SECONDARY METABOLITES FROM MARINE PENICILLIUM BREVICOMPACTUM

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ABSTRACT

In a screening of Basidiomycete cultures isolated from marine invertebrates collected along the Chilean coastline for the production of antibiotics we identified a *Penicillium brevicompactum* strain as a producer of metabolites inhibiting the growth of bacteria and fungi. Bioactivity guided purification resulted in the isolation of four known metabolites. Their structures were elucidated by spectroscopic methods.

Keywords.- *Penicillium brevicompactum*, metabolites

INTRODUCTION

For more than two decades, there has been an ongoing quest to discover new drugs from the sea. Most efforts have been directed to words chemical studies of marine invertebrates. Although these studies have indeed proven that marine invertebrates are an important source of new biomedical leads, a fact well demonstrated by the number of compounds currently in clinical trials, it has proven notoriously difficult to obtain adequate, reliable supplies of these compounds from nature.

Because of these problems, a new avenue of study focusing on marine microorganisms has been garnering considerable attention. Although marine microorganisms are not well defined taxonomically, preliminary studies indicate that the wealth of microbial diversity in the world's oceans, make this a promising frontier for the discovery of new medicines.

Unlike marine in vertebrates which can be dearly defined, there has been some debate as to

what constitutes a marine microorganism. Marine bacteria are most generally defined by their requirements of seawater, or more specifically sodium for growth. In the case of marine fungi, which in general do not display specific ion requirements, obligate marine species are generally considered to be those that grow and sporulate exclusively in a marine habitat (1). Although such definitions can prove useful, they tend to select for a subset of the microorganisms that can be isolated from any one environment.

This problem is compounded in the case of near - shore or estuarine samples where a large percentage of the resident microbes are adapted to varying degrees of marine exposure. For the purpose of microbial drug discovery, it seems only logical to study all microbes that can be isolated from the marine environment. Based on the species studied, most of the new compounds reported from marine microorganisms were obtained from species that can, in principle, be isolated from both land and sea. Although these facultative marine species are clearly a good source of novel metabolites, their ecological roles and degrees of adaptation to the marine environment remain largely in known. In spite of, in souse cases, apparent taxonomic affiliations with terrestrial species, environmental differences are sufficient for novel compound production.

The biomedically relevant secondary metabolites reported to date from marine microorganisms represent diverse structure classes that include terpenes, peptides, polyketides and compounds of mixed biosynthetic origin. The producing strains range from obligate marine bacteria such as *Flavobacterium ulginosum* to ubiquitous fungal genera such as *Penicillium***.** The strains isolated have been obtained from diverse substrates and clearly represent only a small portion of the microbial diversity available for chemical study.

From the mycelia of a strain of *Penicillium sp***.** originally separated from a marine alga were isolated five new cytotoxic metabolites designated penochalasius D - H (2). Previously the same authors isolated the cytotoxic compounds communesins, (3) penochalasius A - C (4) y penostatins A -I (5, 6).

The associations of fungi with marine organisms have been well documented (1) and as part of a comprehensive examination of marine invertebrate associated fungi, we have focused considerable attention on fungi associated with a sponge of genus *Cliona,* collected at Quintay, Chile.

EXPERIMENTAL

General procedures. **-** IR spectra were recorded on a Bruker IFS - 25 spectrophotometer. NMR spectra were recorded on a Bruker AMX - 300 spectrometer, operating at 300 and 75.0 MHz for ^{1}H and ^{13}C respectively, with TMS as on internal reference. EIMS was determined using a V.G. Micromass, Zab-2p mass spectrometer. Liquid chromatography over silica gel Merck was performed in a medium pressure. HPLC was run on a Waters instrument equipped with a differential refractometer. Merck Si gels 7734 and 7729 were used in column chromatography. Compounds were visualized on TLC with a 5% H_2SO_4 solution.

Culturing and isolation of metabolites.- Samples of the sponge *Cliona sp*. were collected by scuba diving in Quintay (V Región), Chile. A voucher fragment is kept under the registration Nº Cl - 2002 at the Facultad de Ciencias, U. 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 15 g/L agar and 24.4 g/L artificial sea salt mixture. Then were incubated at 27ºC and from these a pure fungal culture was isolated after repeated inoculation on fresh malt agar plates. The isolated fungus was identified as *Penicillium brevicompactum* by Prof. E. Piontelli, Universidad de Valparaíso, Chile.

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 12 days incubation at 27ºC with shaking, the mycelium and the broth were extracted with EtOAc and evaporated under reduced pressure. The resulting extract (0.85g) was passed through Sephadex LH - 20 using Hexane -CH_2Cl_2 - MeOH (3:2:1) as the eluent. The third fraction was chromatographed on a silica gel column with hexane - EtOAc gradient as the eluent. Four eluates were collected and purified by HPLC (normal phase) using hexane - EtOAc (3:1) to afford **1** (2mg), **2** (10 mg), **3** (5 mg) and **4** (5 mg).

Compound **1**: oil, IR ν_{max}^{KBr} cm^{-1}: 3691, 2984, 1642, 1470, 1382 and 1095. ^{1}H-RMN and ^{13}C-RMN see table1. HRMS (70 eV): found 138.06810, (Calc. 138.06808 for $C_{20}H_{34}O$). LRMS *m/z*: 380 , 120, 107 (100) and 105.

Compound **2**.- Colourless crystals,mp.139º-140ºC. IRcm^{-1}: 3640, 3550, 2940, 1738, 1452, 1095, 970, 950. MS (*m/z*): $[M]^+$ 320, 302, 261, 260, 247, 245, 229, 219, 207, 159, 152 and 149. ^{1}H-NMR and ^{13}C-NMR, see table 2.

Compound **3**.-Beige solid, mp.96º-97ºC. IRν_{max}^{KBr} cm^{-1}: 3426, 1732. LRMS (*m/z*): $[M]^+$ 334, 303, 275, 261, 260 and 247. ^{1}H-NMR and ^{13}C-NMR, see table 2.

Compound **4**.-White solid, mp. 164º- 169º C. IRν_{max}^{KBr} cm^{-1}: 3376, 3252, 1700, 1600, 1350 and 1300. LRMS(*m/z*): $[M]^+$168 (69), $[M-OCH_3]^+$ 137 (100), $[M-COOCH_3]^+$109 (37). ^{1}H-NMR and ^{13}C-NMR, see table 1.

RESULTS AND DISCUSSION

Penicillium brevicompactum, strain Cl - 2002, collected at -20 m in Quintay, V Region, Chile, was cultured in a sea water based medium. The mycelium and broth were extracted with ethyl acetate and the solvent concentrated to provide a crude extract that showed *in vitro* antibacterial activity against gram-positive bacteria (*Staphylococcus aureus, S. epidermidis*) gram-negative bacteria (*Escherichia coli, Proteus mirabilis, Enterococcus faecalis*) and *Pseudomonas aeruginosa*.

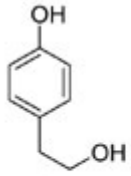
The resulting extract was fractionated by Sephadex LH-20 column chromatography and subjected to purification by liquid chromatography and fractions were combined when it was obvious that the same compounds were involved. After separation into four fractions of different polarity, we obtained four compounds, **1** - **4**.

The IR spectrum of compound **1** presented a band assigned to a hydroxyl group at 3691 cm^{-1}. Its EIMS spectrum showed a peak at *m/z* 138 $[M]^+$ that corresponds to the empirical formula $C_8H_{10}O_2$, (HREIMS calculated: 138,06808). The ^{13}C NMR spectra of **1** indicated the presence of 6 carbons in the molecule whose multiplicities were determined by DEPT spectral data: two methylenes, two unsaturated methines and 2 tetrasustituted carbons. The aromatic nature of this compound was verified by means of ^{1}H NMR spectrum that showed two doublets at d7.10 (2H, d, J = 8.4 Hz) and δ 6.78 (2H, d, J = 8.4 Hz), complete this spectrum two methylenes coupled according to COSY spectrum at d 3.83 (1H, d, J = 6.5Hz) and δ 2.80 (1H, d, J = 6.5Hz). The spectra of ^{1}H NMR and HMQC confirmed the existence of a benzene ring with substitution 1,4. Spectrum HMBC allowed to establish the connectivities of quaternary carbons and to propose structure **1**. Compound **1,** also known as tyrosol, was reported to be antibiotically weakly active against *Saccharomyces cerevisiae*, *Nematospora corlyi*, and is moderately phytotoxic and antifungal (7). The compound is widespread in fungi like *Candida*. The laboratory of J. Clardy (8) identified 4-hydroxyphenethyl alcohol (tyrosol) as a quorum-sensing molecule in the yeast *Candida albicans*. Quorum sensing molecules are released by

cells to monitor their population density and stimulate coordinated behaviour when a threshold concentration of the molecule is reached. In the case of *Candida albicans*, the accumulation of tyrosol in the growth medium increases with increasing cell density. When added to dilute cultures of *C. albicans*, tyrosol reduces the lag phase of growth. Also it was isolated from the bacteria *Rhodospirillum rubrum* (9).

In analogous way we identified the compound **2** as mycophenolic acid (MPA), which was the first secondary metabolite, isolated in 1896 in crystalline form, to exhibit antibiotic properties with respect to gram-positive bacteria. Among MPA producers, only certain strains of fungi of the genus *Penicillium*, belonging to the species *P. brevicompactum* (also known as *P. stoloniferum*), *P. paxilli, P. olivicolor, P. canescens, P. roqueforti*, and *P. viridicatum*, are known (10). Also it was reported a highly efficient synthetic route to mycophenolic acid (11). Among natural products, one microbial metabolites penicillins stands above all others for its profound acid (11,12) that had been discovered as early as 1893 by an Italian physician, B. Gosio (13-15) Moreover, Gosio had observed that his fungal metabolite, a purified, crystalline compound, inhibited the growth of the anthrax bacillus. In consequence, Florey stated that "mycophenolic acid enjoys the distinction of being the first antibiotic produced by a mould to be crystallised" (12). In fact, it is the first purified antibiotic from any source and especially in the last half century, MPA has become something of a cure-all and has been used in the treatment of various disorders.

Compounds **3** and **4** were identified on the basis of a comparison with the data of literature as mycophenolic methyl ester (16) and methyl-3, 5-dihydroxybenzoate (17), respectively. The methyl ester of mycophenolic acid was obtained by Fisher esterification of mycophenolic acid (16). According to the obtained information, it is the first time that methyl ester of mycophenolic acid and methyl -3,5-dihydroxybenzoate had been isolated from natural sources. The antimicrobial activities of *P. brevicompactum* and their extracts can be attributed to the powerful mycophenolic acid antibiotic.



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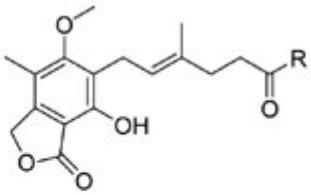

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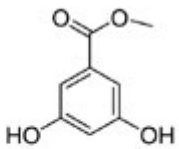

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Table 1.- ^{1}H and ^{13}C NMR* data of compounds **1** and **4** (300 MHz), δ ppm (J), $CDCl_3$

	Compound 1		Compound 4	
Nº	^{1}H - NMR	^{13}C- NMR	^{1}H - NMR	^{13}C- NMR
1		154.2 *s*		132.2 *s*
2	6.78 d (8.4)	115.4 d	6.98 d (2.3)	107.7 d
3	7.10 d (8.4)	130.1 d		158.6 s
4		130.4 s	6.56 d (2.3)	107.0 d
5	7.10 d (8.4)	130.1 d		158.6 s
6	6.78 d (8.4)	115.4 d	6.98 d (2.3)	107.7 d
7	2.80 t (6.5)	38.2 t		166.2 s
8	3.83 t (6.5)	63.4 t	3.80 s	51.3 q
-OH			8.53 s (2H)	

*Multiplicities determined by DEPT experiment.

Table 2. ^{1}H and ^{13}C NMR* data of compounds **2** and **3** (300 MHz), d ppm (J),

	Compound 2			Compound 3		
Nº	^{1}H - NMR	^{13}C- NMR	HMBC	^{1}H - NMR	^{13}C- NMR	HMBC
1		177.3 s			173.0 s	
2	2.43 t (8.5)	31.8 t	C_1, C_3, C_4	2.40 dd (8.0, 6)	32.1 t	C_1, C_3, C_4
3	2.30 t (8.5)	33.5 t	C_1, C_2, C_4, C_5	2.30 t (8.0)	33.8 t	C_1, C_2, C_4, C_5, Me-15
4		133.1 s			133.4 s	
5	5.25 t (6.9)	122.1 d	C_3, Me-15	5.23 t (6.9)	121.9 d	C_3, Me-15
6	3.38 d (6.9)	21.8 t	C_4, C_5, C_7, C_8, C_{12}	3.38 d (6.9)	21.8 t	C_4, C_5, C_7, C_8, C_{12}
7		121.3 s			121.3 s	
8		152.9 s			152.8 s	
9		105.6 s			105.5 s	
10		115.9 s			115.9 s	
11		143.2 s			143.2 s	
12		162.9 s			162.9 s	
13	5.19 s	69.2 t	C_9, C_{10}, C_{11}, C_{14}	5.20 s	69.2 t	C_9, C_{10}, C_{11}, C_{14}
14		172.1 s			172.1 s	
15	1.80 s	15.3 q	C_3, C_4, C_5	1.79 s	15.3 q	C_3, C_4, C_5
16	2.14 s	10.7 q	C_{10}, C_{11}, C_{12}	2.15 s	10.8 q	C_{10}, C_{11}, C_{12}
17	3.75 s	60.2 q	C_{12}	3.76 s	60.2 q	C_{12}
18				3.62 s	50.7 q	C_1
OH				7.67 s		

* Multiplicities determined by DEPT experiment.

$CDCl_3$

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