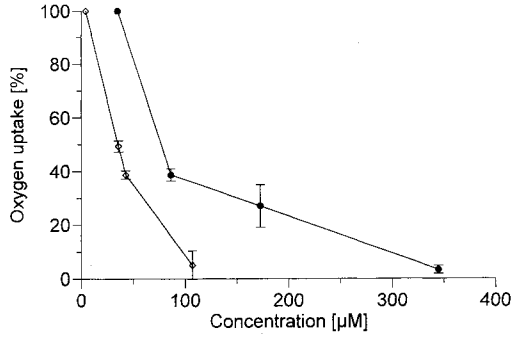
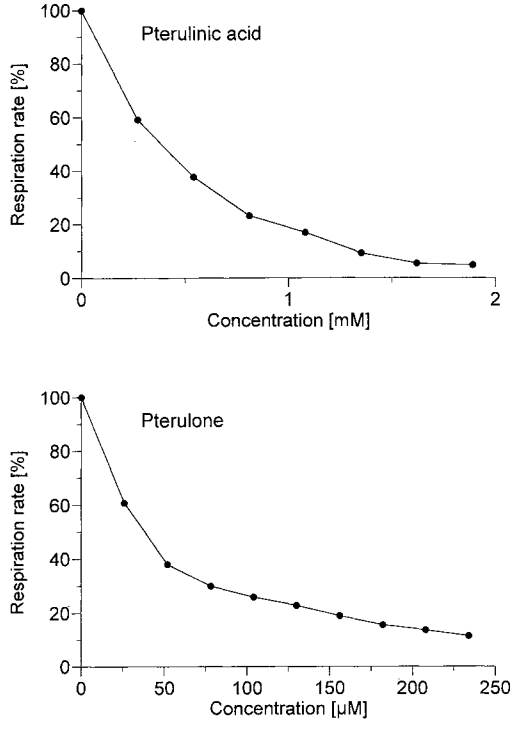


Fig. 3. Effect of pterulinic acid (**1**) and pterulone (**2**) on the respiration rate of isolated bovine heart mitochondria using NADH as substrate.



Results and Discussion

Production of Pterulinic Acid and Pterulone

A typical fermentation of *Pterula* sp. 82168 in YMG medium is shown in Fig. 1. During the fermentation the fungus consumed glucose first and then the maltose of the malt extract. Simultaneously, an increase in the mycelial dry weight was observed. The production of pterulone started after 12 days. The content of pterulone in the mycelia, as detected with HPLC, reached a maximum after 20 days. Pterulinic acid could not be quantified by HPLC in the crude extracts because of overlapping peaks.

Biological Properties

Oxygen uptake by freshly germinated spores of *Penicillium notatum* (30 mg wet weight/ml in 1% glucose solution) was inhibited 60% by 86 μM (25 μg/ml) of pterulinic acid (**1**) or 43 μM (10 μg/ml) of pterulone (**2**) and 99% by >50 μg/ml of **1** and 25 μg/ml of **2** (Fig. 2). **1** and **2** inhibited the respiration rate of bovine heart mitochondria using NADH as substrate with IC_{50} values of 450 μM (130.5 μg/ml) and 36 μM (8.4 μg/ml), respectively (Fig. 3). No effect on the respiration was observed using succinate as substrate. Since NADH is the electron donor for the NADH : ubiquinone oxidoreductase (complex I) and succinate is the electron donor for the succinate : ubiquinone oxidoreductase (complex II), we conclude that pterulinic acid and pterulone selectively

Fig. 4. Scheme of the mammalian mitochondrial respiratory chain and the target of pterulinic acid and pterulone.

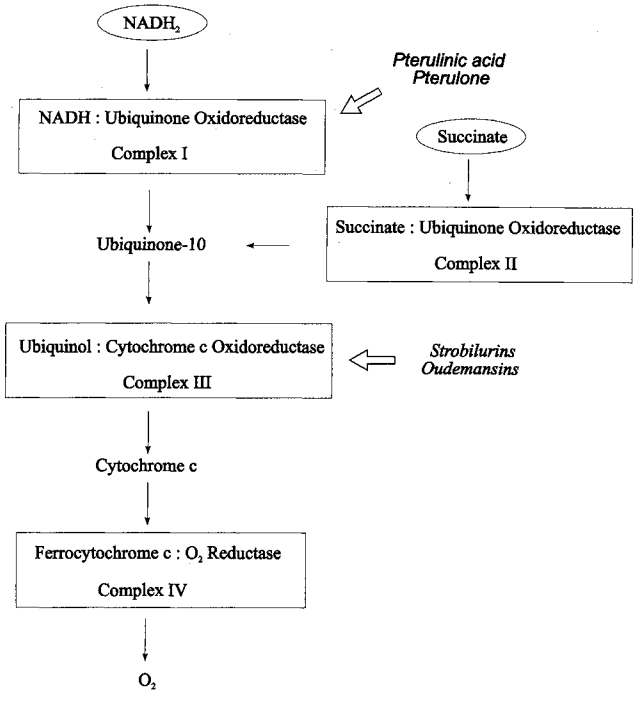


Table 1. Antifungal activity of pterulinic acid (**1**) and pterulone (**2**) in the agar diffusion assay.

Organism	Diameter of inhibition zone [mm]					
	1			**2**		
	1[a]	10[a]	100[a]	1[a]	10[a]	100[a]
Absidia glauca (+)	-	-	18i	20i	33i	N.T.
Absidia glauca (−)	-	-	11	-	12	30
Alternaria porri	-	-	11d	-	-	-
Ascochyta pisi	-	10d	20d	-	12d	23
Aspergillus ochraceus	-	-	9	-	10	20
Botrytis cinerea	-	-	12d	-	-	12d
Curvularia lunata	-	-	14	12	24	N.T.
Epicoccum purpurascens	-	-	12d	-	-	10d
Fusarium fujikuroi	-	-	12d	-	-	-
Fusarium oxysporum	-	-	9	-	-	10
Mucor miehei	-	-	22	-	20i	N.T.
Paecilomyces variotii	-	-	13	-	8	11d
Penicillium notatum	-	8	20i	-	13i	25i
Phoma clematidina	-	-	17	-	18	30
Pythium irregulare	-	13	16d	13	30	N.T.
Rhodotorula glutinis	-	-	12	-	-	14
Saccharomyces cerevisiae.is1[b]	-	10	N.T.	-	12	30
Ustilago nuda	-	23i	32i	-	19	N.T.
Zygorrhynchus moelleri	-	15	25i	-	13	19

-: No inhibition zone.
i: Incomplete.
N.T.: Not tested
[a]: μg/disc, [b]: gift of Prof. F. LACROUTE, Strasbourg.
d: diffuse inhibition zone

inhibit complex I, while the other components of the respiratory chain (complex II, III and IV) are not affected (Fig. 4). It is remarkable that unlike other strobilurin-producing basidiomycetes[16] *Pterula* sp. 82168 simultaneously produces two types of respiration inhibitors interfering with the electron flow at two completely different sites.

Table 1 shows the antifungal activities of pterulinic acid (**1**) and pterulone (**2**) in the plate diffusion assay. **2** exhibits strong antifungal activities at concentrations starting at 1 μg/disc which compare favorably with those of **1**, starting at 10 μg/disc.

The markedly reduced activity of **2** in the serial dilution assay (Table 2) further supports that its prime target is within the respiratory chain. It is well known that fungi exposed to respiration inhibitors in liquid media containing sugars can compensate their energy demands needed for growth to some extent by glycolysis or by the use of alternate electron pathways. In the serial dilution assay **1** exhibits some activity on *Saccharomyces cerevisiae*, which is known to contain only a non proton pumping NADH : ubiquinone oxdioreductase[17]. This seems to indicate that at higher concentrations **1** affects the growth by some other, so far unknown mode of action. The activity of **1** is considered much less selective. In the serial dilution assay both compounds showed no antibacterial activities at concentrations up to 100 μg/ml (*Acinetobacter calcoaceticus*, *Escherichia coli* K12, *Salmonella typhimurium*, *Bacillus brevis*, *Bacillus subtilis*,

Table 2. Antimicrobial spectrum of compounds **1** and **2** in the serial dilution assay.

Organism	MIC (μg/ml)	
	1	**2**
Yeasts:		
Nadsonia fulvescens	25	> 100
Nematospora coryli	> 100	> 100
Saccharomyces cerevisiae S288c	50	> 100
Saccharomyces cerevisiae is1	25	> 100
Rhodotorula glutinis	100	100
Filamentous fungi:		
Fusarium oxysporum	> 100	> 100
Mucor miehei	25	5
Paecilomyces variotii	50	> 100
Penicillium notatum	50	> 100

Table 3. Cytotoxic activities of pterulinic acid (**1**) and pterulone (**2**) towards mammalian cell lines.

Cell line	IC_{50} [μg/ml]	
	1	**2**
L1210	50	> 100
HL 60	20	> 100
BHK	100	> 100
Hela S3	25	> 100

Corynebacterium insidiosum, *Micrococcus luteus*, *Mycobacterium phlei*, *Streptomyces* sp. ATCC 23836).

1 exhibited weak cytotoxic activities towards L1210 and BHK cells but showed moderate toxicity towards HL60 and Hela S3 cells (Table 3). In contrast, **2** was not cytotoxic against L1210, HL60, BHK and Hela S3 cells at concentrations up to 100 μg/ml. **1**, but not **2**, has phytotoxic activities, the germination of *Lepidium sativum* and *Setaria italica* being inhibited at concentrations of 10~50 μg/ml. Both compounds showed no nematicidal activity against *Meloidogyne incognita* and *Caenorhabditis elegans*, and no hemolytical effects at concentrations up to 100 μg/ml.

Acknowledgement

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