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# Two naphthalenone pentaketides from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca of grapevine

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**Summary.** Several phytotoxic metabolites were extracted from culture filtrates of *Phaeoacremonium aleophilum*, a fungus associated with the esca of grapevine and related diseases. Two of these metabolites were identified by chemical and spectroscopic methods as scytalone (23.9 mg $l^{-1}$) and isosclerone (2.4 mg $l^{-1}$), two naphthalenone pentaketides already known as fungal metabolites. Assayed on detached leaves of grapevine cv. Italia, scytalone at 0.05 mg $ml^{-1}$ caused light green to chlorotic, rounded to irregular, interveinal or marginal spots, and isosclerone at 0.1 mg $ml^{-1}$ caused large, coalescent chlorotic and necrotic spots followed by distortion of the lamina and withering. This is the first report on the production *in vitro* of scytalone and isosclerone by *P. aleophilum*, and on the phytotoxic activity of these compounds.

**Key words:** *Phaeoacremonium aleophilum*, scytalone, isosclerone, toxins, esca, grapevine.

## Introduction

Several fungi, including basidiomycetes and hyphomycetes, have been associated with esca and related diseases of grapevine (Larignon and Dubos, 1997). In particular, two species of mitosporic fungi, *Phaeoacremonium chlamydosporum* W. Gams *et al.* (1) and *P. aleophilum* W. Gams *et al.*, consistently isolated from the discoloured wood of esca-affected vines, are assumed to cause an internal symptom, the brown-wood streaking of branches and trunk, as well as a decline of young grapevines (Ferreira *et al.*, 1994; Morton, 1997; Scheck *et al.*, 1998; Graniti *et al.*, 1999; Mugnai *et al.*, 1999).

Recent data demonstrated that *P. aleophilum* produces phytotoxic exopolysaccharides (pullulans) *in vitro*. Tested on different cultivars of grapevine, pullulans induced symptoms reminiscent of those shown by the leaves of esca-affected vines (Sparapano *et al.*, 1998, and this issue).

The aim of this study was to characterise two other secondary metabolites of *P. aleophilum* that could be involved in the symptom expression of infected grapevines.

(1) This species has been redisposed in a new genus *Phaeomoniella* Crous et W. Gams as *P. chlamydospora* (W. Gams *et al.*) Crous et W. Gams. See Crous and Gams in this issue.

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## Materials and methods

### General experimental procedure

Optical rotation was measured in methanol on a JASCO P-1010 polarimeter (Jasco, Tokyo, Japan). Unless otherwise noted, IR and UV spectra were

determined in potassium bromide on a Perkin-Elmer IR FT-1720X spectrometer (Perkin-Elmer, Norwalk, CT, USA) and in ethanol on a Perkin-Elmer Lambda 3B spectrophotometer respectively. The $^{1}H$ NMR spectra were recorded at 500 and 250 MHz; and $^{13}C$ NMR spectra at 125 and 62.5 MHz, in $CDCl_3$, unless otherwise noted, on DRX 500 and AM 250 Bruker spectrometers (Bruker, Karlsruhe, Germany). The same solvent was used as an internal standard. Carbon multiplicities were determined by DEPT [Distortionless Enhancement by Polarisation Transfer] spectra (Breitmaier and Voelter, 1987). The DEPT, COSY-45 [Correlated Spectroscopy] (Bax and Freeman, 1981), HMQC [Heteronuclear Multiple Quantum Correlation] (Bax *et al.,* 1990) and HMBC [Heteronuclear Multiple Bond Correlation] (Bax and Summers, 1986) NMR experiments were performed using Bruker microprograms. The EI- and ES-MS were taken at 70 eV, on a Fisons Trio-2000 (VG Instruments, Manchester, UK) and a Perkin-Elmer API 100 LC-MS spectrometer respectively. Analytical and preparative thin layer chromatography (TLC) was performed on silica gel (Kieselgel 60 $F_{254}$, 0.25 and 0.50 mm respectively; Merck, Darmstadt, Germany) or on reverse phase (KC18 $F_{254,}$ 0.20 mm; Whatman, Clifton, NJ, USA) plates; the spots were visualised by exposure to UV radiation or by spraying with 10% $H_2SO_4$ in methanol and then with 5% phosphomolybdic acid in methanol, followed by heating at 110 °C for 10 min. Column chromatography was performed on silica gel Merck (Kieselgel 60, 0.063-0.20 mm).

**Organism and batch cultures**

Stock cultures of *P. aleophilum* (*Pal*) strain PVFi69-257 (CBS 631.94) isolated from a grapevine in Italy were maintained in slants of malt agar at 4 °C. The fungus was grown in stationary cultures in 1 l Roux flasks each containing 150 ml modified Czapek medium with 0.1% yeast and 0.1% malt extract (pH 5.8), at 25 °C. Each flask was seeded with 5 ml of suspension from three 10-day-old cultures in 50 ml sterile water. The flasks were incubated at 25 °C for 28 days in darkness.

**Extraction and purification of toxins**

At harvest, the mycelial mat was removed from each flask by filtration on a Miracloth (Calbiochem, La Jolla, CA, USA) and Millipore filter (Millipore, Bedford, MA, USA, 0.45 μm). The pooled culture filtrate (3.5 l) was brought from pH 5.9 to pH 4 with 2M HCl and was extracted 4 times with ethyl acetate (1.5 l each). The combined organic extracts were dried on anhydrous sodium sulphate and evaporated under reduced pressure, yielding a dark-brown oily residue. This crude residue was chromatographed on a silica gel column, eluted with chloroform:*iso*-propanol (9:1) (solvent system A) to yield 12 groups of homogeneous fractions. All fractions except group 2 showed strong phytotoxicity when assayed on detached leaves of grapevine cv. Italia (see below).

The residue (58 mg) of combined fraction groups 8-9 was further purified by preparative TLC (solvent system A) to give the main metabolite (10 mg, 2.8 mg $l^{-1}$). In TLC analysis on silica gel with three different systems (solvent system A, chloroform:*iso*-propanol, 85:15; and petroleum ether:acetone, 6:4) and in reverse phase (eluent ethanol:water, 6:4) the metabolite appeared as a homogeneous oily compound and was identified as scytalone. Scytalone crystallised as white prisms from a benzene:ethyl ether (95:5) mixture.

Further purification of the residue (60 mg) of the combined fraction groups 4-6 by preparative TLC on silica gel, eluent chloroform:*iso*-propanol (98:2), yielded two homogenous compounds (6.3 and 1 mg, equivalent to 1.8 and 0.3 mg $l^{-1}$ respectively), the lesser of which was identified as isosclerone.

In a second experiment, the same procedure was applied to purify the crude residue (358 mg) obtained from 3 l culture filtrate, yielding 71.7 mg scytalone and 7.2 mg isosclerone, equivalent to 23.9 and 2.4 mg $l^{-1}$ respectively.

**Scytalone identification**

This compound had: m.p. 163-165 °; $[\alpha]^{25}_{D}$ 0 (*c* 0.4); UV: $\lambda_{max}$ nm (log ε) 326 (3.91), 282 (3.99), 236 (shoulder), 219 (4.05); IR: $\nu_{max}$ 3192, 1648, 1592 $cm^{-1}$; $^{1}H$ and $^{13}C$ NMR, see Table 1. EI-MS *m/z* (rel. int.): 194 $[M]^+$ (99), 176 $[M\text{-}H_2O]^+$ (98), 150 $[M\text{-}CH_3CHO]^+$ (98), 122 $[M\text{-}CH_3CHO\text{-}CO]^+$ (45), 69 (100); ES-MS *m/z* 195 $[MH]^+$(100), 177 $[MH\text{-}H_2O]^+$(20), 149 $[MH\text{-}CH_3CH_2OH]^+$ (18).

**Isosclerone identification**

This compound had: $[\alpha]^{23}_{D}$ +22.2 (*c* 0.1); UV $\lambda_{max}$ nm (log ε) 325 (3.08), 253 (3.43), 2.08 (3.73); IR $\nu_{max}$ (neat): 3422, 1638, 1559, 1456, 1359 $cm^{-1}$; $^{1}H$ δ 7.50

(1H, *t,* *J*=7.5 Hz, H-6), 7.02 (1H, *dd,* *J*=7.5 and 1 Hz, H-5), 6.93 (1H, *dd*, *J*=7.5 and 1 Hz, H-7), 4.92 (1H, *dd*, *J*=7.4 and 3.7 Hz, H-4), 3.05 (1H, *ddd*, *J*=17.8, 8.3 and 4.7 Hz, H-2A), 2.65 (1H, *ddd*, *J*=17.8, 8.3 and 4.7 Hz, H-2B), 2.35 (1H, *m*, H-3A), 2.21 (1H, *m*, H-3B). ES MS *m/z* (rel. int.): 179 $[MH]^+$ (23), 161 $[MH\text{-}H_2O]^+$ (67), 133 $[M\text{-}H_2O\text{-}CO]^+$ (33), 151 $[MH\text{-}C_2H_4]^+$ (13), 121 $[MH\text{-}C_2H_4\text{-}CH_2O]^+$ (100).

**1,3,8-Triacetoxynaphthalene preparation**

Scytalone (**1**, 6 mg) was acetylated with dry pyridine (40 µl) and acetic anhydride (40 µl) at room temperature. After 48 h the reaction was stopped by adding methanol, and the pyridine was eliminated by azeotrope with $C_6H_6$. The residue was chromatographed by preparative TLC on silica gel, eluent chloroform:*iso*-propanol (99:1), to give the 3,6,8-O,O',O"-triacetylderivative of **1** (**3**, Fig. 1, 5.7 mg): UV $\lambda_{max}$ nm (log ε) 2.77 (3.68), 220 (3.78); IR (neat) $\nu_{max}$ 1768, 1638, 1608, 1592 $cm^{-1}$; $^1H$ NMR δ 7.73 (1H, *dd*, *J*=8.0 and 1.0 Hz, H-5), 7.56 (1H, *d*, *J*=2.3 Hz, H-4), 7.46 (1H, *t*, *J*=8.0 Hz, H-6), 7.11 (1H, *dd*, *J*=8.0 and 1.0 Hz, H-7), 7.01 (1H, *d*, *J*=2.3 Hz, H-2), 2.39 (6H, *s*, two MeCO), 2.33 (3H, *s*, MeCO); $^{13}C$ NMR δ 169.5, 169.2 and 168.9 (*s*, three CO), 147.8 (*s*, C-3), 145.8 (*s*, C-1), 145.2 (*s,* C-8), 136.4 (*s*, C-10), 126.8 (*d*, C-6), 126.7 (*d*, C-5), 120.4 (*d*, C-7), 119.2 (*s*, C-9), 117.4 (*d*, C-4), 116 (*d*, C-2), 21.2 (*q*, three Me-CO). ES MS *m/z* (rel. int.) 303 $[MH]^+$ (61), 261 $[MH\text{-}CH_2CO]^+$ (35), 243 [MH-AcOH]$^+$ (100), 219 $[MH\text{-}2xCH_2CO]^+$ (28), 201 $[MH\text{-}CH_2CO\text{-}AcOH]^+$ (27), 177 $[M\text{-}3xCH_2CO]^+$ (63).

**Grapevine bioassay**

Samples of culture filtrate of *Pal*, crude organic extracts, and the purified components scytalone and isosclerone, were assayed on detached leaves of grapevine cv. Italia. The leaves with their petioles were immersed in 3 ml toxic solution until complete absorption, which usually took a few hours, and were then transferred to distilled water. Toxicity symptoms were recorded 48 h later. Throughout the assay, the leaves were kept in a growth chamber at relatively low temperature (23 °C), RH (60%), and illumination (150 µE $m^{-2}$ $s^{-1}$).

The culture filtrate was assayed after 1:100 dilution with distilled water. The crude extracts from culture filtrate were tested at 0.05, 0.1 and 1 mg $ml^{-1}$. The purified compounds scytalone and isosclerone were assayed at 0.01, 0.05 and 0.1 mg $ml^{-1}$. Controls included distilled water and 1:100 diluted culture medium**.**

## Results

**Scytalone [3,4-dihydro-3,6,8-trihydroxy-1(2H)-naphthalenone] (1**, Fig. 1**)**

Preliminary TLC analysis of crude organic extracts from culture filtrate of *Pal,* performed on silica gel using solvent system A, showed the presence of several metabolites, the most important of which, scytalone, had a $R_f$ of 0.40.

The oily residue (360 mg) extracted from 3.5 l culture filtrates was chromatographed on a silica gel column to give 12 groups of homogenous fractions, of which only the fraction group 2 did not exhibit phytotoxicity.

The residue of the combined fraction groups 8-9 was further purified by preparative silica gel TLC to give as its main metabolite a homogeneous oil (10 mg, equivalent to 2.8 mg $l^{-1}$), which crystallised as white prisms from a benzene-ethyl ether (95:5) mixture.

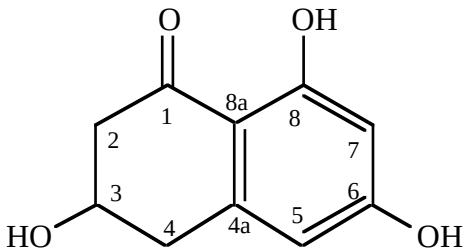
In a second batch-culture experiment, a higher yield (23.9 mg $l^{-1}$) of this compound was obtained. Its was identified as scytalone, a naphthalenone pentaketide.

Both the physical (m.p. and $([\alpha]_D$) and the spectroscopic (IR, UV, $^1H$ and $^{13}C$ NMR) data were in good agreement with those already reported for this compound (Findlay and Kwan, 1973; Aldridge *et al.*, 1974; Bell *et al.*, 1976a; McGraw and Hemingway, 1977). Furthermore, 1 and 2D $^1H$ and $^{13}C$ NMR data, in particular the COSY, HMQC, and HMBC spectra, allowed us to attribute the chemical shifts to all the protons and corresponding carbons (Table 1).

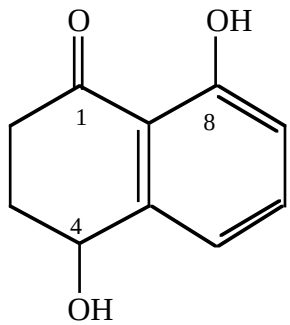
**Isosclerone [3,4-dihydro-4,8-dihydroxy-1(2H)-naphthalenone] (2**, Fig. 1**)**

Two minor metabolites (6.3 and 1 mg, equivalent to 1.8 and 0.3 mg $l^{-1}$ respectively) were purified from the residue (60 mg) of fraction groups 4 to 6 of the initial column. The second of these metabolites was identified as isosclerone (**2**). Its spectroscopic data (IR, UV $^1H$ NMR and ES-MS) were in very good agreement with those reported in the literature (Findlay and Kwan, 1973a; Morita and Aoki, 1974).

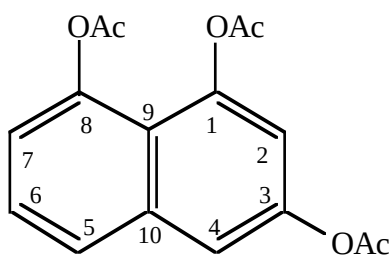
From the second batch-culture, compound **2** was obtained at a concentration eight times higher (2.4 mg $l^{-1}$).




**1**




**2**




**3**

Fig. 1. Structure formulae of scytalone (**1**), isosclerone (**2**), and 1,3,8-triacetoxynaphthalene (**3**).

Isosclerone is closely related to scytalone. It is known as a metabolite of *Scytalidium* sp. (Findlay and Kwan, 1973a) and *Sclerotinia sclerotiorum* (Lib.) de Bary, and affects plant growth (Morita and Aoki, 1974).

**1,3,8-Triacetoxynaphtalene (3**, Fig. 1**)**

The structure assigned to scytalone was confirmed by its conversion to 1,3,8-triacetoxynaphtalene (**3**). Scytalone was dissolved in pyridine by a reaction with acetic anhydride at room temperature, for a time (48 h), longer than that reported in the literature (Findlay and Kwan, 1973), to achieve a complete aromatisation of the molecule. The spectroscopic data of derivative **3**, integrated with the data of the $^{13}C$ NMR spectrum, were in full agreement on those reported in the literature (Findlay and Kwan, 1973).

**Biological activity**

The crude organic extract from the culture filtrates of *Pal*, when assayed at concentrations ranging from 1 to 0.05 mg $ml^{-1}$, was toxic to grapevine cv. Italia leaves. The fractions collected from the chromatographic column also proved to be phytotoxic.

Symptoms appeared on the foliar lamina 6-8 h after absorption of the toxic solutions (up to 0.1 mg $ml^{-1}$), and their severity increased during the next two days. The leaves first showed irregular, pale green areas located among the main veins or at the leaf margins. These areas subsequently became chlorotic, then necrotic, turning yellow-brown or red-brown. Finally, the affected tissue dried and the entire lamina was detached from the petiole.

When scytalone was assayed at 0.05 mg $ml^{-1}$ on leaves of the same grapevine cultivar, light green to chlorotic, round to irregular, interveinal or marginal spots eventually becoming coalescent or diffused to large areas were produced on the leaf lamina (Fig. 2A).

Similarly, isosclerone assayed at 0.1 mg $ml^{-1}$ caused large yellowish spots, slowly becoming coalescent and necrotic, followed by distortion and withering of the leaf lamina (Fig. 2B).

## Discussion

This is the first report on the production *in vitro* of scytalone and isosclerone by *P. aleophilum* ([2]), and on the phytotoxic activity of these compounds.

Scytalone and isosclerone, as well as other closely related naphthalene pentaketides, are known as fungal metabolites (Stipanovic and Bell,

([2]) Recently, *P. chlamydosporum* has been found to produce in culture the same two naphthalenone pentaketides (Tabacchi, this issue).

Table 1. $^{1}H$ and $^{13}C$ NMR data ($CDCl_3/CD_3OD$, 99/1) for scytalone.

| Scytalone[a] | | | | | | |
|---|---|---|---|---|---|---|
| C | δ | m[b] | δH | *m* | *J*(Hz) | HMBC |
| 1 | 200.8 | *s* | | | | 4.21, 2.81, 2.59 |
| 2 | 46.2 | *t* | 2.81 | *d* | 17.1 | 3.01, 2.78 |
| | | | 2.59 | *dd* | 17.1, 8.3 | |
| 3 | 65.3 | *d* | 4.21 | *m* | | 3.01, 2.81, 2.78, 2.59 |
| 4 | 37.9 | *t* | 3.01 | *dd* | 16.0, 4.0 | 2.81, 2.59 |
| | | | 2.78 | *dd* | 16.0, 3.5 | |
| 4a | 144.2 | *s* | - | - | - | 4.21, 3.01, 2.78 |
| 5 | 108.4 | *d* | 6.16 | *d* | 2.4 | |
| 6 | 164.7[c] | *s* | | | | |
| 7 | 100.8 | *d* | 6.11 | *d* | 2.4 | |
| 8 | 164.8[c] | *s* | | | | |
| 8a | 110.4 | *s* | | | | 6.16, 6.11, 3.01, 2.78 |

[a] The chemical shifts are in δ-values (ppm) from TMS. 2D $^{1}H$, $^{1}H$ (COSY) and 2D $^{13}C$, $^{1}H$ (HMQC) NMR experiments delineated correlations of all protons and corresponding carbons.
[b] Multiplicities were determined by DEPT spectrum.
[c] These attribution can be exchanged.

1976; Turner and Aldridge, 1983; Ayer *et al.,* 1989, 1993). Both are considered precursors to melanin (Bell *et al.*, 1976; Wheeler *et al.*, 1976; Butler and Day, 1998).

Scytalone is produced by several microscopic fungi, including some plant pathogens. The compound purified from *P. aleophilum* showed physical and spectroscopic properties very similar to those reported in the literature for scytalone preparations from *Scytalidium* sp. (Findlay and Kwan, 1973), *Phialophora lagerbergii* (Melin et Nannf.) Conant (Aldridge *et al.*, 1974), *Verticillium dahliae* Kleb. (Bell *et al.*, 1976a) and *Ceratocystis minor* (Hedgc.) Hunt. (McGraw and Hemingway, 1977). It proved

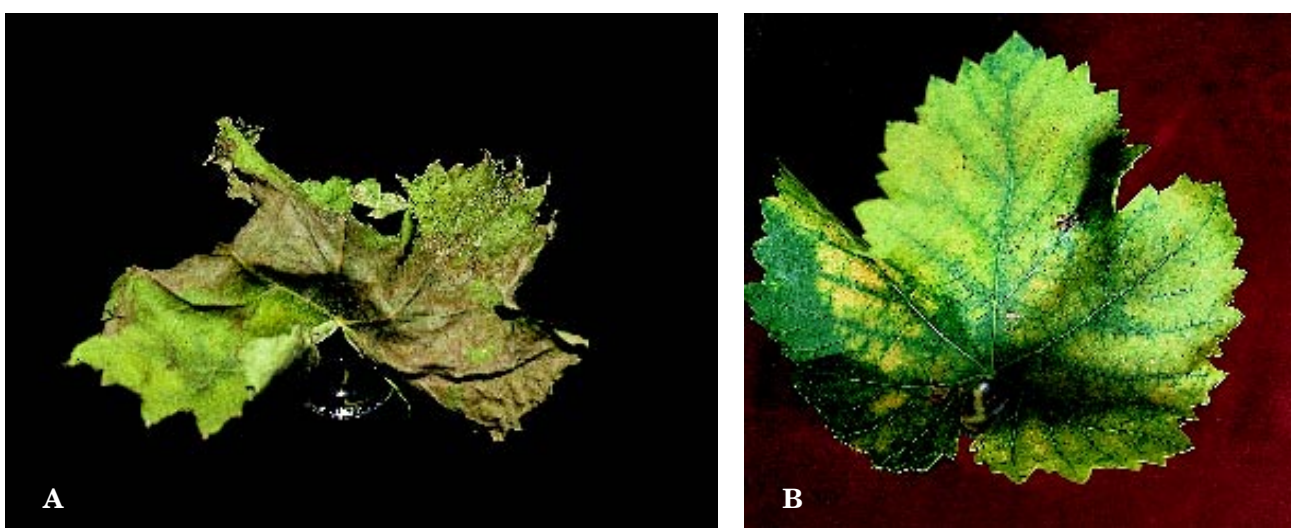



Fig. 2. Effect of the absorption for a few hours of 3 ml of 0.05 mg $ml^{-1}$ scytalone (A) or 0.1 mg $ml^{-1}$ isosclerone (B) on detached leaves of grapevine cv. Italia.

to be (+)-scytalone since its optical rotation was identical to that described for the enantiomer produced by *P. lagerbergii* and *Scytalidium* sp.

The spectroscopic data of isosclerone were also in agreement with those reported in the literature (Findlay and Kwan, 1973a; Morita and Aoki, 1974). In particular, the high resolution conditions (500 MHz) used to record its $^1$H NMR spectrum allowed us to assign the chemical shift and the multiplicity to the protons of the two methylene groups of the cyclohexenone ring. Moreover, the optical rotation of isosclerone ($[\alpha]^{23}_D$+22.2°) was in agreement with that ($[\alpha]^{15}_D$+19°) reported for the (+)-enantiomer produced by *Sclerotinia sclerotiorum* (Lib.) De Bary (Morita and Aoki, 1974), and ruled out its identity with regiolone, the (-)-enantiomeric metabolite ($[\alpha]^{23}_D$-3.3°) isolated from *Juglans regia* L. (Talapatra *et al.*, 1988). A further confirmation of this result came from the different $^1$H NMR data recorded for **2** in comparison with those reported for (-)-regiolone (Talapatra *et al.*, 1988), and in particular the constant measured for the coupling of H-4 with the two protons of $H_2$C-3.

The occurrence of scytalone and isosclerone as phytotoxic metabolites of *P. aleophilum* can contribute to a better understanding of the development of symptoms in esca and related diseases of grapevine (Mugnai *et al.*, 1999; Sparapano *et al.*, this issue). Assayed on detached leaves of the host plant, both compounds caused symptoms similar to those shown by the leaves of vines with brown wood-streaking. Brown wood-streaking is associated with wood infection by *P. aleophilum* and *P. chlamydosporum* (Graniti *et al.*, this issue).

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