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Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities

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Abstract

Isopestacin is an isobenzofuranone obtained from the endophytic fungus *Pestalotiopsis microspora*. While a few other isobenzofuranones are known from natural sources, isopestacin is the only one having a substituted benzene ring attached at the C-3 position of the furanone ring. The compound was isolated from culture broths of the fungus and crystallized and its structure was determined by X-ray crystallography. Both proton and carbon NMR spectral assignments are also reported for isopestacin. This compound possesses antifungal activity and, as measured by electron spin resonance specroscopy, it also behaves as an antioxidant scavenging both superoxide and hydroxy free radicals. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Isobenzofuranone; Antioxidant; Antimycotic; Endophyte; Fungus; X-ray; NMR

1. Introduction

An isolate of *Pestalotiopsis microspora* was obtained as an endophyte from a combretaceaous plant—*Terminalia morobensis* growing in the Sepik river drainage of Papua New Guinea (Womersley, 1995). Strains of this fungus are some of the most commonly isolated endophytic fungi associated with rainforest plants (Strobel, 2002). These strains differ widely in the degree and diversity of secondary metabolites that they may produce. Bioactive compounds such as taxol, jesterone, ambuic acid, torreyanic acid, pestaloside, pestalotiopsins and 2-α-hydroxydimeniol are but a few examples that illustrate this point (Strobel, 2002). However, the isolate of *P. microspora* studied in this report, showed both antimycosis, and antioxidant effects. Both bioactivities are seldomly observed in *P. microspora*, regardless of the origin of the culture. Herein, is described and presented the isolation and characterization of one component of the culture fluid, isopestacin. Antioxidant activity of isopestacin against a variety of free radicals is given together with details regarding its antimycotic activity.

2. Results and discussion

The isolate of *P. microspora* used in this study was obtained as an endophyte from a stem of *Terminalia morobensis*. It was identified on the basis of its 5-celled conidium possessing characteristic appendages (Worapong, 2001). Its ITS1, 5.8S, and ITS2 sequences have been deposited in GenBank as AF 377301 and the relationship of this fungus to other *Pestalotia* spp. and *Pestalotiopsis* spp.on the basis of genetic and structural characteristics have been described by Worapong (2001). This particular isolate of *P. microspora* was of interest because of its antifungal properties, as well as the multitude of secondary products that it was producing. The culture was designated as 12–30 and stored as hyphae and mycelia in 15% glycerol at −70 °C in the Montana State University mycological collection.

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The fungus was grown on the M1D medium with the culture fluid extracted with 2 equal volumes of methylene chloride (Strobel et al., 1996). Biological activity against *Pythium ultimum* was used to guide purification of a product that was crystallized after purification by silica gel column chromatography. The product was shown to be homogeneous by thin layer chromatography in a number of solvent systems. The yield of this product was about 20 mg/l of culture medium. After structural elucidation by X-ray crystallography, the compound was shown to be an isobenzofuranone and was termed, isopestacin **1**.

Compound **1** appears as slightly yellowish crystalline plates. HREIMS of **1** revealed a MW for this compound at 270.051292. The best fit calculated mass for this molecular species is 270.052824, which accounts for $C_{15}H_{10}O_5$.

The structure of isopestacin **1** was solved by X-ray crystallographic analysis. Isopestacin **1** was found to have crystallized as two molecules in an asymmetric unit (see Figs. 1 and 2). Carbon shifts for structure **1** were established using the 2D INADEQUATE analysis. The NMR spectral analysis primarily emphasized obtaining unambiguous shift assignments as reference data rather than for structural elucidation. Additional structural information needed to complete NMR shift assignments (i.e. heteroatom positions and connections) was obtained from the X-ray analysis. Complete ^{1}H assignments were made from HMQC analysis. Longer- range proton carbon correlation experiments (i.e. HMBC) were unnecessary for assignments as all connections were known unambiguously from INADEQUATE or X-ray analyses. Complete NMR shift assignments are given in Table 1.

Fourteen 3,5,7-substituted isobenzofuranones have been previously isolated as natural products from such sources as fungi, liverworts and higher plants (Arone et al., 1989, 1990; Asakawa et al., 1986; Dekker et al., 1997; Kraut et al., 1994). The substituents at positions 5 and 7 are always either –OH, $-CH_3$, or $-OCH_3$ functionalities. Likewise, there are a variety of different substituents at

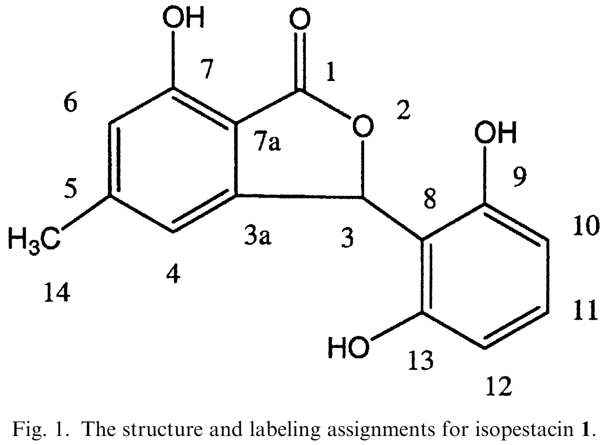

Fig. 1. The structure and labeling assignments for isopestacin **1**.

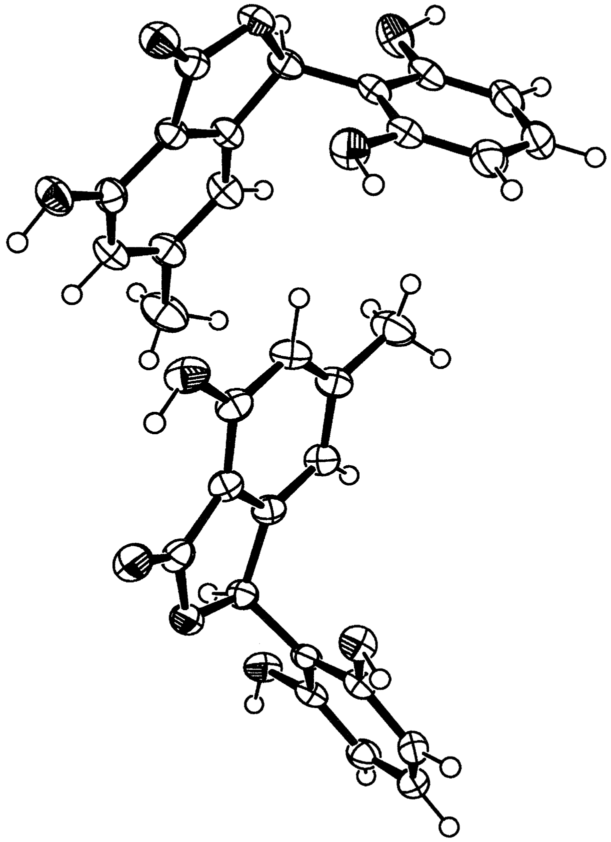

Fig. 2. The structure and labeling assignments for isopestacin **1**. The arrangement of two molecules of isopestacin **1** in the asymmetric unit of the crystal.

Table 1
Isopestacin NMR spectral shift assignments

Position	$\delta^{13}C$[a]	$\delta^{1}H$[b]
1	173.84	–
3	77.24	6.92 (*s*)
3a	154.87	–
4	114.54	6.52 (*s*)
5	149.00	–
6	116.53	6.64 (*s*)
7	157.33	–
7a	111.71	–
8	110.32	–
9	158.95[a]	–
10	107.96[b]	6.29 (*d*, J=8.2 Hz)
11	131.53	6.96 (*t*, J=8.2 Hz)
12	107.96[a]	6.27 (*d*, J=8.2 Hz)
13	158.95[b]	–
14	22.14	2.3 (*s*)

[a] These isotropically degenerate peaks were demonstrated to contain two carbons by integration of a 1D ^{13}C spectrum collected with ^{1}H decoupling present only during the free induction decay and a 20 s recycle time.

[b] These isotropically degenerate peaks were demonstrated to contain two carbons by integration of a 1D ^{13}C spectrum collected with ^{1}H decoupling present only during the free induction decay and a 20 s recycle time.

position C-3 however the direct attachment of a substituted benzene ring at this location has no precedent. Therefore, isopestacin **1**, having the basic structural features of an isobenzofuranone, possesses a 3-benzo-substituent, making it novel.

Structural similarities of isopestacin to the flavonoids suggest that **1** may possess antioxidant activity. Testing confirms that isopestacin **1** indeed has antioxidant activity as determined by ESR measurements described in the Experimental. The compound is able to scavenge superoxide $O_2^-\cdot$ and OH· radicals in solution. The superoxide was generated in our experimental set-up by the action of hypoxanthine on water and oxygen via the catalysis of xanthine oxidase. On the other hand, OH· was generated via the Fenton reaction of hydrogen peroxide on ferrous iron. Simultaneously, EPR spectra are recorded of the free electrons associated with the free radicals. Since **1** has a very low solubility in water we dissolved it in a small amount of methanol in prior to its placement in the reaction mixture. The spin trapper EMPO was used to trap $O_2^-\cdot$, and the effect of Vitamin C (ascorbic acid), a well known scavenger of free radicals along with **1** under similar conditions, was studied. Thus, in order to reduce the free radical $O_2^-\cdot$ in the sample by 50%, a concentration of 0.013 mM of Vitamin C was needed. In a comparable experiment, at least 0.185 mM of **1** was required to produce a 50% reduction of the ESR signal. Thus, ascorbic acid is at least 14.2 times as effective as **1** as an antioxidant for scavenging the superoxide radical. It should be noted that since superoxide was generated via the xanthine oxidase reaction it was not possible to accurately calculate the exact molar concentration of $O_2^-\cdot$ in these reactions. However, **1** is extremely capable of scavenging the hydroxyl free radical (OH·) with 0.22 mM required to reduce this free radical (0.18 mM OH·—chemically generated) by 50%. The ability of **1** to scavenge OH· is comparable to that of ascorbic acid. This is an important biological and chemical property of **1** since OH· is believed to be more detrimental to cell death directly than $O_2^-\cdot$. On the other hand, **1** has no effect in reducing the lipid free radical $LOO^-\cdot$.

Isopestacin **1** is moderately antimycotic, with total inhibition of *Pythium ultimum*, a plant pathogenic oomycete, at 40 μg/ml at 48 h. This activity compares with a value of 0.03 μg/ml of oocydin A on *P. ultimum* (Strobel et al., 1999). However,**1** has no inhibitory effect on some other plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. The culture fluid of this isolate of *P. microspora* also appears to contain several other compounds that are more antifungal than **1**. These more active constituents appear later in the culture period of this organism (i.e. 4–5 weeks) and in much smaller quantities than **1**. Preliminary NMR spectroscopic studies suggest that these compounds are related to **1**. Besides these compounds, this isolate of *P. microspora* contains the greatest array of secondary metabolites of any of the myriad of *P. microspora* isolates yet examined. For instance, the crude methylene chloride extract, besides possessing antimycotic and antioxidant activity, also has antimalarial and general antibiotic activities, indicating the presence of additional potentially novel compounds.

3. Experimental

3.1. *P. microspora and isopestacin isolation*

An isolate of *Pestalotiopsis microspora* was obtained as an endophyte associated with *Terminalia morobensis* located in the Sepik river drainage of Papua New Guinea (Womersley, 1995). The host plant of this fungus was collected on the shores of the Karawari River on the north coast of Papua New Guinea at 4° 23′ 39″South and 143 ° 18′ 49″East. *P. microspora,* isolate 12–30, was only one of many endophytes recovered from this tree. It was acquired from a section of a small tree stem (0.75 cm in dia) using previously described techniques (Strobel et al., 1996). This fungal culture was ultimately purified from surface treated limb pieces that had been plated on water agar. After fungal growth from these pieces, hyphal tips of the fungi were removed and plated on potato dextrose agar.

P. microspora was grown in 500 ml volumes in 2 l flasks for 35 days at 23 °C under still culture conditions on the M1D medium (Strobel et al., 1996). The mycelium of the fungus was separated from the culture fluid by passing the culture through 4 layers of cheesecloth. The filtrate was extracted with 2 equal volumes of CH_2CL_2 and ultimately taken to dryness by flash evaporation. Approximately 90 mg of residue remained with no more than 50 mg of the residue $CHCl_3$ soluble. This $CHCl_3$ solution was placed on a silica gel column (1×8 cm), and the column was rinsed with 25 ml of chloroform, followed by 25 ml of a mixture of chloroform: ethyl acetate; 10:1 v/v, and then 25 ml of chloroform: ethyl acetate; 5:1 v/v, respectively. Fractions (5ml) were collected and those containing compound **1**, as checked by thin layer chromatography were saved, pooled and applied to a second silica column (1×8 cm). Pure compound **1** appeared in these fractions eluted with chloroform: ethyl acetate (10:1, v/v). The fractions were pooled, dried and redissolved in a minimum volume of chloroform and allowed to slowly evaporate at 23 °C until crystals appeared. Purity of the compound was determined by thin layer chromatography in multiple systems.

3.2. *Thin layer chromatography*

Thin layer chromatography of fungal fermentation products was performed on Merck silica gel plates (0.25

mm thick) using various solvent systems. Each system is indicated along with the respective R_F value of **1** in it as follows: chloroform: methanol 10:1 v/v ($R_F = 0.28$); chloroform: acetonitrile 6:3 v/v ($R_F = 0.28$); chloroform: ethyl acetate 9:1 v/v (R_F 0.03); isopropanol: ethyl acetate 9:1 v/v ($R_F = 0.83$); methylene chloride: *n*-butanol 5:1 v/v ($R_F = 0.81$); ethyl acetate: tetrahydofuran 3:1 v/v ($R_F = 0.83$) and chloroform: methanol: acetic acid 10:1: 0.1 v/v/v ($R_F = 0.40$). Compound **1** could be detected by its absorption under a short-wave UV lamp and by the bright red coloration obtained after spraying with vanillin in sulfuric acid reagent followed by gentle heating (Cardellina, 1991).

3.3. Isopestacin *1*

Isopestacin **1** was crystallized by slow evaporation from a chloroform solution at room temperature; mp (uncorrected) 218–220 °C. UV λ max (methanol) (log ϵ): 214 (3.57) 247 (3.63) 287 (3.59) 298 (3.55). HR CIMS (*m/z*): 270.051292 calc. for $C_{15}\ H_{10}\ O_5$: 270.052824. Isopestacin crystallizes in a centrosymmetric space group (i.e. P21/c) and thus, consists of a racemic mixture of the 3S and 3R enantiomers. No optical rotation was therefore observable for isopestacin.

3.4. X-ray crystallography

A colorless plate shaped crystal 0.32×0.23×0.15 mm in size was mounted on a glass fiber with traces of viscous oil and then transferred to a Nonius Kappa CCD diffractometer equipped with Mo$K\alpha$ radiation ($\lambda = 0.71073$ Å). Ten frames of data were collected at 200±1 K with an oscillation range of 1°/frame and an exposure time of 20 s/frame (Nonius, 1998). Indexing and unit cell refinement based on all observed reflection from those ten frames, indicated a monoclinic *P* lattice. A total of 9451 reflections ($\lambda_{max} = 27.5°$) were indexed, integrated and corrected for Lorentz polarization and absorption effects using DENZO-SMN and SCALEPAC (Otwinowski and Minor, 1997). Post refinement of the unit cell gave $a = 11.8852(2)$ Å, $b = 8.1240(3)$ Å, $c = 26.9689(8)$ Å, $\beta = 101.8214(19)$, and $V = 2548.76(13)$ Å^3. Axial photographs and systematic absences were consistent with the compound having crystallized in the monoclinic space group $P\,2_1/c$.

A combination of direct methods and heavy atom methods were employed using SIR 97 (Altomare et al., 1997). All of the non-hydrogen atoms were refined with anisotropic displacement coefficients. Hydrogen atoms were located and refined isotropically using SHELXL97 (Sheldrick, 1997). The weighting scheme employed was $w = 1/[\sigma^2(F_o^2) + (0.0399P)^{2+}0.6937P]$ where $P = (F_o^2 + 2F_c^2)/3$. The refinement converged to $R1 = 0.0462$, $wR2 = 0.0934$, and $S = 1.018$ for 3866 reflections with $1 > 2\sigma(I)$, and $R1 = 0.0825$, $wR2 = 0.1071$, and $S = 1.018$ for 5716 unique reflections and 459 parameters. In this case, $\mathrm{R1} = \Sigma(\|F_o|-|F_c\|)/\Sigma|F_o|$, $\mathrm{wR2} = \left[\Sigma\left(\mathrm{w}\left(F_o^2 - F_c^2\right)^2\right)/\Sigma\left(F_o^2\right)^2\right]^{1/2}$, and S = Goodness-of-fit on $F^2 = \left[\Sigma\left(w\left(F_o - F_c^2\right)^2/(n-p)\right)\right]^{1/2}$, where n is the number of reflections and p is the number of parameters refined. The maximum Δ/σ in the final cycle of the least-squares was <0.001, and the residual peaks on the final difference-Fourier map ranged from −0.204 to 0.229 e/Å^3. Scattering factors were taken from the International Tables for Crystallography, Volume C (Maslen et al., 1992). Structural figures were prepared using ORTEP3 for windows (Farrugia, 1997). A complete list of refined coordinates together with other revelant data are deposited at the Cambridge Crystallographic Centre.

3.5. NMR spectroscopic analyses

A 2D INADEQUATE spectroscopic analyses was performed on **1** (57mg) dissolved in CD_3OD (0.2 ml), using a Varian INOVA 500 MHz spectrometer operating at 125.76 MHz, and using CD_3OD as reference. All ^{13}C assignments were established using a computer program designed to enhance signal detection (Dunkel et al., 1990, 1992).

The HMQC analysis was performed on **1** (8 mg) dissolved in CD_3OD, with analysis being performed on a Varian INOVA 500 MHz spectrometer using a Varian indirect detection probe at an operating frequency of 500.62 MHz. The spectrum was acquired without ^{13}C decoupling and all signals were evaluated visually.

3.6. Electron spin resonance measurements

EPR techniques used in this work are primarily those reported by Sheu et al. (2000) and Wen and Fung (2000). All EPR spectra were recorded on a Bruker model ESP 300E spectrometer, X-band operating at a microwave frequency of 9.787 GHz, a microwave power of 10 mW, a modulation frequency of 100.0 kHz, and a modulation amplitude of 0.367 mT. The magnetic field range was 334±80 mT, a time constant of 40.96 ms, a receiver gain of 2×10^4 and a magnetic fieldsweep rate of 0.48 mT/s. The magnetic field spacings are similar to those reported by Zhang et al. (2000). The superoxide $O_2^-\cdot$ was generated by the action of hypoxanthine or xanthine on water and oxygen through the catalysis of xanthine oxidase. The hydroxyl free radical $OH\cdot$ is generated by the well-known Fenton reaction: $Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH\cdot$, where 0.09 mM ferrous sulfate and 0.18 mM H_2O_2 were used. All enzymes and their respective substrates were purchased from Sigma Chemical Co., while other reagents were acquired from Calbiochem, and Life-Tech. The reaction mixture was transferred to an EPR flat tube for signal detection. Also placed in the tube was a spin trap compound 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) to trap the superoxide. On the other hand, 2-diethoxyphosphoryl-2-methyl-3, 4-dihydro-2H-pyrrole-1-oxide

(DEPMPO) (Zhang et al., 2000) was used to trap the hydroxyl free radical (Zhang et al., 2000). Test compounds were added to the tube and their effects on signal reduction (antioxidant activity) were monitored. Because of the water insolubility of **1** it was necessary to dissolve it first into a small amount of methanol and then add it to the reaction mixture. Signal intensity was plotted as a function of antioxidant concentration to get an estimate of the amount of compound needed to reduce either the superoxide or the free radical hydroxide concentration by 50%.

3.7. *Antimycotic activity*

The concentration of **1** required to give a 100% reduction of growth of a test fungus over a course of two days was determined by dissolving **1** in 1 ml of potato dextrose broth (PDB). An agar plug, 1 cm dia, containing the test fungus was then added to the broth and the organisms incubated at 23 °C for 48 h. At that time the plate containing the fungi was examined visually for growth and the concentration of compound reducing growth by 100% was recorded. Fungi tested were each plant pathogenic and included an oomycete — *Pythium ultimum*, an ascomycete — *Sclerotinia sclerotiorum*, and a basidiomycete- *Rhizoctonia solani*.

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