Metabolites from *Microsphaeropsis olivacea*, an Endophytic Fungus of *Pilgerodendron uviferum*

Emilio Hormazabal[a], Guillermo Schmeda-Hirschmann[a,*], Luis Astudillo[a], Jaime Rodríguez[b], and Cristina Theoduloz[b]

[a] Universidad de Talca, Instituto de Química de Recursos Naturales, Laboratorio de Química de Productos Naturales, Casilla 747, Talca, Chile. Fax: +56 71 200448. E-mail: schmeda@utalca.cl

[b] Universidad de Talca, Facultad de Ciencias de la Salud, Departamento de Ciencias Básicas Biomédicas, Casilla 747, Talca, Chile

* Author for correspondence and reprint requests

Z. Naturforsch. **60 c**, 11–21 (2005); received June 23/August 4, 2004

Seven compounds belonging to different structural skeletons were isolated from *Microsphaeropsis olivacea* grown in liquid and solid media. The enalin derivative 7-hydroxy-2,4-dimethyl-3(2*H*)-benzofuranone is reported for the first time, while additional spectroscopic information is provided for the acetates of botrallin and ulocladol.

The activity of the isolated compounds was assessed towards the enzyme acetylcholinesterase (AChE) and their cytotoxicity against human lung fibroblasts. Graphislactone A and botrallin presented a moderate activity towards AChE, with IC_{50} of 8.1 and 6.1 μg/ml (27 and 19 μM, respectively). Under the same experimental conditions, the IC_{50} of the standard inhibitor galanthamine was 3 μg/ml. The cytotoxicity of both compounds was > 1000 and 330 μM, respectively. None of the compounds was promising as antibacterial or antifungic against phytopathogenic fungi and bacteria.

Botrallin and graphislactone A were detected in the liquid potato-dextrose and yeast extract/malt extract/dextrose as well as on a solid substrate (rice). Butyrolactone I was obtained from the fungus growing on solid medium.

Key words: Microsphaeropsis olivacea, Botrallin, Graphislactone A

Introduction

The gymnosperm tree *Pilgerodendron uviferum* (D. Don) Florin shows association with endophytic fungi, some of them could be grown in culture from wood samples. One of the endophytes isolated from a sample collected in Chiloe island (Chile) was *Microsphaeropsis olivacea* (Bonord.) Hohn. This fungus has been previously isolated from the peduncle bark and xylem of *Eucalyptus globulus* (Bettucci and Saravay, 1993; Lupo *et al.*, 2001), from lichens (Seephonkai *et al.*, 2002) as well as from marine spongi including *Agelus* sp., collected from Sombrero Key East, Florida, *Myxila incrustans*, from the island of Helgoland, Germany, and *Aplysina aerophoba*, from the Mediterranean Sea.

Endophytic fungi are eukaryotic organisms that live inside the plant tissues and behave as plant hosts (Petrini, 1996). The association is symbiontic and both organisms profit from the relationship. Endophytes are presumably ubiquitous in plants with populations dependent on host species and location. Some endophytes isolated from gymnosperms have been shown to produce secondary metabolites with very strong fungicidal and bactericidal activity (Tan and Zou, 2001). Endophytes are considered as a potential source of bioactive metabolites (Strobel and Daisy, 2004).

In Chile, the native gymnosperms comprise 4 families with 8 genera and 9 species, most of them restricted to the southern part of the country and the eastern Andean slopes in Argentina. The genus *Pilgerodendron* (Cupressaceae) is monotypic and endemic with a distribution in the South American subantarctic forests. The trees grow at an altitude of 0–600 m above sea level. According to the conservation status, the species has to be considered vulnerable (Marticorena and Rodriguez, 1995).

Search for active compounds from endophytes in Chilean gymnosperms is a challenge since the native species belonging to this plant group have a restricted distribution, unique evolutionary traits and their conservation status is vulnerable. The aim of the present work was to take into culture, to isolate and identify active secondary metabo-

0939–5075/2005/0100–0011 $ 06.00

lites from the endophytic fungus *Microsphaeropsis olivacea* isolated from *Pilgerodendron uviferum* (D. Don) Florin ("Cipres de las Guaitecas").

Materials and Methods

Isolation and culture of the endophytic fungus

The fungus *Microsphaeropsis olivacea* (Bonord.) Hohn was isolated from the phloem of the native tree *Pilgerodendron uviferum* (D. Don) Florin (Cupressaceae). The plant sample consisting of branches and leaves was collected in Chiloe island on march 04, 2002. The samples were surface-disinfected with 5% sodium hypochlorite and 70% ethanol and placed in PDA medium (potato/dextrose agar: 20 g dehydrated mashed potatoes, 20 g glucose, 15 g agar in 1 l water, pH 5.5) supplemented with antibiotics (penicillin G, 30 mg/l; streptomycin, 30 mg/l) and cultured at 25 °C according to the methodology described by Bills (1996). A voucher specimen of the tree was deposited at the Herbarium of the Universidad de Talca under reference number 2732. The fungus was identified by Prof. Eduardo Piontelli, Universidad Católica de Valparaíso and it is kept at the microbial strain collection from the Universidad de Talca.

The fungus was cultured in two different liquid media, potato/glucose (PG: 20 g dehydrated mashed potatoes and 20 g glucose in 1 l water, pH 5.5) and yeast extract/malt extract/glucose (YMG: 4 g yeast extract, 10 g malt extract, 10 g glucose, in 1 l water, pH 5.5), as well as on solid medium (rice). The liquid cultures were maintained at 25 °C under shaking (150 rpm). After glucose consumption, the cultures were filtered to obtain the mycelium and culture filtrate. Both were separately extracted with ethyl acetate (EtOAc) to afford a culture filtrate and the mycelium extract. The extraction yields of the cultures in different liquid (PG and YMG) and a solid medium (rice) were 470 mg/l, 245 mg/l and 23.3 g/kg, respectively.

Antibacterial and antifungal activity

The isolated compounds were assessed for antimicrobial activity by the agar diffusion method at a concentration of 50 μg/disk towards the following microorganisms: Gram positive bacteria: *Bacillus subtilis* (ATCC 6633) and *Micrococcus luteus* (ATCC 381); fungi: *Mucor miehei* (Cooney et Emerson Tü 284), *Paecilomyces variotii* (ETH 44646) and *Penicillium notatum* (Collection University of Kaiserslautern, Germany).

The activity of the extracts was estimated by the growth inhibition (in mm) as follows: < 8 mm: inactive; 8–12 mm: weak activity; 13–15 mm: moderate activity; > 15 mm: strong activity. As a reference, the commercial antifungal Benlate [benomyl, methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate, 50% active ingredient, 50 μg benomyl/disk] and chloramphenicol (30 μg/disk) were used.

Furthermore, the antifungal activity of the compounds against *Alternaria alternata*, (Centro Micológico, Universidad de Rosario, Rosario, Argentina) and *Botrytis cinerea* (Instituto de Investigaciones Agropecuarias del Gobierno de Chile, INIA) was determined by the microdilution assay (Favre *et al.*, 2003) in 96-well microtiter plates. Stock solutions of compounds in DMSO were diluted to give serial twofold dilutions that were added to each medium resulting in concentrations ranging from 1.92 up to 250 μg/ml. The final fraction of DMSO in the assay did not exceed 2%. The plates were incubated for 7 d at 25 °C. Minimal inhibitory concentration (MIC) was defined as the lowest extract concentration showing no visible fungal growth after the incubation time. As a reference, the commercial antifungal Rukon (iprodione, 50% active ingredient) was used.

The activity of the compounds against the phytopathogenic Gram negative bacteria *Erwinia carotovora* (INIA) and *Pseudomonas syringae* (INIA) was assessed by the microdilution assay (Eloff, 1998) in 96-well microtiter plates. The bacterial inoculum was prepared to give approx. 10^4–10^5 colony forming units (CFU) per well. Stock solutions of compounds in DMSO were diluted to give serial twofold dilutions that were added to each medium resulting in concentrations ranging from 1.92 up to 250 μg/ml. The final fraction of DMSO in the assay did not exceed 2%. The plates were incubated overnight at 25 °C. To assess bacterial growth, the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was dissolved in water (0.5 mg/ml), added to the microplate wells and incubated at 25 °C for 30 min. The absorbance was read in an universal microplate reader (Bio-Tek Instruments, INC, Vermont, USA) at 515 nm. The results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves. Penicillin G and streptomycin were used as standard antibacterials.

Acetylcholinesterase inhibition activity

The assay for measuring acetylcholinesterase (AChE) activity was carried out according to López *et al.* (2002). Briefly, some 50 μl of AChE solution (0.25 U/ml) in phosphate buffer (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) and 50 μl of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature before the addition of 100 μl of the substrate solution [0.1 M Na_2HPO_4, 0.5 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.6 mM acetylthiocholine iodide (ACTI) in distilled water, pH 7.5]. The absorbance was read in a Bio-Tek Instrument microplate reader at 405 nm after 3 min. Enzyme activity was calculated as the percentage compared to a control using buffer and enzyme solution only. The IC_{50} values were calculated from three individual determinations.

Cytotoxicity

The cytotoxic effect of the compounds, expressed as cell viability, was assessed on the permanent fibroblast cell line derived from human lung (MRC-5) (ATCC Nr CCL-171). MRC-5 fibroblasts were grown as monolayers in minimum essential Eagle medium (MEM), with Earle's salts, 2 mM L-glutamine and 1.5 g/l sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cells were seeded at a density of 2.5×10^3 cells per well in 96-well plates. Confluent cultures were treated with medium containing the compounds at concentrations ranging from 3.8 up to 1000 μM. The substance was firstly dissolved in DMSO and then in the medium supplemented with 2% FBS. The final content of DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to test medium with or without the compound (control). Each drug concentration was tested in quadruplicate, and repeated three times in separate experiments. At the end of the incubation, the neutral red uptake (NRU) assay was carried out as described by Rodríguez and Haun (1999). To calculate the IC_{50} values the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves.

Fungal culture and isolation of secondary metabolites

Solid culture

Microsphaeropsis olivacea was cultured in some 6 flat culture bottles (Kolle flasks) containing 50 g rice and 50 ml distilled water each, autoclaved at 121 °C for 20 min. Each flask received 20 ml of a spore suspension (10^5 CFU/ml) under sterile conditions. After 30 d growing at 25 °C, ethyl acetate (3×300 ml) was added to each bottle, sonicated for 20 min, filtered and taken to dryness to afford 8.1 g of a crude extract. The extract was successively partitioned with chloroform and methanol to yield some 7.0 g of $CHCl_3$ and 1.1 g of MeOH extract, respectively.

The $CHCl_3$ extract (7.0 g) was chromatographed on a silica gel column (70 cm, internal diameter 4.5 cm) with a petroleum ether (PE), PE/ethyl acetate (EtOAc), acetone and methanol (MeOH) gradient. Some 46 fractions of 250 ml each were collected and pooled as follows: PE (0.75 l; fractions 1, 2); PE/EtOAc 9:1 v/v (1 l; fractions 3–6); PE/EtOAc 8:2 v/v (1 l; fractions 7–10); PE/EtOAc 7:3 v/v (1 l; fractions 11–13); PE/EtOAc 6:4 v/v (1 l; fractions 14–18); PE/EtOAc 1:1 v/v (1 l; fractions 19–27); PE/EtOAc 4:6 v/v (1 l; fractions 28–31); EtOAc (1 l; fractions 32–34); EtOAc/Acetone 1:1 v/v (1 l; fractions 35–38); Acetone (1 l; fractions 39–42) and MeOH (1 l; fractions 43–46).

Fractions 25–27 (308 mg) afforded after gel permeation on Sephadex LH-20 (MeOH) and preparative TLC (SiO_2, $CHCl_3$/acetone 9:1 v/v, two developments) 4.3 mg butyrolactone I (compound **1**, Rf 0.32). Fraction 29 (200 mg) was acetylated (Ac_2O, pyr). After the usual work-up and preparative TLC on silica gel (SiO_2, $CHCl_3$, two developments) 15.6 mg of graphislactone A diacetate (compound **2a**, Rf 0.5), 7.2 mg of ulocladol diacetate (compound **3a**, Rf 0.4), 19.0 mg of ulocladol triacetate (compound **3b**, Rf 0.3) and 7.5 mg of botrallin diacetate (compound **4a**, Rf 0.325) were obtained. Fractions 32 and 33 (340 mg) yielded after gel permeation on Sephadex LH-20 (MeOH) and preparative TLC (SiO_2, $CHCl_3$/acetone 9:1 v/v, two developments) 3.0 mg ulocladol (compound **3**, Rf 0.22), 4.0 mg graphislactone A (compound **2**, Rf 0.475) and 2.8 mg botrallin (compound **4**, Rf 0.60).

Culture in liquid media

The fungus was cultured in potato/glucose (PG) medium at 25 °C under agitation (150 rpm) in 2 l Erlenmeyer flasks (9 × 0.8 l) to a total volume of 7.2 l. The glucose was consumed after 13 d. Mycelium (ML) and culture filtrate (CF) were separated by filtration and extracted with EtOAc to afford 3.38 g of a CF and 3.74 g of a ML extract. The CF (800 mg) extract was chromatographed on a silica gel column (70 cm, internal diameter 3.5 cm) with a petroleum ether (PE), PE/EtOAc and MeOH gradient. Some 8 fractions of 200 ml each were collected as follows: PE (fraction 1, 0.6 mg); PE/EtOAc 9:1 v/v (fraction 2, 7.8 mg); PE/EtOAc 8:2 v/v (fraction 3, 17.7 mg); PE/EtOAc 7:3 v/v (fraction 4, 28.9 mg); PE/EtOAc 1:1 v/v (fraction 5, 231 mg); EtOAc (fraction 6, 175.9 mg); EtOAc/MeOH 1:1 v/v (fraction 7, 243.8 mg) and MeOH (fraction 8, 35.5 mg).

Fraction 5 (230 mg) was re-chromatographed on a medium pressure column (silica gel) using PE/EtOAc 6:4 v/v as the mobile phase. Some 160 fractions of 4 ml each were obtained and pooled according to their TLC patterns in eight groups: fraction I (7 mg); fraction II (24.4 mg); fraction III (21.3 mg); fraction IV (37.3 mg); fraction V (17.5 mg); fraction VI (24.0 mg); fraction VII (19.2 mg); fraction VIII (56.6 mg). From fractions IV (37.3 mg) and V (17.5 mg), after preparative TLC on silica gel using $CHCl_3$ as the mobile phase (two developments) 7.7 mg botrallin (compound **4**, Rf 0.625) and 6.5 mg graphislactone A (compound **2**, Rf 0.45) were obtained, respectively. Preparative TLC of fraction VI (silica gel, PE/EtOAc 6:4 v/v) yielded 2.0 mg of 2,5-diacetylphenol (compound **5**, Rf 0.625), 2.2 mg of 7-hydroxy-2,4-dimethyl-3(2*H*)-benzofuranone (compound **6**, Rf 0.55) and 4.9 mg enalin (compound **7**, Rf 0.40).

The fungus was also cultured in YMG medium to compare the production of secondary metabolites under different nutrient conditions by HPLC.

Identification of the main secondary metabolites by analytical HPLC

The extracts obtained from liquid and solid cultures were compared by analytical HPLC to identify and quantify the main compounds present in each medium. The quantitative determination of each compound in the samples was performed as reported by Sánchez-Rabaneda *et al.* (2003) with some modifications. HPLC analysis was performed using a HPLC-DAD Merck-Hitachi (Darmstadt, Germany) equipment consisting of a L-6200 pump, a L-4000 UV detector and a D-2500 chromato-integrator. A Luna C18 column (250 × 4.60 mm. i.d., 5 μm film thicknes) was used. The compounds were monitored both at 254 nm and in the range of 200–400 nm. The mobile phase used was water/acetonitrile 60:40 (v:v) and 0.1% formic acid at a flow rate of 0.8 ml/min. The correlation between concentration/peak area was assessed by the ordinary least square regression model. The correlation coefficient r^2 was 0.9998. The identity of the compounds was checked by UV spectra and coinjection with the isolated products. The results are presented as mg/l for the PG and YMG media and as mg/kg for the solid medium (rice). The content and composition of the extracts are summarized in Table IV.

Butyrolactone I (**1**): Colorless crystals, m.p. 95 °C. – MS (EI): *m/z* = 424.449 (calcd. for $C_{24}H_{24}O_7$: 424.449) (1), 380 (44), 348 (37), 320 (7), 302 (7), 291 (18), 175 (100). – $[\alpha]_D$ +84° (^{c} = 0.5, EtOH). The spectroscopic data are in agreement with those reported by Rao *et al.* (2000).

Graphislactone A (**2**): Colorless crystals, m.p. 220–225 °C. – MS (EI): *m/z* = 302.079 (calcd. for $C_{16}H_{14}O_6$: 302.079) (100), 287 [M-CH_3] (12), 259 [287-CO] (10), 231 [259–28] (14), 203 [231–28] (11). – For ^{1}H NMR: see Table I.

Graphislactone A diacetate (**2a**): Colorless crystals, m.p. 221–225 °C. – MS (EI): *m/z* = 386.352 (calcd. for $C_{20}H_{18}O_8$: 386.352) (1), 344 (27), 302 (100), 287 (6), 259 (6), 231 (3). – For ^{1}H NMR: see Table I.

The spectroscopic data for compounds **2** and **2a** are in agreement with those reported in literature (Tanahashi *et al.*, 1997, 2003).

Ulocladol (**3**): Colorless crystals, m.p. 108–110 °C. – MS (EI): *m/z* = 318.222 (calcd. for $C_{16}H_{14}O_7$: 318.222). – For ^{1}H NMR: see Table I. The spectral data are in agreement with those published by Tanahashi *et al.* (1997) and Höller (1999).

Ulocladol diacetate (**3a**): Colorless crystals, m.p. 194–198 °C. – MS (EI): *m/z* = 402.352 (calcd. for $C_{20}H_{18}O_9$: 402.352) (26), 360 (28), 318 (70), 300 (100), 274 (20). – For ^{1}H NMR: see Table I.

Ulocladol triacetate (**3b**): Colorless crystals, m.p. 85 – 88 °C. – MS (EI): *m/z* = 444.388 (calcd. for $C_{22}H_{20}O_{10}$: 444.388) (3.6), 402 (20), 360 (60), 318 (72.7), 300 (100), 274 (6.4). – For ^{1}H NMR: see Table I. The spectroscopic data agree with those reported by Höller (1999).

Table I. ^{1}H NMR data of compounds **2**–**4** [400 MHz, $CDCl_3$, δ (ppm) *J* (Hz)] and ^{13}C NMR data of compound **4** [100 MHz, $CDCl_3$, δ (ppm)].

H	**2**	**2a**	**3**	**3a**	**3b**	**4**	**4a**	C	**4**
1	–	–	6.90 d (2.45)	6.59 d (2.45)	6.91 d (2.5)	–	–	1	133.86 s
2	6.71 s	6.75 s	–		–	–	–	2	176.76 s
2 OMe	–	–	3.85 s	3.83 s	3.83 s	–	–		
3	–	–	6.54 d (2.45)	6.55 d (2.45)	6.73 d (2.5)	–	–	3	148.64 s
3 OMe	3.91 s	3.93 s	–		–	3.84 s	3.75 s	3 OMe	55.68 q
4	–	–	–		–	6.17 s	5.95 s	4	118.29 d
								4 a	78.30 s
								6	168.10 s
								6a	99.74 s
7	–	–	4.77 d (12.0); 5.09 d (12.0)	4.87 d (12.2); 5.11 d (12.2)	4.84 d (12.2); 5.04 d (12.2	–	–	7	166.27 s
8	6.56 d (2.3)	6.73 d (2.5)	6.56 s	6.94 s	6.97 s	6.58 d	6.77 d (2.4)	8	102.13 d
								9	164.61 s
9 OMe	3.97 s	3.90 s	3.94 s	3.91 s	3.91 s	3.94 s	3.90 s	9 OMe	55.79 q
10	7.27 d (2.3)	7.59 d (2.5)	–	–	–	7.38d	7.25 d (2.4)	10	108.98 d
								10a	142.46 s
								10b	119.07 s
11	2.78 s	2.83 s	–		–	1.80 s	1.70 s	11	30.0 q
1-OH	–	–	–		–	7.30 s*	–		
4-OH		–	10.28 s	10.29 s	–	–	–		
7-OH	11.86 s	–	–	–	–	11.39 s*	–		
1-Ac			–	–	–		2.36 s		
4-Ac	–	2.41 s	–	–	2.20 s	–			
7-Ac	–	2.39 s	–	–	–	–	2.37 s		
10-Ac			–	2.31 s	2.31 s				
11-Ac			–	2.14 s	2.30 s				

* Exchangeable with D_2O.

H	**5**	**6**	**7**
2	–	4.63 q (7.09) (1H)	–
3	7.15 d (8.1)	–	–
4	6.96 dd (8.1, 2.7)	–	–
5	–	6.68 dd (8.07, 0.73)	6.65 d (7.9)
6	7.09 d (2.7)	7.02 d (7.83)	6.98 d (7.9)
10	–	1.54 d (7.09)	1.53 s
11	–	2.49 s	2.43 s
2-$COCH_3$	2.50 s	–	–
5-$COCH_3$	2.43 s	–	–

Table II. ^{1}H NMR data of compounds **5**–**7** (compounds **5**–**6** in $CDCl_3$, compound **7** in MeOH-d_4,) [400 MHz, δ (ppm) *J* (Hz)].

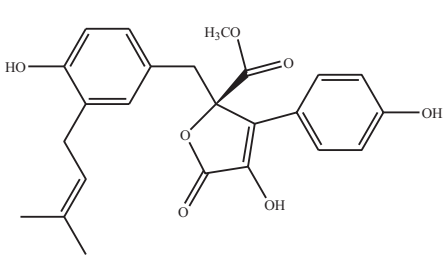
Botrallin (**4**): Colorless crystals, m.p. 171–172 °C. – MS (EI): *m/z* = 318.222 (calcd. for $C_{16}H_{14}O_7$: 318.222) (18.5), 302 (41.8), 274 (9), 259 (15), 249 (22.5), 189 (51), 121 (100). IR (KBr): υ = 3370, 2921, 1655, 1631 cm^{-1}. – The ^{1}H NMR data (see Table I) of compound **4** nicely agree with those reported by Kameda *et al.* (1974) for botrallin. The ^{13}C NMR assignments are reported for the first time (Table I).

Botrallin diacetate (**4a**): Colorless crystals, m.p. 199–206 °C. – MS (EI): *m/z* = 402.352 (calcd. for $C_{20}H_{18}O_9$: 402.352). – For ^{1}H NMR: see Table I.

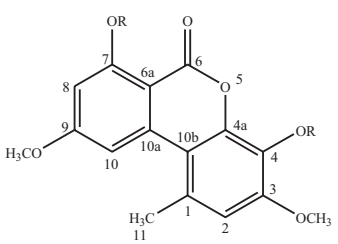
2,5-Diacetylphenol (**5**): Colorless resin. – MS (EI): *m/z* = 178.0674 (calcd. for $C_{10}H_{10}O_3$: 178.0674), 178 (10), 151 (11), 135 (100), 107 (38). – For ^{1}H NMR: see Table II.

7-Hydroxy-2,4-dimethyl-3(2H)-benzofuranone (**6**): Colorless crystals, m.p. 160–164 °C. – MS (EI): *m/z* = 178.0629 (calcd. for $C_{10}H_{10}O_3$: 178.0629), 178 (100), 163 (28), 151 (20), 131 (11), 106 (22). – For ^{1}H NMR: see Table II.

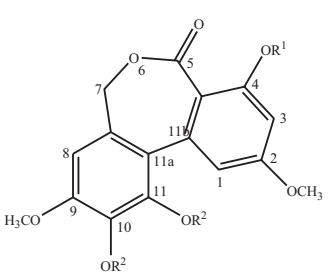
Enalin [2,7-dihydroxy-2,4-dimethyl-3(2*H*)-benzofuranone] (**7**): Colorless crystals, m.p. 175–180 °C. – MS (EI): *m/z* = 194.184 (calcd. for $C_{10}H_{10}O_4$: 194.184) (9), 177 (5), 151 (100), 106 (14), 77 (14). – For ^{1}H NMR: see Table II. The spectroscopic data are in agreement with those reported by Lin *et al.* (2002).



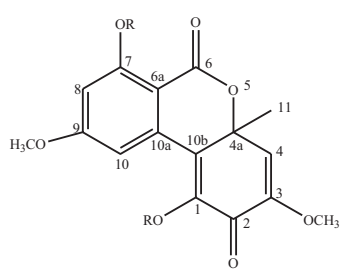
1 Butyrolactone I



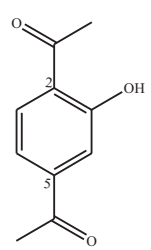
	R	
2	H	Graphislactone A
2a	Ac	Graphislactone A diacetate



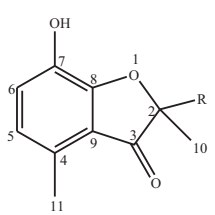
	R^1	R^2	
3	H	H	Ulocladol
3a	H	Ac	
3b	Ac	Ac	



	R	
4	H	Botrallin
4a	Ac	Botrallin diacetate



5 2,5-Diacetylphenol



	R	
6	H	7-Hydroxy-2,4-dimethyl-3(2*H*)-benzofuranone
7	OH	Enalin

Fig. 1. Compounds isolated from *Microsphaeropsis olivacea* (Bonord.) Hohn cultures.

Table III. Antibacterial and antifungal activity, inhibition of the enzyme acetylcholinesterase (AChE) and cytotoxicity against human lung fibroblasts (IC_{50}) of compounds isolated from *Microsphaeropsis olivacea*.

	Erwinia carotovora	*Pseudomonas syringae*	*Botrytis cinerea*	*Alternaria alternata*	Inhibition of AChE	Inhibition of AChE	Cytotoxicity
Compound	IC_{50} [μg/ml]	IC_{50} [μg/ml]	MIC [μg/ml]	MIC [μg/ml]	IC_{50} [μg/ml]	IC_{50} [μM]	IC_{50} [μM]
Butyrolactone I (**1**)	94.5	> 250	250	> 250	> 100	> 236	247
Graphislactone A (**2**)	> 250	> 250	> 250	250	8.1	27	> 1000
Graphislactone A diacetate (**2a**)	> 250	> 250	> 250	250	88	228	> 1000
Ulocladol diacetate (**3a**)	–	–	125	62.5	82	204	> 1000
Υλοχλαδολ τριαχετατε (**3b**)	> 250	> 250	62.5	62.5	37	83	734
Botrallin (**4**)	> 250	> 250	> 250	62.5	6.1	19	330
Botrallin diacetate (**4a**)	> 250	> 250	> 250	250	27	67	773
2,5-Diacetylphenol (**5**)	> 250	> 250	> 250	> 250	–	–	> 1000
7-Hydroxy-2,4-dimethyl-3(2*H*)-benzofuranone (**6**)	> 250	> 250	> 250	> 250	–	–	> 1000
Enalin (**7**)	> 250	> 250	> 250	125	89	459	> 1000
(*R*)-Rukon	–	–	31.3	3.9	–	–	–
(*S*)-Systane	–	–	15.6	31.3	–	–	–
Penicillin G	15.6	122.7	–	–	–	–	–
Streptomycin	11.1	15.6	–	–	–	–	–
Galanthamine	–	–	–	–	3	–	–

–: not done.

The structure of compound **5** followed from the ^{1}H NMR spectrum as well as from MS that indicate an 1,2,5-trisubstituted benzene ring with two $COCH_3$ and a hydroxy group. Related compounds have been reported from *Artemisia campestris* (De Pascual *et al.*, 1981; González *et al.*, 1983). Compound **5** was not found in databases like the Dictionary of Natural Products on CD-ROM (2004) but it was described as a synthesis intermediate (Cao *et al.*, 2002).

The ^{1}H NMR spectrum of compound **6** was similar to that of enalin (**7**), differing mainly due to the presence of a q at δ 4.63 which coupled with a methyl d at δ 1.54, instead of the s at δ 1.53 of enalin. The HRMS of **6** showed a molecular ion at *m/z* 178 which agrees with the molecular formula $C_{10}H_{10}O_3$ and supports the proposed structure. The spectroscopic data assigned to the 7-hydroxy derivative of enalin was not previously reported as a natural product.

Results and Discussion

Some seven compounds belonging to different structural skeletons (Fig. 1) were isolated from *Microsphaeropsis olivacea* grown in liquid as well as in solid media. The enalin derivative **6** is reported for the first time while additional spectroscopic information is provided for the compounds **3a**, **3b** and **4a**.

At 50 μg/disk, compounds **1**–**7** were inactive against the Gram positive bacteria *Bacillus subtilis*, *Micrococcus luteus* and the fungi *Mucor miehei, Paecilomyces variotii,* and *Penicillium notatum.* The IC_{50} value of the compounds was determined against *Erwinia carotovora* and *Pseudomonas syringae* as well as the MIC values towards *Botrytis cinerea* and *Alternaria alternata.* Butyrolactone I (**1**) presented some activity against *E. carotovora* with an IC_{50} of 94.5 μg/ml while none of the compounds can be regarded as promising against *Pseudomonas syringae* (IC_{50} > 250 μg/ml). Ulocladol triacetate (**3b**) was quite active towards *Botrytis cinerea* and *Alternaria alternata*, with MIC values of 62.5 μg/ml while botrallin (**4**) presented a selective effect against *A. alternata* (Table III).

Compounds **2** and **4** presented a moderate activity towards AChE, with IC_{50} of 8.1 and 6.1 μg/ml (27 and 19 μM, respectively). Under the same experimental conditions, the IC_{50} value of the standard inhibitor galanthamine was 3 μg/ml. The cy-

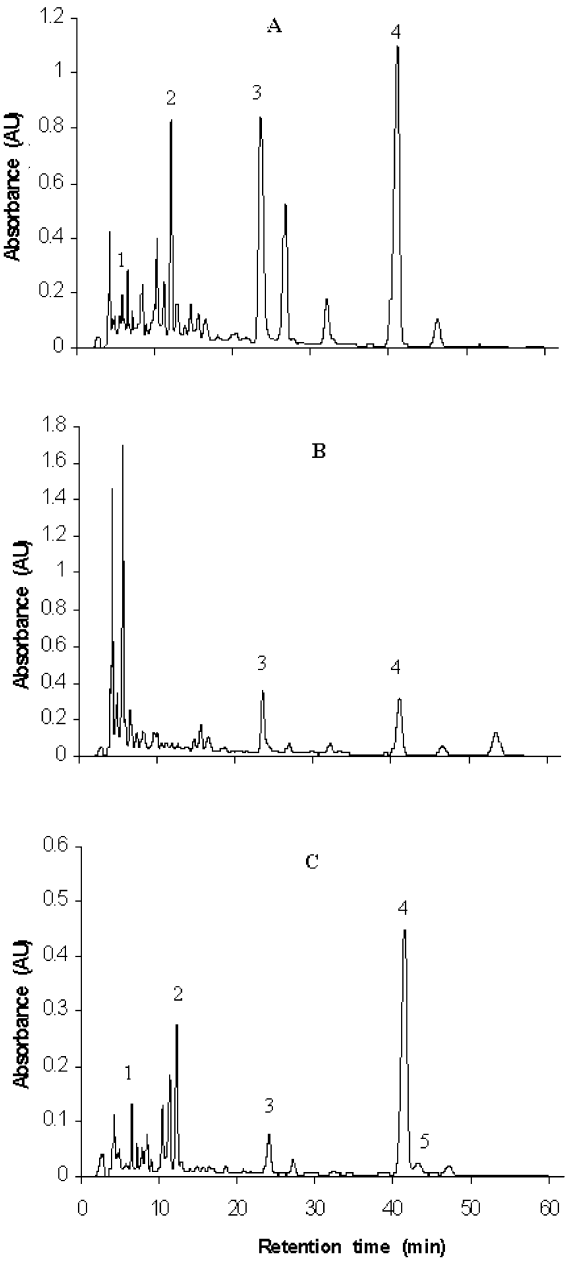


Fig. 2. HPLC trace of *Microsphaeropsis olivacea* extracts grown in different culture media. Liquid media: (A) potato/glucose (PD); (B) yeast extract/malt extract/dextrose (YMG). Solid medium (C): rice. Compounds: 1, enalin; 2, 2,5-diacetylphenol; 3, botrallin; 4, graphislactone A; 5, butyrolactone I.

Table IV. Production of the main secondary metabolite by *Microsphaeropsis olivacea* grown in liquid and solid media. Results are presented as mg/l for the potato-glucose (PG) and yeast extract/malt extract/glucose (YMG) media and as mg/kg for the solid medium (rice). Data are presented as mean + SD.

Compound	R_t [min]	Culture medium		
		PG [mg/l]	YMG [mg/l]	Rice [mg/kg]
Enalin (**7**)	6.64	6.8 ± 0.4	–	215.5 ± 9.5
2,5-Diacetylphenol (**5**)	12.19	29.7 ± 1.2	–	784.3 ± 104.3
Botrallin (**4**)	23.57	174.8 ± 9.9	21.3 ± 1.7	788.7 ± 55.9
Graphislactone A (**2**)	40.37	67.1 ± 3.8	14.8 ± 2.1	3,465.9 ± 330.9
Butyrolactone I (**1**)	42.11	–	–	497.9 ± 51.9

–: not detected.

totoxicity of compounds **2** and **4** were > 1000 and 330 μM, respectively.

Butyrolactone I (**1**) has been previously obtained by Rao *et al.* (2000) from *Aspergillus terreus*. It displayed antibacterial and antifungal activity. It is a selective inhibitor of eukaryotic cell kinases dependant from cyclin. Butyrolactone inhibits the cyclin-dependant kinases cdk1 and cdk2 from mammalian cells with an IC_{50} of 2.6 and 0.8 mM, respectively (Schimmel *et al.*, 1998). Butyrolactone I was able to inhibit several CDK/cyclin complexes *in vitro* as well as *in vivo* (Schütte *et al.*, 1997).

Graphislactone A (**2**) was isolated from a spore culture of the mycobiont lichen *Graphis scripta* var. *pulverulenta* (Tanahashi *et al.*, 1997) and from *G. prunicola* (Tanahashi *et al.*, 2003). Ulocladol (**3**) was previously reported as a metabolite from the fungus *Ulocladium botrytis*, isolated from the sponge *Callispongia vaginalis* (Faulkner, 2001). The compound proved to be active towards the tyrosine kinase ($p56^{lck}$) (Höller *et al.*, 1999a; Kirsch, 1999).

Botrallin (**4**) was first isolated from *Botrytis allii* (Kameda *et al.*, 1974) and *Ulocladium botrytis* (Höller *et al.*, 1999a; Kirsch, 1999). The compound presented activity as an inhibitor of tyrosine kinase (Höller *et al.*, 1999a).

Enalin (**7**) was isolated from the mycoparasite *Coniothyrium minitans* by Machida *et al.* (2001) as well as from *Verruculina enalia*, a fungus growing on decayed wood in a salt lake in the Bahamas (Lin *et al.*, 2002).

Microsphaeropsis olivacea has been reported to produce several biologically active compounds including cerebrosides (Keusgen *et al.*, 1996), unusual fatty acids and its glycerides (Yu *et al.*, 1996; Höller, 1999), microsphaeropsisin, (*R*)-mellein, (3*R*,4*S*)-hydroxymellein, (3*R*,4*R*)-hydroxymellein, 4,8-dihydroxy-3,4-dihydro-(2*H*)-naphthalen-1-one (Höller *et al.*, 1999b), microsphaerones A and B (Wang *et al.*, 2002), preussomerins (Seephonkai *et al.*, 2002), anthraquinones and betaenone derivatives (Brauers *et al.*, 2000).

The fungus *Microsphaeropsis olivacea* has been recently reported as the agent of a human skin infection (Guarro *et al.*, 1999) and as a very rare ocular pathogen (Shah *et al.*, 2001).

The HPLC trace of the crude extracts and the content of the main compounds in different media is shown in Fig. 2. Under our working conditions, the R_t of enalin (**7**), 2,5-diacetylphenol (**5**), botrallin (**4**), graphislactone A (**2**) and butyrolactone I (**1**) was 6.64, 12.19, 23.57, 40.37 and 42.11 min, respectively. The production of the main secondary metabolites by *Microsphaeropsis olivacea* grown in liquid and solid medium is presented in Table IV. High variability in the production of the secondary metabolites associated with the culture medium was observed. The best yields were obtained when the fungus was grown on solid medium.

This is the first report on the production of natural products previously obtained from marine fungi by an endophytic fungus of a native South American gymnosperm. This fact pointed out to the potential of endophytes from native species when looking for new sources of bioactive compounds.

Acknowledgements

E. Hormazabal thanks the Universidad de Talca for a Doctoral grant. We thank the Centro de Investigación en Biotecnología Silvoagrícola de la Universidad de Talca and the Programa "Investigación y Desarrollo de Productos Bioactivos", Universidad de Talca, for financial support.

Bettucci L. and Saravay M. (1993), Endophytic fungi of *Eucalyptus globulus*: a preliminary study. Mycol. Res. **97**, 679–682.

Bills G. F. (1996), Isolation and analysis of endophytic fungal communities from woody plants. In: Endophytic Fungi in Grases and Woody Plants. Systematics, Ecology and Evolution (Redlin S. C. and Carris L. M., eds.). APS Press, St. Paul, MN, USA, pp. 31–65.

Brauers G., Edrada R. A., Ebel R., Proksch P., Wray V., Berg A., Gräfe U., Schächtele C., Totzke F., Finkenzeller G., Marme D., Kraus J., Münchbach M., Michael M., Bringmann G., and Schaumann K. (2000), Anthraquinones and betaenone derivatives from the sponge-associated fungus *Microsphaeropsis* species: novel inhibitors of protein kinases. J. Nat. Prod. **63**, 739–745.

Cao S. X., Bounaud P. Y., Chen H. H., Dumas D. P., Sunil K., Min C., Yang J. Y., and Long M. C. (2002), Preparation of phenol and hydroxynaphthalene inhibitors of protein kinase for the treatment of disease (LG Biomedical Institute, USA). PTC Int. Appl. 286 pp. Coden: PIXXD2 WO 2002096867 A2 20021205 Designated States W.

De Pascual T. J., Bellido I. S., Gonzalez M. S., Muriel M. R., and Hernandez J. M. (1981), Aromatic compounds from *Artemisia campestris* subsp. *glutinosa*. Phytochemistry **20**, 2417–2420.

Dictionary of Natural Products on CD-ROM (2004), Version 12:2. Chapman and Hall/CRC, Boca Raton, FL, USA.

Eloff J. (1998), A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. **64**, 711–713.

Faulkner J. (2001), Marine natural products. Nat. Prod. Rep. **18**, 1–49.

Favre B., Hofbauer B., Hildering K. S., and Ryder N. (2003), Comparison of *in vitro* activities of 17 antifungal drugs against a panel of 20 dermatophytes by using a microdilution assay. J. Clin. Microbiol. **41**, 4817–4819.

González A. G., Bermejo J., Estevez F., and Velásquez R. (1983), Phenolic derivatives from *Artemisia glutinosa*. Phytochemistry **22**, 1515–1516.

Guarro J., Mayayo E., Tapiol J., Aguilar C., and Cano J. (1999), *Microsphaeropsis olivacea* as an etiological agent of human skin infection. Medical Mycology **37**, 133; doi:10.1046/j.1365–280X.1999.00188.x

Höller U. (1999), Isolation, biological activity and secondary metabolite investigation of marine-derived fungi and selected host sponges. Dissertation, TU Braunschweig, Germany. http://www.biblio.tu-bs.de/ediss/data/19990601a/19990601a.html

Höller U., König G., and Wright A. (1999a), A new tyrosine kinase inhibitor from a marine isolate of *Ulocladium botrytis* and new metabolites from the marine fungi *Asteromyces cruciatus* and *Varicosporina ramulosa*. Eur. J. Org. Chem. **11**, 2949–2956.

Höller U., König G., and Wright A. (1999b), Three new metabolites from marine-derived fungi of the genera *Coniothyrium* and *Microsphaeropsis*. J. Nat. Prod. **62**, 114–118.

Kameda K., Aoki H., and Namiki M. (1974), An alternative structure for botrallin a metabolite of *Botrytis allii*. Tetrahedron Lett. **1**, 103–106.

Keusgen M., Yu C. M., Curtis J. M., Brewer D., and Ayer S. W. (1996), A cerebroside from the marine fungus *Microsphaeropsis olivacea* (Bonord.) Höhn. Biochem. Syst. Ecol. **24**, 465–468.

Kirsch G. (1999), Marine Makro- und Mikroorganismen als Quellen für HIV-1-RT und $p56^{lck}$-PTK-Inhibitoren. Dissertation, TU Braunschweig. http://opus.tu-bs.de/opus/volltexte/2000/76/pdf/20000113a.pdf

Lin Y., Wu X., Deng Z., Wang J., Zhou S., Vrijmoed L., and Jones E. B. G. (2002), The metabolites of the mangrove fungus *Verruculina enalia* Nº 2606 from a salt lake in the Bahamas. Phytochemistry **59**, 469–471.

López S., Bastida J., Viladomat F., and Codina C. (2002), Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. Life Sci. **71**, 2521–2529.

Lupo S., Tiscornia S., and Bettucci L. (2001), Endophytic fungi and flowers capsules and seed of *Eucalyptus globulus*. Rev. Iberoam. Mycol. **18**, 38–41.

Machida K., Trifonov L., Ayer W., Lu X., Laroche A., Huang H., Cheng K., and Zantige J. L. (2001), 3(2*H*)-Benzofuranones and chromanes from liquid cultures of the mycoparasitic fungus *Coniothyrium minitans*. Phytochemistry **58**, 173–177.

Marticorena C. and Rodriguez R. (1995), Flora de Chile. Vol. I. Pteridophyta – Gymnospermae. Universidad de Concepción, Concepción, Chile, pp. 321–322.

Petrini O. (1996), Ecological and physiological aspects of host-especificity in endophytic fungi. In: Endophytic Fungi in Grases and Woody Plants. Systematics, Ecology and Evolution (Redlin S. C. and Carris L. M., eds.). APS Press, St. Paul, MN, USA, pp. 87–93.

Rao K. V., Sadhukhan A. K., Veerender M., Ravikumar V., Mohan E. V. S., Dhanvantri S. D., Sitaramkumar M., Babu J. M., Vyas K., and Reddy G. (2000), Butyrolactones from *Aspergillus terreus*. Chem. Pharm. Bull. **48**, 559–562.

Rodríguez J.A. and Haun M. (1999), Cytotoxicity of *trans*-dehydrocrotonin from *Croton cajucara* (Euphorbiaceae) on V79 cells and rat hepatocytes. Planta Med. **65**, 522–526.

Sánchez-Rabaneda F., Jáuregui O., Casals I., Andrés-Lacueva C., Izquierdo-Pulido M., and Lamuela-Raventós R. (2003), Liquid chromatographic/electrospray ionization tandem mass spectometric study of the phenolic composition of cocoa (*Thebroma cacao*). J. Mass Spectrom. **38**, 35–42.

Schimmel T., Coffman A., and Parsons S. (1998), Effect of butyrolactone I on the producing fungus *Aspergillus terreus*. Appl. Environ. Microbiol. **64**, 3707–3712.

Schütte B., Nieland L., Van Engeland M., Henfling M., Meijer L., and Ramaekers F. (1997), The effects of the cyclin-dependent kinase inhibitor olomoucine on cell cycle kinetics. Exp. Cell Res. **236**, 4–15.

Seephonkai P., Isaka M., and Kittakoop P. (2002), Evaluation of antimycobacterial, antiplasmodial and cytotoxic activities of preussomerins isolated from the lichenicolous fungus *Microsphaeropsis* sp. BCC 3050. Planta Med. **68**, 45–48.

Shah C. V., Jones D. B., and Holz E. R. (2001), *Microsphaeropsis olivacea* keratitis and consecutive endophthalmitis. Am. J. Ophthalmol. **131**, 142–143.

Strobel G. and Daisy B. (2004), Bioprospecting for microbial endophytes and their natural products. Microbiol. Molec. Biol. Rev. **67**, 491–502.

Tan R. X. and Zou W. X. (2001), Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. DOI: 10.1039/b100918o.

Tanahashi T., Kuroishi M., Kuwahara A., Nagakura N., and Hamada N. (1997), Four phenolics from the cultured lichen mycobiont of *Graphis scripta* var. *pulverulenta*. Chem. Pharm Bull. **45**, 1183–1185.

Tanahashi T., Takenaka Y., Nagacura N., and Hamada N. (2003), 6*H*-Dibenzo[b,d]pyran-6-one derivatives from the cultured lichen mycobionts of *Graphis* spp., and their biosynthetic origin. Phytochemistry **62**, 71–75.

Wang C.-Y., Wang B.-G., Brauers G., Guan H.-S., Proksch P., and Ebel R. (2002), Microsphaerones A and B, two novel γ-pyrone derivatives from the sponge-derived fungus *Microsphaeropsis* sp. J. Nat. Prod. **65**, 772–775.

Yu C. M., Curtis J. M., Wright J. L., Ayer S., and Fathi-Afshar Z. R. (1996), An unusual fatty acid and its glyceride from the marine fungus *Microsphaeropsis olivacea*. Can. J. Chem. **74**, 730–735.