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ANTIBIOTIC ACTIVITY OF THE MARINE FUNGUS LEPTOSPHAERIA ORAEMARIS[1]

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Two new pigments, obioninene and oraemarin from the lignicolous marine fungus *Leptosphaeria oraemaris* are described. The effect of salinity on the production of antibiotic compounds from this organism was investigated. Activity of extracts in the HeLa cell bioassay was greatest for fermentations of lower salinity. Toxicity of extracts to brine shrimp larvae was greatest at 14, 18 and 22$^{0}/_{00}$. In contrast, activity against a marine bacterium was greatest in extracts from fermentations from higher salinities (>26$^{0}/_{00}$). The results are discussed in relation to the ecology of *L. oraemaris*.

Deux nouveaux pigments, l'obioninene et l'oraemarin, obtenus du fungus marin lignicole *Leptosphaeria oraemaris*, sont décrits. L'effet de la salinité sur la production de composés antibiotiques par cet organisme a été étudié. L'activité biologique, telle que mesurée par la toxicité envers les cellules HeLa, fut inversement proportionnelle à la salinité de la fermentation. La toxicité des extraits envers les larves de crevettes de mer fut plus élevée à 14, 18 et 22$^{0}/_{00}$. Par contre, l'activité contre une bactérie fut plus élevée pour les fermentations à haute salinité (>26$^{0}/_{00}$). Les résultats sont discutés en de l'écologie de *L. oraemaris*.

Introduction

Miller & Whitney (1981) recorded lignicolous marine fungi from the three classes of wood in the sea: drift wood, intertidal wood and sterile panels of wood used as and showed that fungal diversity decreases among these three, a fact often reported others (Kohlmeyer & Kohlmeyer 1979). Close examination of such wood reveals signs of interference competition between some of the marine fungi present, something that appears also to occur under controlled conditions (Miller *et al.* 1985, Strongman et al. 1987).

Interference competition in lignicolous fungi can be mediated by the production of antibiotic metabolites including volatiles and possibly by the production of compounds that sequester nutrients (Wicklow 1981, Bruce *et al.* 1984). The substrate on which aquatic lignicolous fungi can be found, i.e. wet or waterlogged wood, would appear to limit interference competition. Compounds that are produced might be expected to leach away before having any effect. None the less, the production of

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Marine fungi growing on intertidal wood are exposed to variations in moisture, temperature and, depending on rainfall and land run-off, salinity. There are average differences between sites, and often profound differences on a daily basis. This is particularly true for sites in eastern Canada such as the Bay of Fundy and the shore of Prince Edward Island, locations with extreme tidal range and ice in the winter, respectively (Miller & Whitney 1981, Strongman *et al.* 1985). The optimum salinity for growth of lignicolous marine fungi is approximately $28^0/_{00}$ at 25°C. Regulation of osmoticum in higher marine fungi is thought to involve the production of sugar alcohols, particularly glycerol (Jennings 1983, Wethered *et al.* 1985). In filamentous fungi, the production of antibiotics involves the diversion of various primary metabolites to secondary metabolism (Bu'Lock 1975). The effects of salinity stress could be postulated therefore to include effects on secondary metabolism.

This strain of *Leptosphaeria oraemaris* Linder has been reported to produce the antifungal sesquiterpene diol culmorin (Strongman *et al.* 1987). This species has also been reported to produce the aminohexose leptosphaerin, and other metabolites (Miller *et al.* 1984; Schiehser *et al.* 1986; White *et al.* 1989). The purpose of this report is to describe experiments characterizing a new metabolite from *L. oraemaris* and to demonstrate the effect of salinity on the production of crude extracts toxic in various bioassays.

Materials and Methods

Fermentations. Leptosphaeria oraemaris (Strongman *et al.* 1987) was maintained on seawater agar (1 g glucose, 1 g yeast extract, 1 g peptone (all Difco) per L seawater $28^0/_{00}$; Johnston & Sparrow 1961). A seawater agar slant of the culture was macerated in 50 mL sterile artificial seawater (without N or P; Churchland & McLaren 1976). The resulting suspension was used to inoculate (5% v/v) six 250 mL Erlenmeyer flasks containing 50 mL of the following medium: 2 g NH_4Cl, 0.2 g $FeSO_4$, 2 g KH_2PO_4, 2 g peptone, 2 g yeast extract, 2 g malt extract and 20 g glucose per L of $28^0/_{00}$ artificial seawater. The flasks were put on a rotary shaker at 220 rpm (3.81 cm throw in a horizontal plane) at 25°C for three days. The resulting mycelium was macerated, washed in sterile artificial seawater ($28^0/_{00}$) by centrifugation and resuspended to deliver 2 mg dry weight in 1 mL sterile artificial seawater (Miller *et al.* 1984). This was used to inoculate (5% v/v) each of six 250 mL Erlenmeyer flasks containing 50mL of 10 g glucose, 1 g yeast extract and 1 g peptone per L artificial seawater at salinities of 14, 18, 22, 26, 30 or $34^0/_{00}$, respectively. These were incubated at 25°C for 28 d.

At the end of the incubation period, cultures were filtered through Whatman filter paper (#1), washed and dry weights determined. The mycelia from each salinity were pooled and macerated in 100 mL methyl alcohol, the solution was filtered, taken to dryness and weighed. The extract was then subjected to vacuum liquid chromatography to separate the material into 5 fractions of increasing polarity as follows. Silica gel 60 was put in a 30 mL sintered glass funnel and the extract dissolved in chloroform was layered on the silica. This was eluted with 100 mL aliquots of chloroform/methanol: fraction 1 (100/0), 2 (95/5), 3 (90/10), 4 (85/15), 5 (80/20); fraction 1 was discarded and the rest were dried. These fractions are, henceforth, described as e.g. 14-2, 26-5, the first two digits referring to salinity, the last to fraction number.

Bioassays. The above fractions were tested in three bioassays involving brine shrimp, HeLa cells and a gram-negative marine bacterium freshly isolated on

seawater agar from *Fucus vesiculosus* L. obtained from McLaren's Beach, New Brunswick. (Miller & Whitney 1981). Brine shrimp eggs were obtained from a tropical fish store and 20 mg added to artificial seawater ($28^0/_{00}$) containing 6 g/L glycine (100 mL in a 250 mL Erlenmeyer flask). These were incubated in the light with sterile air bubbled into the seawater for 24 h at 30°C. Extracts (2-5) dissolved in ethyl alcohol and added to sterile 24-well plates in triplicate at 1, 10, 100 and 1000 μg plus solvent controls. The solvent was allowed to dry in a laminar flow hood and 1 mL of artificial seawater/glycine solution with 25 live brine shrimp was added to each well. The plates were sealed with Parafilm and put in an incubator at 30°C in the light. The wells were inspected each day for three days and those containing >50% dead brine shrimp were recorded as toxic. Control wells contained >95% living brine shrimp after three days under these conditions.

The bioassay involving the *Fucus* bacterium was done by the paper disc method. (Vincent & Vincent 1944). A lawn of the marine bacterium was prepared on seawater agar and the agar surface allowed to dry. Antibiotic assay discs (10 mm) were sterilized, 100 μg of each extract in ethanol put on two discs and the solvent was allowed to evaporate under sterile conditions before placement on the agar. After 1 and 2 days, the presence of a zone of inhibition around each disc was recorded (as + or -).

HeLa cells were cultured in Minimal Essential Medium (Gibco #320-1095) plus 5% heat inactivated fetal bovine serum, 14.7 mg per 100 mL glutamic acid and 100 μg/mL gentamycin. Aliquots (1 ml at 10^5 cells/mL) were dispensed into a 24 well plate and allowed to grow for 24 h at 37°C in 4% CO_2. A 2 mg/mL ethyl alcohol solution of each extract was prepared and 1 μL added to each of the three wells (i.e. 2 μg/mL medium). Ethyl alcohol controls and a deoxynivalenol positive control (1 μg/ml) were included in each assay. The cells were examined at 24 and 48 h and extracts that resulted in the death of all or most of the cells were recorded as toxic. Each extract was assayed in two experiments on different days. Results from the bioassays indicated the presence of an active compound(s) in the $18^0/_{00}$ fermentation.

Characterization of pigments. ^1H and ^{13}C spectra were recorded at 500 MHz and 125 MHz, respectively on a Bruker AM-500 spectrometer or at 250 MHz and 62.9 MHz on a Bruker AM-250 spectrometer. Chemical shifts are referenced to residual $CHCl_3$ at 7.24 ppm for ^1H spectra and C^2HCl_3 at 77.0 ppm for ^{13}C spectra and reported relative to tetramethylsilane. Mass spectra were obtained on either a Finnigan MAT 312 mass spectrometer or Finnigan MAT 4500 GC/ms system. Accurate mass measurements were determined by peak matching with an ion in the spectrum of perfluorokerosene.

Isolation of pigments. The mycelium (21 g) from 3 L of culture broth grown at $18^0/_{00}$ salinity was collected by filtration. It was dried in air, macerated with ethyl acetate (6 x 200 mL) and the combined ethyl acetate extracts evaporated to give a brown oil (792 mg) which was applied to silica gel and the column eluted with ethyl acetate-hexane (1:1) to provide a red pigment (413 mg). Further chromatography on Sephadex LH-20 using dicholoromethane-hexane (1:1) as the eluant, followed by recrystallization from ethyl acetate-hexane (1:1) gave the naphthoquinone (Fig 2), m. p. 160-162°(dec.), $[\alpha]^{24}_D$=+52° (c: 0.01, $CHCl_3$), λ_{max} (MeOH) 242, 311, 382 nm (ε 13900, 10200, 10700 $dm^3\ mol^{-1}\ cm^{-1}$, λ_{max} (MeOH + NaOH) 280, 402, 530 nm (ε 10500, 5100, 720 $dm^3mol^{-1}\ cm^{-1}$), IR ($CHCl_3$) 3090, 2400, 1510, 1425, 1210, 930 cm^{-1}, m/z 354.1494 (100; $C_{21}H_{22}O_5$ requires 354.1467), 339, 325, 307, 297, 285, 269, 257, 214, 149, 109, n.m.r. see Table II. The quinoxaline derivative was prepared in 37% yield by treatment of the metabolite (20 mg) with 1,2-diaminobenzene (40 mg) in ethyl alcohol (17 mL). The reaction was heated under reflux for 15 minutes, cooled and the precipitated quinoxaline (m. p. 233-235°, $[\alpha]^{24}_D$=+78° (c: 0.08, $CHCl_3$), m/z 426 (100), n.m.r. see Table I) recrystallized from diethyl ether.

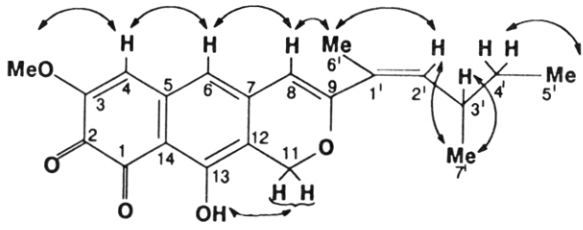

Fig 2 Structure of obioninene; arrows indicate demonstrated nuclear Overhauser effects.

Table I Nuclear magnetic resonance data (^1H & ^{13}C) for obioninene and its quinoxaline derivative. The numbers in column 1 are locants (Fig 2). Coupling constants (*J*) are given in parentheses in hertz (Hz). Chemical shifts are given in parts per million of field strength downfield from the signal of tetramethylsilane.

	Obioninene		Quinoxaline	
	^1H	^{13}C	^1H	^{13}C
1	-	178.8		144.9
2	-	177.0		140.7
3	-	151.7		151.6
4	6.25, s*, 1H	113.9	6.92, s, 1H	108.4
5	-	134.8		134.1
6	6.38, s, 1H	118.0	7.06, s, 1H	113.1
7	-	144.4		136.4
8	5.83, s, 1H	100.4	6.01, s, 1H	100.6
9	-	161.9		157.9
11	5.16, d (13.9 Hz), 1H	62.7	5.47, d (13.2 Hz), 1H	63.7
	5.12, d (13.9 Hz), 1H		5.44, d (13.2 Hz), 1H	
12		112.6		112.2
13	-	162.1		155.0
14	-	111.1		109.1
1'	-	126.5		126.9
2'	6.22, dq (10.6, 0.9 Hz), 1H	141.2	6.14, dq (9.8, 1.3 Hz), 1H	137.6
3'	2.45, m, 1H	34.9	2.46, m, 1H	34.7
4'	1.42, m, 1H; 1.34, m, 1H	30.0	1.43, m, 1H; 1.36, m, 1H	30.3
5'	0.85, t, (7.3 Hz), 3H	12.0	0.88, t (7.4 Hz), 3H	12.0
6'	1.85, d (0.9 Hz), 3H	12.9	1.91, d (1.4 Hz), 3H	13.0
7'	1.00, d (6.5 Hz), 3H	20.2	1.02, d (6.6 Hz), 3H	20.4
OCH_3	3.80, s, 3H	55.7	4.17, s, 3H	56.3
OH	12.32, s, 1H		14.43, s, 1H	
				138.1
			8.15, d (8.2 Hz), 1H	127.1
			7.84, m	129.5
			7.84, m	130.0
			8.40, d (8.3 Hz), 1H	131.0
				138.9

* Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet

A second red pigment was obtained from the combined fractions 14-4, 14-5, 18-4, and 18-5. It was purified by flash chromatography on silica gel (230-400 mesh). The column was developed with ethyl acetate-hexane (1:9, 15 mL) and the pigment (3 mg, m/z 366 (100), 351 (53), 337 (58), 322 (23), 311 (8), 284 (19), 279 (20); δ_H (C^2HCl_3) 12.69 (H), 9.38 (H, *J* 0.9 Hz), 8.09 (H, *J* 0.9 Hz), 7.44 (H), 6.54 (H, *J* 9.8 Hz, *J* 1.3 Hz), 4.66 (2H), 4.03 (3H), 2.60 (H, m), 2.18 (3H, d, J 1.2 Hz), 1.45 (2H, m), 1.07 (3H, J 6.7 Hz), 0.90 (3H, t, J 7.5 Hz), δ_c (C^2HCl_3) 148.4, 142.9, 114.8, 104.2, 56.2, 35.4, 30.1, 20.1, 14.4, 12.0) eluted with ethyl acetate-hexane (1:4, 20 mL).

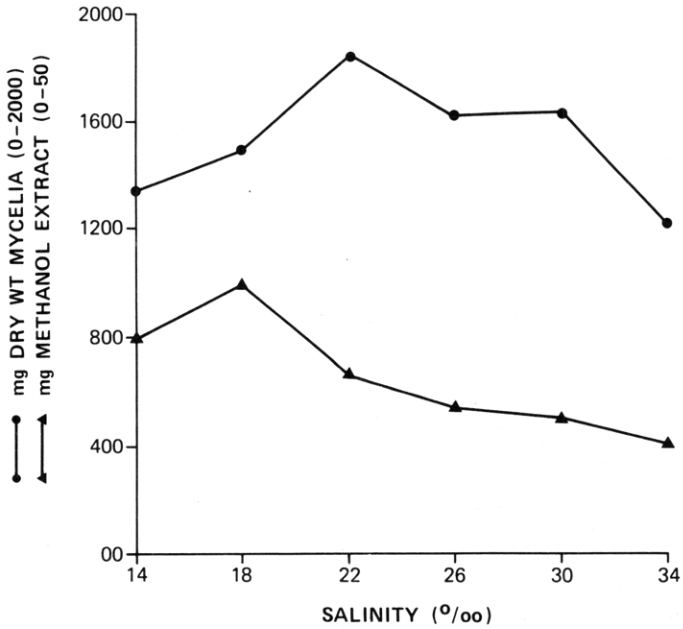

Fig 1 Mycelial dry weight and weight of methyl alcohol extract of cultures of *L. oraemaris* grown at different salinities for 28 days at 25°C. Standard deviations of mycelial and extract weights at each point varied from 5-10% of the mean (n=6).

Results and Discussion

Effects of salinity on antibiotic production

Growth of *Leptosphaeria oraemaris* at different salinities is shown in Fig 1. Maximum dry weight production occurred at ca. 22°/oo at 25°C for this strain. The ratio of the weight of the methanol extract to the mycelia peaked at ca. 18°/oo. Production of red pigment was also maximum at 18°/oo salinity by a factor of two or more compared to that at 34°/oo.

The results of the various bioassays are summarized on Table II. A remarkable pattern of biological activity emerged. The salinities at which the greatest activity occurred for each bioassay were different. HeLa cell toxicity of the fractions decreased as salinity was changed from 14 to 34°/oo. Toxicity to brine shrimp occurred in fractions from fermentations at 14, 18 and 22°/oo, with the maximum occurring at 18°/oo. In contrast, the fractions active against the *Fucus* bacterium came from the

Table II Bioassays of fermentation extracts

Fraction	Brine shrimp larvae[1] (μg/mL)				HeLa cell[2] (2 μg/mL)	Marine bacterium[3] 100 (μg/disc)
‰-#	1000	100	10	1		
14-2	+	-	-	-	+++++	+
14-3	-	-	-	-	-	-
14-4	-	-	-	-	+	-
14-5	-	-	-	-	+++	-
18-2	-	-	-	-	-	+
18-3	-	-	-	-	+	-
18-4	+	+	-	-	+++++	-
18-5	+	+	+	-	+	-
22-2	+	-	-	-	+++	-
22-3	+	-	-	-	-	-
22-4	+	-	-	-	-	+
22-5	+	-	-	-	-	-
26-2	-	-	-	-	-	+
26-3	-	-	-	-	-	+
26-4	-	-	-	-	-	+
26-5	-	-	-	-	-	+
30-2	-	-	-	-	+	+
30-3	-	-	-	-	++	-
30-4	-	-	-	-	-	+
30-5	-	-	-	-	-	+
34-2	-	-	-	-	++	-
34-3	-	-	-	-	-	+
34-4	-	-	-	-	-	+
34-5	-	-	-	-	-	+
culmorin	-	-	-	-	-	+

[1] 25 live brine shrimp added to each well of a 24 well plate containing artificial seawater/glycine medium. The plates were inspected each day for 3 days and those wells containing >50% dead brine shrimp were recorded as toxic (+). Control wells and ethanol controls contained >95% living brine shrimp).

[2] HeLa cell assay: - = no damage, +++++ damage equal to 1 μg/mL deoxynivalenol.

[3] Definite zone of inhibition observed = +.

fermentations in the higher salinities. Although the strain of *L. oraemaris* has been reported to produce culmorin, this compound was not produced under these fermentation conditions. Culmorin was not bioactive in the brine shrimp and Hela cell assays at the concentrations tested (Table II).

Bolton *et al.* (1988) reported the effect of salinity on antibiotic production in 17 species of lignicolous marine fungi. Methanol extracts of the mycelia grown at different salinities were tested for activity against a number of medically important bacteria, *Saccharomyces* and *Mucor*. For most of the fungi tested, the use of full strength seawater resulted in decreased antibiotic production. In the case of *Aniptodera marina* Shearer and *Lulworthia* sp., antifungal activity was only expressed or increased markedly, respectively, in the fermentation with seawater. An unidentified *Leptosphaeria* species produced similar activity against *Bacillus subtilis* (Ehrenberg) Cohn regardless of salinity. In general, these authors reported that salinity affected the qualitative and quantitative distribution of antibiotic production in the bioassays used. This is consistent with the present data.

L. oraemaris is distributed in cool waters on saltmarsh plants, drift and intertidal wood and test panels. On the basis of distribution and habitat analysis, this species has been further characterized as eryhalothermic or found in areas with salinity change >5% (Booth & Kenkel 1986). This assignment is consistent with reports from Atlantic Canada i.e. the fungus has been reported from the Bay of Fundy and coastal Prince Edward Island but not from homeohaline Halifax or Argentia (Meyers & Reynolds 1960; Miller & Whitney 1981; Strongman *et al.* 1985). The response to salinity with respect to the production of toxic metabolites demonstrated by this fungus accords with the environmental conditions in the preferred habitat. *L. oraemaris* in this habitat will be exposed to a range of salinity and hence the antibiosis observed *in vitro* could be expected to be produced *in vivo*. These data argue that consideration of salinity as a variable is required in studies of the production of metabolites from higher marine fungi. Additionally, *L. oraemaris* produces metabolites which are active against microbial competitors (bacteria and fungi) and fauna that might be expected to consume mycelia (Miller 1986; Strongman *et al.* 1987).

Characterization of pigments. The main pigment obtained, $C_{21}H_{22}O_5$ was shown to be an orthoquinone by its reaction with 1,2-diaminobenzene to give a quinoxaline whose molecular weight was 426. The absorption of the quinone at 482 nm, shifted to 530 nm in alkaline solution, indicated the presence of a phenol (δ_H 12.32) and a fourth oxygen atom was accounted for by the methoxyl resonance at 3.80 p.p.m. The nature of the asymmetry in the molecule ($[\alpha]_D$+58°) was indicated by the coupling of the proton at 2.45 p.p.m. with the methyl group at 1 p.p.m. and with a methylene group (δ_H 1.34, 1.42) which in turn was coupled to a second methyl group at 0.85 p.p.m. The proton at 2.45 p.p.m. was also coupled to an olefinic proton (δ_H 6.22) which was allylically coupled to a third methyl group (^4J 0.9 Hz). These data, fully consistent with ^{13}C n.m.r. data indicate the presence of a 1,3-dimethylpent-1-enyl moiety analogous to the 1,3-dimethylpentane side chain thought to be present in obionin A (Poch & Gloer 1989). Evidence that the orientation of the methoxyl group was the same as in obionin A was obtained by nuclear Overhauser effect experiments; positive effects are indicated in Fig 2 by arrows. Thus the pigment is probably an unsaturated obionin A and the formula in Fig 2 is consistent with the data obtained.

A small quantity of a second, unstable red pigment, named oraemarin, was isolated from fractions 4 and 5 of cultures grown in $14^0/_{00}$ and $18^0/_{00}$ salinity. Some of its physical properties are given above, and they suggest that it is related to obioninene.

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