METABOLITES FROM THE MARINE FUNGUS *CLADOSPORIUM CLADOSPORIOIDES*

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Abstract

p-Methylbenzoic acid and peroxyergosterol were isolated and identified by spectroscopic methods from cultures of the fungus Cladosporium cladosporioides isolated from a marine sponge. To the best of our knowledge, this is the first time that p-methylbenzoic acid had been isolated as a natural product.

Resumen

El ácido p-metilbenzoico y el peroxiergosterol fueron aislados e identificados por métodos espectroscópicos en el cultivo del hongo Cladosporium cladosporioides, obtenido de una esponja marina. En la medida de nuestro conocimiento este es el primer informe del aislamiento como producto natural del ácido p-metilbenzoico.

Introduction

The search for new drugs from fungi started with the discovery of penicillin [1], a potent antibiotic against Gram-positive bacteria, which was produced by *Penicillium notatum*. Other important therapeutic agent is the immunosuppressant cyclosporine, which is produced by *Tolypocladium inflatum* and *Cylindrocarpon lucidum* [2] that it was first used as an antifungal metabolite and later found to be immunosuppressive [3]. These two compounds together with the antifungal agent griseofulvin being isolated from *Penicillium griseofulvum* [4] and the cholesterol biosynthesis inhibitor lovastatin isolated from *Aspergillus terreus* [5] are examples supporting today's great interest in new secondary metabolites from fungi. Marine fungi do not represent specific Taxa, but are a group defined by their ecology and physiology. They can be divided into obligate and facultative marine fungi. Kohlmeyer [6] defined obligate marine fungi as those "that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine are fungi from freshwater or terrestrial areas also able to grow in the natural marine environment."*Cladosporium* (Hyphomycetes; mitosporic fungi) is the most frequently found genus of fungi in outdoor air in temperate climates. It has been isolated from many different types of soil and is a major colonizer of plant litter. It is found indoors a swell, but usually in less numbers, unless there is an indoor source of contamination. Indoors this fungus is often encountered

in dirty refrigerators, especially in reservoirs where condensation is collected. The ability to sporulate heavily, ease of dispersal, and buoyant spores makes this fungus an important fungal allergen. Due to its ability to rapidly invade many different ecological niches (including marine habitat), *Cladosporium* is ubiquitous and therefore sometimes problematic. From *C. cladosporioides* [7] and *C. tenuissimum* [8] it has been isolated lactones such as cladospolide A, B and C. Cladospolide A and B were shown to be phytotoxic [8]. From *C. herbarum* it has been described the isolation of a new 12-membered macrolide which contain a side chain with sulfur and a new macrolide dimer [9]. In our search for new interesting secondary metabolites, we collect, isolate and determine species of marine-derived fungi, improve the cultivation for a productive fermentation in a 10 or 20-liter scale and characterize the obtained metabolites. In a chemical analysis of *C. cladosporioides*, we now report the isolation of peroxyergosterol and p- methyl benzoic acid.

Results and Discussion

From sponges and other marine organisms (molusks and algae), which were collected on the Chilean coast, several fungi strain were purified. The strains were cultured in small scale (500 mL) liquid medium, filtered and extracted separately the mycelium and the filtrate with dichloromethane-methanol (1:1) mixture and ethyl acetate, respectively. The organic extracts were tested on larvae of *Artemia salina,* a small crustacean highly sensitive to chemicals (results not informed). As *Cladosporium cladosporioides* shown the greatest activity, it was cultivated with agitation during 20 days at 20° C and then filtered. After partitioning with organic solvents, a crude organic extract was obtained. Fractionation of the extract by chromatographic methods (Sephadex LH-20 column chromatography, followed by normal phase, HPLC) the compounds **1** and **2** were obtained.

Compound **1** was obtained as a white crystal. Its molecular formula was determined by a combination of NMR data and EIMS to be $C_8H_8O_2$. Thus, compound **1** has four degrees of unsaturation. Its structure was established through 1D (^1H, ^{13}C, and DEPT) and 2D (COSY and HMBC) NMR. The IR absorption at 3450, 3440-3100 and 1699 cm^{-1} were consistent with both oxygenated functionalities: one hydroxyl and one acid carbonyl. The IR spectrum of compound **1** shown a wide absorption band at 3200- 2800 cm^{-1} and a strong band at 1700 cm^{-1} which were assigned to a carboxylic acid. The ^{13}C NMR and DEPT spectra revealed only 6 carbon atoms: one carbonyl, four aromatic carbons, and one methyl group. Of the aromatic carbons, two were unsubstituted, and of the substituted carbons no one was oxygen-bound. The signal at δ 172.0 was assigned to a carboxylic acid. At δ 130.0 a signal was observed which belong to aromatic carbons orto to a carboxylic group. The signal at δ 129.1 (CH) was assigned to carbons meta with respect to a carboxylic group. A sp^3 carbon appeared at δ 20.9 correspond to a methyl attached to an aromatic system. The ^1H NMR spectrum showed two pairs of orto coupled protons at δ 7.75 (J = 7.6 Hz) and δ 7.19 (J = 7.6 Hz), two protons each, a broad singlet at δ 8.60 (1H) and a methyl group at δ 2.35 (s,3H). Therefore, compound **1** was identified as *p*-methylbenzoic acid by comparison of the spectral data with published values [10]. This compound is well known and it could be a solvent contaminant or it could be present in the growth media. A careful blank growth experiment and extraction procedure allowed to discard these possibilities. To our best knowledge, this is the first report of isolation of compound **1** from any microorganism or living life .

Compound **2** was obtained as a white crystals and by analysis of its EIMS, NMR spectra and physical data was shown to be the ergosterol peroxide (5α, 8α- epidioxyergosta-6,22-dien-3β-ol) [11]. Ergosterol peroxide is also frequently obtained from fungi and it has been suggested that it is an important metabolite in the biosynthesis of ergosterol [12]. It is a known natural product which has been obtained from a variety of fungi, lichens, sponges and marine organisms [13,14,15]. It was reported that ergosterol peroxide from *I. obliquus* not only inhibited the growth of cancer cells but also killed them in anti-tumor tests. Ergosterol peroxide showed a potent inhibition on lipid peroxidation and exhibited higher antioxidant activity than well-known antioxidants, α-tocopherol and thiourea. A recent study also revealed its inhibitory effects on induced inflammation and tumor promotion in mouse skin [16].

Compound **1** and ergosterol peroxide were inactive when tested against Gram- positive and Gram- negative bacteria in agar plate diffusion assay. Both compounds were inactive against *Artemia salina*.

Experimental Section

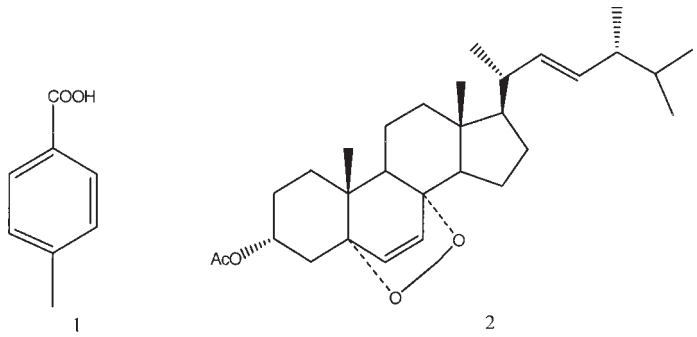
General experimental procedures. ^{1}H and ^{13}C NMR, HMQC, HMBC, NOESY and ^{1}H-^{1}H COSY spectra were measured employing a Bruker AMX-300 instrument operating at 300 MHz for ^{1}H-NMR and at 75 MHz for ^{13}C NMR. Mass spectra were recorded on a V.G. Micromass, ZAB-2R. Infrared spectra were measured on a Bruker IFS-25 spectrometer. Merck Si gels 7734 and 7741 were used in column chromatography. The spray reagent for TLC was H_2SO_4-H_2O-AcOH (1:4:20).

Animal Material-Samples of the marine sponge *Cliona sp.* were collected by scuba diving (-20 m) in the IV Region (Los Molles), Chile in april of 2004. A voucher fragment is kept under registration, Universidad de Chile. Under sterile conditions, a piece of tissue from the inner part of the freshly collected sponge was cut and inoculated on malt agar slants consisting of 2% agar, 0.5% glucose, 0.5% yeast extract in filtered sea water. The inoculated agar slants were incubated at 20 °C, and from these a pure fungal culture was isolated after repeated inoculation on fresh malt agar plates. The Prof. E. Piontelli, Universidad de Valparaiso, Valparaiso, Chile, identified the isolated fungus as *Cladosporium cladosporioides*. Mass cultivation of the fungus was carried out in Erlenmeyer flasks in malt extract broth consisting of 15 g/L malt extract in distilled water supplemented with 24.4 g/L artificial sea salt mixture. After 20 days incubation at 20°C with shaking, the fermentation broth was filtered under suction, obtaining a mycelium and the broth (filtrate).

Extraction and Isolation. The filtrate was partitioned with EtOAc. This extract (2.0g) was fractionated by column chromatography with Sephadex LH-20 using methanol as solvent system. Some fractions were subjected to column chromatography with medium pressure employing *n*-hexane–EtOAc (6:4) as a solvent, yielding compounds **1** (120 mg) and **2** (90 mg). The mycelium collected was extracted with CH_2Cl_2 – MeOH (1:1). This extract was evaporated under reduced pressure and then chromatographed yielding compound **2** (14 mg).

Compound **1**. White crystals, mp. 180° - 181° C. IR (KBr) ν max, 3200-2800, 1700, 1390, 1200, 765 cm^{-1} ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 8.60 (bs, 1H); 7.75 (2H, d, J = 7.6 Hz), 7.19 (2H, d , J = 7.6 Hz) and 2.35 (s, 3H) ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 172.0, 142.9, 130.0, 129.1, 127.6 and 20.9. m/z (EI): 136.1 (M^{+}84%), 119 (68%), 91 (100).

Compound **2.** White crystals, mp 178°- 179° C. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ:0.61 (s), 0.66 (s) 0.91(d, J=6.7 Hz), 0.99 (d, J=6.8 Hz), 1.00(d, J=6.5), 3.92(m), 5.14(dd, J=8.4, 15.3 Hz), 5.25(dd, J=7.6, 15.3 Hz), 5.95(d, J=8.4 Hz), 6.29 (d, J=8.4 Hz). ^{13}C-NMR($CDCl_3$, 75 MHz): δ12.9, 17.5, 18.7, 19.6, 19.9, 20.7, 20.9, 23.4 28.6, 30.1, 33.0, 34.7, 36.9, 39.4, 39.4, 39.7, 42.8, 44.6, 51.2, 51.7, 56.3, 66.3 , 79.4, 82.7, 130.7, 132.3, 135.2, 135.4 m/z (EI): 428 (M^+), 410, 396, 363, 337.



1 2

Compounds 1 and 2

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