Chemistry of the cytotoxic principles of the marine fungus *Lignincola laevis*

S. P. Abraham¹, T. D. Hoang¹, M. Alam¹*, and E. B. Gareth Jones²

¹Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, Texas 77204 and ²School of Biological Sciences, University of Portsmouth, Portsmouth, England

Abstract: A methylene chloride : methanol (2:1v/v) extract of the marine fungus *Lignincola laevis* was found to inhibit the growth of mouse murine leukemia cells (L 1210). Large scale culturing followed by fractionation of the extract on silica gel has resulted in the isolation of a cytotoxic 1 along with 7-hydroxyergosterol 2 and a ceramide 3. Structures of the isolated compounds were established with the help of two-dimensional NMR spectroscopy.

During the last decade a plethora of compounds with novel structures have been isolated from marine invertebrates[1]. A number of studies have shown that many of these compounds have had their origin either in the dietary sources of these invertebrates or they are metabolic products of compounds derived from dietary sources. Investigations dealing with the secondary metabolites of the marine cyanobacteria (blue-green algae) and phytoflagellates (belonging to the phylum Mastigophora), which form the basis of the food chain in marine environments, have confirmed that in many instances the real sources of compounds with unusual structures that have been isolated from marine coral and mollusks, are derived from marine microorganisms [1, 2].

During the last decade only ten marine fungal metabolites have been reported in the literature; these include siccaynes (1) from *Halocynthia villosa* [3], leptoshaerin (2) from *Leptosphaeria oraeanis* [4], gliovictin (3) from *Asteromyes cruciatus* [5], phomactin A (4) from a fungus of *Phoma* sp. [6], helicascolides (5) from *Helicascus kanaloanus* [7], melinacidins (6), a number of dendryphielins (7) from marine deuteromycetes *Dendryphiella salina* [8], fumiquinazolines (8)

from *Aspergillus fumigatus* [9], phenazine L-quinovose (9) esters from a marine Actinomycetes [10] and auranticins (10) from *Preussia aurantiaca* [11] and gancidin W from *Corollospora pulchella* [12]. In contrast to the secondary metabolites of phytoflagellates and blue-green algae, all, but two, of the above mentioned compounds, produced by fungi, are secreted into the medium
During the last two years we have been working to improve the culturing techniques and media for marine fungi and have developed a method that has increased the production of mycelial biomass by about 50 percent. We now report the isolation and structures of an unusual dimerized phosphorohydrazide thioate (11), 7-hydroxyergosterol (12) and a common ceramide (13) from *L. laevis*. It is quite possible that 11 is derived from a pesticide, however, attempts to find a correlation between the structure of 11 and of known pesticides were unsuccessful.

**EXPERIMENTAL**

**LARGE-SCALE CULTURING:**

Starter cultures (obtained from the culture collection of Dr. E. B. Gareth Jones, Portsmouth Polytechnic, Portsmouth, England) are maintained on solid agar (in sea water). Plugs of solid agar supporting mycelial growth are cut and transferred aseptically to a 125 mL erlenmeyer flask containing 50 ml of liquid medium (13). The flasks are incubated at 25°C on a rotary shaker for 14 days. The mycelia are separated by decantation, washed with distilled water and homogenized in sterilized sea water. The homogenate is used to inoculate 8L of enriched sea water medium. Cultures are incubated for 14 to 21 days at 25°C with continuous aeration with 0.22μ filtered air.

**EXTRACTION AND SEPARATION OF METABOLITES:**

Grown cultures are filtered through cheese cloth. Excess culture medium, from the mycelia, is removed by compression. The separated mycelia was homogenized in CH2CI2; MeOH (2 : 1 v/v) in a blender and filtered and the filtrate was evaporated to dryness. The organic residue was chromatographed on a silica gel 60 (230 - 400 mesh) column (1 x 60 cm) which was eluted with a linear gradient of MeOH (0 - 30 %) in CHC13. Fraction of 5 ml volume were collected and combined on the basis of tlc [solvent ranged from 2.5 % MeOH in CHC13 (for 11) to 20% MeOH in CHCl3 (for 12 and 13).

**RESULTS AND DISCUSSION**

Repeated chromatography of the combined fraction 40 - 49 resulted in the isolation of 1 as colorless crystalline needles, mp. 110°C. The molecular formula of C16H36N4O4S2P2 was deduced for 1 based on a HRMS (m/z 474, 1604, Δ1.2ppm). The 1H NMR spectrum of 1 showed the presence of two n-propoxy moieties [δ 0.954 (6H, t, J = 7.25 Hz), 1.721 (4H, dq, J = 7.25 Hz)] and 4.020 (4H, m)]. The presence of a two isochronous methyls attached to vinylic carbons was evident from the resonance at δ 2.007 [6H, s]. The 1H NMR spectrum had two additional resonances belonging to exchangeable protons, at δ 6.812 and δ 6.687 (1H, s each). The 13C NMR spectrum of 1 showed the presence of two methyls (δ 8.97 and 10.08), two similar, but nonequivalent, methylenes (δ 23.17 and 23.47), two oxygen bearing carbons (δ 69.09 and 69.30) and two vinylic carbons resonating at δ 148.00 and 148.61. A 1H COSY spectrum established the presence of a propoxy moiety in 1. A HMBC spectrum established the connectivities between the carbons of the propoxy group along with the relationship between two methyls on vinylic carbon. The protons of vinylic methyls showed correlations with carbons resonating at δ 148.10 and 148.61. On the basis of the 2-dimensional NMR spectral data structure 1 was tentatively assigned to 1.

Fractions 87 - 96 were found to contain only a single compound which was further purified by rechromatography on silica gel to yield a colorless solid. A HR EIMS established the molecular formula C28H44O2 (found 412. 33490 calc. for 412.445089 ) for 2. The 1H NMR spectrum of 2, in pyridine, was reminiscent of a sterol [six methyls δ 0.499 (3H, s.), 0.689 (3H,
Lignicola laevis

The 1H NMR spectrum confirmed the presence of six vinylic carbons in 2 (δ 120.21, 122.81, 131.81, 131.88, 141.23, 141.27) which were also required by the molecular weight as determined by a HREIMS. A 1H COSY spectrum was used to discern the connectivities between protons resonating at δ 0.916 and 1.782. The proton at δ 1.782 was in turn connected to a proton resonating at 5.051. The proton at δ 5.051 showed connectivity to the proton at δ 5.000 which was linked to the proton at δ 1.881. The proton at δ 1.881 was in turn connected to a methyl resonating at δ 1.010. These data established the presence of A in the sterol side chain. On the basis of 1H 1H COSY and HMBC spectra 2 was identified as 7-hydroxyergosterol.

Fractions 108 - 115 were found to contain a major and a minor compounds. The major component was separated by reversed-phase HPLC. An FABMS established the molecular weight at m/z 757. A 500 Mz 1H NMR spectrum (in pyridine) suggested the presence of a galactose [δ 4.01 (1H, m, H-5'), 4.12 (1H, t, J = Hz, H-2'), 4.21(1H, t, J = Hz, H-4'), 4.33 (1H, t, J = Hz, H-3'), 4.36 and 4.57 (1H each, m, H-6a' and H-6b') and 4.96 (1H, d, J = 8.0 Hz, h-1') with β-stereochemistry at C1'] and a sphingosine [δ 4.26 and 4.28 (1H each, m, H-1a and H-1b), 4.83 (1H, m, H-3), 4.86 (1H, m, H-2), 6.05 (1H, m, H-5) and 6.13 (1H, dd, J = 15.6, 5.8 Hz, H4) partial structure B] moiety in 3. The presence of the galactose and sphingosine moieties were confirmed by the mass spectral fragments at m/z 163, 506 (M - 251, allylic cleavage) respectively. The 1H 1H COSY and 1H 13C COSY spectra also confirmed the presence of a galactose and a sphingosine moieties in 3. A HMBC spectrum established the linkage between the galactose and sphingosine units (carbon resonating at 67.5 (C1) was linked to the H-1' (δ 4.96). Similarly the linkage between the hydroxy fatty acid and the sphingosine nitrogen was established by the presence of a connectivity between H-2 (δ 4.86) and carbonyl carbon (δ 174.5) of the fatty acid. On the basis of the 1H 1H COSY, HMBC and HMQC spectral data the ceramide was assigned structure 13. Attempts are underway to elucidate the chemical structure of the minor component of the combined fraction 108 - 115.

Compound 11 showed cytotoxicity against L1210 cell line at a 0.25 µg/ml level, while the minor component of the the combined fraction 108 - 115 showed cytotoxicity against the above cell
line at a 1.12 μg/ml level. Although a number of marine fungi have been reported to possess novel compounds, we report for the first time a cytotoxic compound from the extract of the marine fungus *Lignincola laevis*. It is quite possible that I 1 is a biotransformation product of an insecticide which was present in the sea water. However, attempts to locate an insecticide with a comparable structure through the chemical registry have been unsuccessful. This also constitute the first report on the presence of 7-hydroxyergosterol in a marine fungus.

ACKNOWLEDGMENTS

The HRMS were recorded at the Midwest Center for Mass Spectrometry which is partially supported by a grant from the NSF, Biology Division (Grant # DIR9017262). We thank Dr. Gary Martin, Burroughs Wellcome Company for recording the 500 MH, HMQC and HMBC NMR spectra. This work was supported in part by a grant from the University of Houston Coastalk center.

REFERENCES