JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Botrytone, a New Naphthalenone Pentaketide Produced by *Botrytis fabae*, the Causal Agent of Chocolate Spot Disease on *Vicia faba*

Alessio Cimmino,[†,‡] Angel M. Villegas-Fernández,[†] Anna Andolfi,[‡] Dominique Melck,[§] Diego Rubiales,[†] and Antonio Evidente*,[‡]

[†]Institute for Sustainable Agriculture, CSIC, Apartado 4084, 14080 Córdoba, Spain

[‡]Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy

[§]Istituto di Chimica Biomolecolare del CNR, Comprensorio Olivetti, Edificio 70, Via Campi Flegrei 34, 80078 Pozzuoli, Italy

ABSTRACT: A strain of *Botrytis fabae* isolated from faba bean (*Vicia faba* L.) plants displaying clear chocolate spot disease symptoms produced phytotoxic metabolites in vitro. The phytotoxins isolated from the culture filtrate organic extract were characterized by spectroscopic and optical methods. A new naphthalenone pentaketide, named botrytone, was isolated and characterized as (4*R*)-3,4-dihydro-4,5,8-trihydroxy-1(2*H*)-naphthalenone together with other well-known closely related naphthalenones such as regiolone and *cis*- and *trans*-3,4-dihydro-2,4,8-trihydroxynaphthalen-1(2*H*)-ones. When tested on leaves of the host plant, with the *cis*- and *trans*-3,4-dihydro-2,4,8-trihydroxynaphthalen-1(2*H*)-ones assayed in mixture, regiolone demonstrated the highest level of phytotoxicity together with *cis*- and *trans*-3,4-dihydro-2,4,8-trihydroxynaphthalen-1(2*H*)-ones. Botrytone showed moderate phytotoxic activity at 1 mg/mL and was still phytotoxic at 0.5 mg/mL.

KEYWORDS: *Botrytis fabae*, *Vicia faba*, phytotoxins, naphthalenone pentaketides, botrytone

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the oldest legume crops mainly grown as a valuable protein-rich food for both human food and animal feed.[1] Among the several biotic stresses it is subjected to, chocolate spot stands out as one of the most destructive diseases.[2] The pathogen is the necrotrophic fungus *Botrytis fabae* Sard., which is present in nearly all faba bean cultivation areas. The first symptoms are discrete dark brown spots surrounded by an orange-brown ring on leaves, flowers, and stems. When temperatures are mild (15−22 °C) and relative humidity is high (>80%), the limited-lesion stage may be followed by an aggressive phase in which necrosis spreads rapidly, defoliating and then killing the plant, sometimes within as little as 2 days.[3]

B. fabae kills plant cells as it grows inside its host. This process probably is carried out by means of the production of enzymes and phytotoxins that degrade plant tissue.[3] As far as we know, no phytotoxin produced by *B. fabae* has been identified so far. Only Harrison[3] reported the presence of some metabolites with phytotoxic activity in extracts from infected leaf tissue and from liquid cultures of *B. fabae*, but did not carry through full isolation and identification of these compounds.

The objective of this work was to identify metabolites produced by *B. fabae* that possess phytotoxic activity on *V. faba*.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotation was measured in EtOH, unless otherwise noted, on a JASCO (Tokyo, Japan) polarimeter, whereas the CD spectrum was recorded on a JASCO J-175 in MeOH; ^{1}H and ^{13}C NMR spectra were recorded at 600 or 300 MHz and at 125 or 75 MHz, respectively, in $CDCl_3$, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra. DEPT, correlation spectroscopy (COSY)-45, heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed using standard Bruker microprograms. ESI and HRESI MS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent 1100 coupled to a JEOL AccuTOF (JMS-T100LC) (Milford, MA) instrument. Analytical and preparative thin layer chromatographies (TLC) were performed on silica gel, Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively (Merck, Darmstadt, Germany) plates. The spots were visualized by exposure to UV radiation (254 nm) or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on a silica gel column, Kieselgel 60, 0.063−0.200 mm (Merck). Isoclerone and scytalone were isolated from the culture filtrates of *Phaeoacremonium aleophilum* as previously reported.[4]

Fungal Strain, Culture Medium, and Growth Conditions. A monoconidial isolate of *B. fabae*, isolated from infected faba bean tissues collected in a Cordoba field and deposited in the Collection of Institute for Sustainable Agriculture, CSIC, Cordoba, Spain, No. Bf-CO-05, was identified as *Botrytis fabae* on the basis of morphological traits, mainly the size of macroconidia. These traits do not correspond with those of *Botrytis fabiopsis* as they have been reported.[5] The fungus was grown in V8 medium (270 mL of V8 juice, 30 mL of deionized water, and 3 g of agar, at pH 5.5) for 7 days at 19 °C under a cycle of 12 h of darkness and 12 h of exposure to visible light plus near-UV radiation, until a carpet of sporulating mycelium was clearly visible. A spore suspension

Received: May 25, 2011
Revised: July 21, 2011
Accepted: July 22, 2011
Published: July 22, 2011

 © 2011 American Chemical Society

Table 1. ^{1}H and ^{13}C NMR Data of Botrytone (1)[a]

position	δC[b]	δH	J (Hz)
1	203.1 s		
2	35.5 t	2.84 ddd	17.2, 9.8, 4.7
		2.64 ddd	17.2, 12.9, 4.7
3	31.8 t	2.45 dddd	14.7, 12.9, 9.8, 4.8
		2.19 ddt	14.7, 9.6, 4.7
4	67.9 d	5.28 dd	9.6, 4.8
4a	126.2 s		
5	147.2 s		
6	126.9 d	7.09 d	9.0
7	118.7 d	6.86 d	9.0
8	156.6 s		
8a	114.6 s		

[a] The chemical shift are in δ values (ppm) from TMS. [b] Multiplicities determined by DEPT spectra.

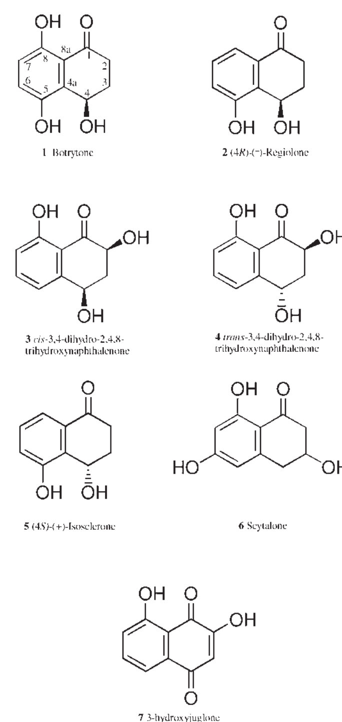

Figure 1. Structures of botrytone, **1**; regiolone, **2**; *cis*- and *trans*-2,4,8-trihydronaphthalenones, **3** and **4**, isolated from *B. fabae*, and of the closely related naphthalenones isosclerone, **5**; scytalone, **6**; and 3-hydroxyjuglone, **7**.

(1×10^{6} spores/mL) was obtained from these cultures and added to 250 mL flasks (1 mL per flask) containing 100 mL of a modified Czapek−Dox medium (5% glucose, 0.1% yeast extract, 0.05% K_2HPO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$). These liquid cultures were incubated for 24 days at 20 °C in the dark on an orbital shaker at 200 rpm. Then the content of the flasks was centrifuged at 7000 rpm, and the supernatant was filtered and lyophilized.

Extraction and Purification of Phytotoxic Naphthalenone Pentaketides. The lyophilized culture filtrates (3.5 L) were dissolved in $^{1}/_{10}$ of the initial volume with distilled water and extracted with EtOAc (3×500 mL). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure to yield a brown solid residue (428 mg). The residue tested at a concentration of 5 mg/mL as described below was found to be phytotoxic against *V. fabae*, and it was then submitted to bioassay-guided fractionation through column chromatography (750 mm × 30 mm) on silica gel, eluted with $CHCl_3$/*i*-PrOH (9:1, v/v). Two hundred tubes (10 mL each) were collected, monitored by TLC using the same solvent, and combined. Nine homogeneous fraction groups were collected and screened for their phytotoxic activity. The residue (31.1 mg) of a third fraction was further purified by two successive steps by TLC on silica gel, eluted with the $CHCl_3$/*i*-PrOH (92:8, v/v) and petroleum ether/acetone (6:4, v/v), yielding a homogeneous yellow amorphous solid [**1**, R_f 0.50, eluent $CHCl_3$/*i*-PrOH (92:8, v/v), R_f 0.42, eluent petroleum ether/acetone (6:4, v/v), 7.7 mg, 2.2 mg/L], named botrytone. The residue (36.0 mg) of the second fraction, containing the main metabolite, was further purified by TLC on silica gel, eluted with the $CHCl_3$/*i*-PrOH (94:6, v/v), yielding a homogeneous amorphous solid (**2**, R_f 0.44, 22.0 mg, 6.3 mg/L), which slowly crystallized from $CHCl_3$ and was identified as regiolone. The residue (57.3 mg) of a fourth fraction was further purified by CC on silica gel, eluted with $CHCl_3$/*i*-PrOH (9:1, v/v). The residue of the first fraction of the last column (23 mg) was purified by TLC on silica gel, eluted with EtOAc/*n*-hexane (6:4, v/v), and the main fraction of the latter (10.0 mg) was further purified by TLC on silica gel eluted with $CHCl_3$/*i*-PrOH (9:1, v/v), yielding a homogeneous amorphous solid [**3** and **4**, R_f 0.45, eluent EtOAc/*n*-hexane (6:4, v/v); R_f 0.47, eluent $CHCl_3$/*i*-PrOH (9:1, v/v), 8.8 mg, 2.5 mg/L], which slowly crystallized from $CHCl_3$, identifed as a mixture in a ratio of 1:3 of *cis*- and -*trans*-3,4-dihydro-2,4,8-trihydroxynaphthalen-1(2*H*)-ones, and an orange polar metabolite [**7**, 1.2 mg, R_f 0.1, eluent $CHCl_3$/*i*-PrOH (9:1, v/v)], which was identified as 3-hydroxyjuglone.

Botrytone (1). $[\alpha]^{25}_{D}$ −20 (*c* 0.15); CD (*c* 7.68×10^{-5}, 25 °C) $[\theta]_{266}$ +2451.0 (for the (4*S*)-3,4-dihydro-4,5,8-trihydroxy-1(2*H*)-naphthalenone, lit. ref 6, $[\alpha]^{25}_{D}$ +13 (*c* 0.1, EtOH); lit. ref 7, $[\alpha]^{25}_{D}$ +12 (*c* 0.3 EtOH) CD (*c* 7.68×10^{-5}, 25 °C) $[\theta]_{266}$ −2418.8); UV λ_{max} nm (log ε), 236 (4.0), 266 (3.7) 374 (3.5) (for the (4*S*)-3,4-dihydro-4,5,8-trihydroxy-1(2*H*)-naphthalenone, lit. ref 6, UV λ_{max} nm (log ε), 235 (4.0), 265 (3.7) 372 (3.5); ^{1}H and ^{13}C NMR spectra, see Table 1; HRESI MS(−) spectrum, *m/z* 193.0512 [$C_{10}H_9O_4$, calcd 193.0501, M − H]$^{-}$, 175 [M − H − H_2O]$^{-}$.

Regiolone (2). $[\alpha]^{25}_{D}$ −7 (*c* 0.66) (lit. ref 8, $[\alpha]_{D}$ −3.3° (*c* 0.077, EtOH); ^{1}H and ^{13}C NMR spectra were very similar to those previously reported;[7] ESI MS(+), *m/z* 217 [M + K]$^{+}$, 201 [M + Na]$^{+}$, 179 [M + H]$^{+}$, 160 [M − H_2O]$^{+}$.

***cis*- and *trans*-3,4-Dihydro-2,4,8-trihydroxynaphthalen-1(2*H*)-ones (3 and 4).** Mixture in a ratio of 1:3; ^{1}H and ^{13}C NMR spectra were very similar to those previously reported;[9,10] ESI MS(+), *m/z* 217 [M + Na]$^{+}$, 195 [M + H]$^{+}$.

3-Hydroxyjuglone (7). NMR data were very similar to those previously reported;[11] ESI MS(−), *m/z* 189 [M − H]$^{-}$.

Bioassays. Organic extract from the liquid culture filtrates of *B. fabae*, the chromatographic fractions, and pure compounds botrytone, regiolone, *cis*- and *trans*-3,4-dihydro-2,4,8-trihydronaphthalenone (**1**−**4**), and 3-hydroxyjuglone (**7**) were bioassayed on *V. faba*. The general procedure for all bioassays was as follows: plants of *V. faba* (var. Baraka) were grown in 1 L plastic pots filled with a mixture of sand and peat and grown for 3 weeks (until they had seven expanded leaves) in a growth chamber at 20 °C with a photoperiod of 14 h of visible light (150 μmol/m^{2}/s photon flux density) and 10 h of darkness. Then, one leaflet was detached from the fifth leaf of each plant, and the epidermis of its abaxial surface was removed. The leaflets were then placed onto a square Petri dish (15 × 15 cm) containing water−agar (0.4% w/v) medium, the abaxial surface facing up. Samples were dissolved in MeOH and brought to a final concentration of 1 mg/mL with distilled water; botrytone was additionally tested at three different concentrations (1.0, 0.5, and 0.25 mg/mL). The concentration of MeOH was 5% v/v, which is nontoxic to leaves of *V. faba* in the control. A drop of test

solution (20 μL) was placed onto each leaflet. In all cases, the dishes were subsequently covered with their lids and kept in darkness at 20 °C for 72 h, after which they were exposed to a photoperiod of 14 h of visible light and 10 h of darkness as described above for growing plants. Phytotoxicity was recorded as the area of the necrosis surrounding the site of application of the inoculum drops. Assessments were made at 24, 48, 72, and 96 h after treatment.

Each leaflet from a single plant was considered a replication. Organic extract and chromatographic fractions were tested in three to four replications. Pure compounds were tested in eight replications, whereas botrytone was tested in triplicate at three different concentrations.

Statistical Analysis. Analyses of variance (ANOVA) were carried out for phytotoxicity records, with the different tested solutions and metabolites as fixed factors of their respective bioassays. Phytotoxicity values were transformed using the square root transformation to increase the normality of their distribution. Means were compared by least significance difference (LSD) tests at $p < 0.05$.

All statistical analyses were performed using Statistix 8 (Analytical Software, Tallahassee, FL).

RESULTS AND DISCUSSION

The liquid culture filtrate of *B. fabae* was exhaustively extracted with ethyl acetate, and the organic extract, showing high phytotoxic activity on the host plant, was purified by combined column and TLC as described under Materials and Methods, affording four different metabolites, **1**−**4** (Figure 1), all as homogeneous amorphous solids and two, **3** and **4**, in a mixture (ratio 1:3). The preliminary NMR investigations, both ^{1}H and ^{13}C NMR spectra, showed that the four metabolites are all closely related and belong to the family of naphthalenone polyketides, which are very well-known as bioactive fungal and plant metabolites.[12,13]

In particular, the metabolite **1**, having a molecular weight of 194 as deduced from its HR ESI MS spectrum, showed ^{1}H and ^{13}C NMR spectra (Table 1) very similar to those of a racemic mixture of this triol prepared by selective reduction of 5-hydroxyjuglone in a research project aimed to prepare some bisepoxides, closely related dimers with antitumor activity, isolated from a culture broth of *Nattrassia mangiferae*.[14] In particular, the ^{1}H NMR spectrum of **1** showed the presence of a para-disubstituted benzene ring for the presence of the two ortho-coupled protons (H-6 and H-7) appearing as two doublets (J = 9.0 Hz) at δ 7.09 and 6.86, respectively.[15] The two substituents were two hydroxy groups as shown by the typical chemical shift values of the geminal carbons resonating in the ^{13}C NMR spectrum at δ 156.6 and 147.2 (C-8 and C-5) as well as those of the respective *ortho*-located carbons at δ 118.7 and 114.6 (C-7 and C-8a) and 126.9 and 126.2 (C-6 and C-4a), respectively.[16] The hydroxy group at C-8 was hydrogen bonded to the adjacent carbonyl group (O═C-1) as shown by the typical singlet observed in the proton spectrum at δ 12.02.[15] The carbonyl group, which appeared in the ^{13}C NMR spectrum as expected at δ 203.1,[16] belonged to an α,β-unsaturated cyclohexenone ring joined to the *p*-dihydroxy-substituted benzene as shown by other signal systems observed in both ^{1}H and ^{13}C NMR spectra. In fact, the ^{1}H NMR spectrum showed two doublet of double doublets (J = 17.2, 9.8, and 4.7 Hz and J = 17.2, 12.9, and 4.7 Hz) at δ 2.84 and 2.64, which are typical chemical shift values of the two nonequivalent ketomethylene (H_2C-2) protons.[15] The latter were coupled with the protons of another methylene group (H_2C-3) appearing as doublets of double doublets (J = 14.7, 12.9, 9.8, and 4.8 Hz) and a doublet of double triplets (J = 14.7, 9.6, and 4.7 Hz) at δ 2.45 and 2.19. These latter protons, in turn, also

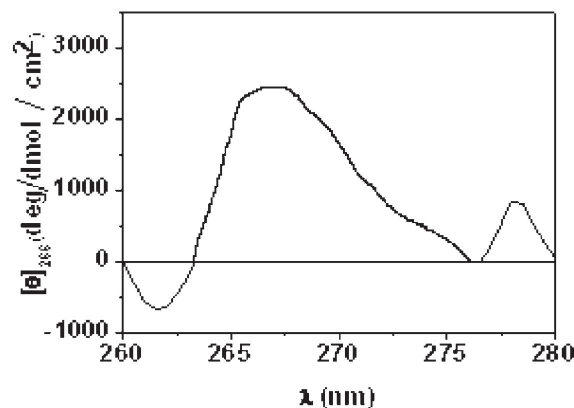

Figure 2. CD spectrum of botrytone recorded in MeOH.

coupled with the proton (H-4) of a secondary hydroxylated carbon (C-4) appearing as a double doublet (J = 9.6 and 4.8 Hz) at the typical chemical shift value of δ 5.28.[15]

These partial structures were confirmed by the typical chemical shift values observed for the two methylene and the methyne carbons of the α,β-unsaturated cyclohexenone ring observed in the ^{13}C NMR spectrum at δ 35.5, 31.8, and 67.9 (C-2, C-3, and C-4).[16] On the basis of these data metabolite **1** was identified as 3,4-dihydro-4,5,8-trihydroxynapthalenone, and its structure was confirmed by the peaks observed in the HRESI mass spectrum, recorded in negative ion mode, at m/z 193.0512 and 175 for the pseudolecular ion $[M - H]^-$ and the ion $[M - H - H_2O]^-$ generated from it by loss of water. It was also known as 4,5,8-α-trihydroxytetralone, when it was isolated as the 4*S* stereoisomer from *Juglans sigillata*,[17] together with (4*S*)-4-hydroxy-α-tetralone, (4*S*)-4,5-dihydroxy-α-tetralone (1*S*)-1,2,3,4-tetrahydro-4-oxonaphthalen-1-yl-6-*O*-[(3,4,5-trihydroxyphenyl)carbonyl]-β-D-glucopyranoside, (1*S*)-1,2,3,4-tetrahydro-8-hydroxy-4-oxo-naphthalen-1-yl-6-*O*-[(3,4,5-trihydroxyphenyl)carbonyl]-β-D-glucopyranoside, and three new α-tetralone galloylglucosides. It was also isolated as three 5-*O*-galloylglucosides[6] and then as 4-*O*- and 5-*O*-glucosides from *Juglans madshurica*,[7] together with other galloyl glucosides and glucosides of different naphthalenones and flavanoids. The two above-cited plants belong to the *Juglans* genus (Juglandaceae), which comprise about 20 species widely distributed in temperate and subtropical areas of the world.[18] The seeds of the *Juglans* species, particularly walnuts (*Juglans regia*), are excellent sources of unsaturated fatty acids and polyphenols and are used as remedies for cancer, kidney, and stomach diseases in Asia and Europe. The absolute 4*S* configuration to 4,5,8-trihydroxytetralone was assigned by circular dichroism study carried out on its tribenzoate.[5] As expected, the spectroscopic data (^{1}H and ^{13}C NMR) of **1** were very similar to those of 4,5,8-trihydrotetralone, but the ^{1}H NMR of the latter, recorded in $CDCl_3$ plus CD_3OD, significantly differed in the coupling of H-4 (J = 4.4 Hz), which resonated as a triplet at δ 5.27[5] instead of a double doublet (J = 9.6 and 4.8 Hz) at δ 5.28 observed for **1** when the same spectrum was recorded in $CDCl_3$. The same latter couplings were observed for H-4 in the corresponding 5-*O*-galloyl glucoside, and the hydroxy group at C-4 is α-oriented axial positioned and its geminal proton consequently β-equatorial with the cyclohexanone ring that assumes a half-chair conformation.[6] Consequently, the same orientation of the hydroxyl group at C-4 should be suggested for 3,4-dihydro-4,5,8-trihydroxynaphthalenone (**1**) isolated from *B. fabae*, in which

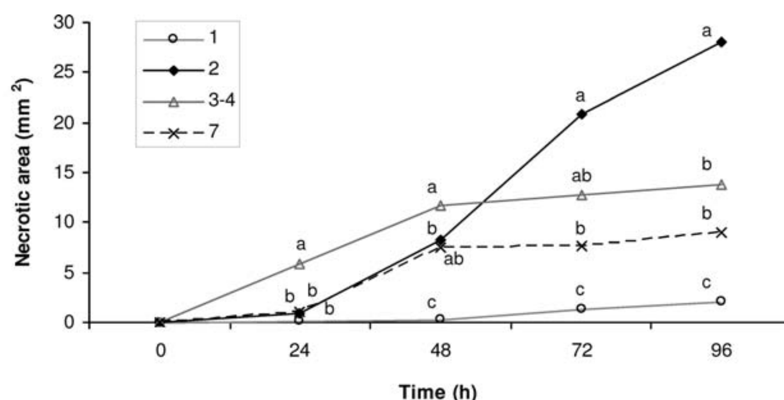

Figure 3. Necrotic area (mm^2) incited by metabolites **1**, **2**, **3**, **4** and **7** at different times (h) after treatment of leaves of *V. faba*. Values followed by the same letter at each time are not significantly different (LSD test, $p < 0.05$).

the cyclohexenone ring should assume the same a half-chair conformation.

However, when the optical rotation of **1** (levorotatory, $[\alpha]^{25}_{D}$ -20 (c 0.15), recorded in the same conditions, was compared with that of (4*S*)-4,5,8-trihydrotetralone (dextrorotatory, $[\alpha]^{25}_{D}$ +13;[5] $[\alpha]^{25}_{D}$ +12[7]), it appeared quite equal in value but opposite in sign. This result indicated that the two compounds are enantiomers and that botrytone has a 4*R*-configuration. This absolute configuration was confirmed by recording the CD spectrum (Figure 2), which, recorded in the same conditions, showed a positive Cotton effect at 266 nm ($[\theta]_{266}$ +2451.0) nm, opposite to the negative one ($[\theta]_{266}$ -2418.8) reported for (4*S*)-4,5,8-trihydroxytetralone.[7] This result was also supported by the fact that their spectroscopic properties are very similar.[6,7] Therefore, **1** from *B. fabae* appeared to be a new phytotoxic fungal metabolite, which was named botrytone.

Th result did not surprise us, as, for example, sapinopiridione has recently been isolated as a phytotoxin produced by *Sphaeropsis sapinea*, a toxigenic pathogen of native and exotic conifers, which appeared to be the enantiomer of FRT-A toxin isolated from unidentified species of the genus *Macrophoma* causing the fruit rot of apple.[19]

Metabolite **2** showed the same molecular weight of 178, as deduced from its ESI MS spectrum, and the same ^{1}H NMR spectrum of isosclerone **5** (Figure 1), previously isolated together with the closely related scytalone **6** (Figure 1), by some of us as the main lipophilic phytotoxins of both *Phaeoacremonium aleophilum*[4] and *Phaeomoniella chlamydospora*,[20] extensively studied as tracheomycotic fungi involved in the grapevine esca disease.[21] However, it showed also the same ^{1}H and ^{13}C NMR data and identical but opposite optical rotation (levorotatory) of isosclerone (dextrorotatory), to which was assigned an absolute 4*S*-configuration when it was isolated as a new bioactive metabolite in plant growth regulating tests from *Sclerotinia sclerotium*.[22] Therefore, metabolite **2** appeared to be regiolone, the enantiomer of isosclerone, with a *R*-configuration at C-4. Subsequently, isosclerone was also isolated as a metabolite of *Botrytis cinerea*, known as a pathogen of a number of crops[23] and as a phytotoxin of *Tubiaka dryina*, the causal agent of red oak (*Quercus rubra*) leaf spot.[24] As regiolone on its first isolation from *Juglans regia* was wrongly assigned an *S*-configuration,[8] a comparison between regiolone isolated from *B. fabae* and isosclerone isolated from *P. aleophilum* was carried out, recording their CD spectra in the same conditions. The CD data (not shown) confirmed their enantiomeric relationship and the absolute configuration 4*R* and 4*S* assigned to regiolone and isosclerone, respectively.

Finally, metabolites **3** and **4** were isolated as a 1:3 mixture, as deduced by integration of the peaks observed in their ^{1}H NMR spectra, which could not be separated using normal or reverse-phase HPLC techniques. From the ^{1}H NMR and ^{13}C NMR spectra they appeared to be two diastereomeric naphthalenones. The spectra did not change with temperature, ruling out the possibility that they are a mixture of two conformers. The identification of both compounds from the mixture was possible because of the well-separated resonance signals observed in the ^{1}H and ^{13}C NMR spectra. The chemical shifts and multiplicities of all protons and corresponding carbons of **3** and **4** were assigned by the couplings observed in the COSY, HSQC, and HMBC spectra. On the basis of these data, **3** and **4** were identified as *cis*- and *trans*-3,4-dihydro-2,4,8-trihydroxynaphthalen-1(2*H*)-ones, as shown by ^{1}H and ^{13}C NMR data very similar to those previously reported from one (the *cis*-($-$)-diastereomer) of these two naphthalenones, when it was isolated for the first time from *Penicillium diversum* var. *aureum*.[25] Successively, both *cis*-(+) and *trans*-($-$)-2,4,8-trihydronaphthalenones were isolated as phytotoxins, together with other naphthalenones and isocoumarins, from *Ceratocystis fimbriata* f. sp. *platani*, a peritecial ascomycete that infects high-value crops and trees such as hevea, sweet potato, coffee, cacao, oak, coconut palma, and plane tree.[10] The 2,4,8-trihydronaphthalenone was also isolated, together with isosclerone, from *Mycosphaerella fijiensa*, causing Black Sigatoka disease on banana,[26] and only the *trans*-stereoisomer from the wood-inhabiting cup fungus, *Urnula craterium*.[9] Their total synthesis from juglone,[27] as well as their asymmetric synthesis,[10] was also realized. In particular, the last synthesis allowed them to obtain the enantiomers of both *cis*- and *trans*-3,4-dihydro-2,4,8-trihydroxynaphthalenones, the absolute stereochemistries of which were also determined. The comparison of the CD spectra of the two natural 3,4-dihydro-2,4,8-trihydronaphthalenones and synthetic ones allowed them to establish that the natural *trans*- and *cis*-isomers were the ($-$)-(2*S*,4*S*)-isomer and the (+)-(2*S*,4*R*)-isomer, respectively.[10] Unfortunately, we failed to separate the natural *cis*- and *trans*-2,4,8-trihydroxytetralones produced by *B. fabae*, so it was not possible to record the optical properties of each diastereomer. Attempts to separate them also failed, because of the low yield in which they are produced by the fungus (0.62 and 1.87 mg/L) and

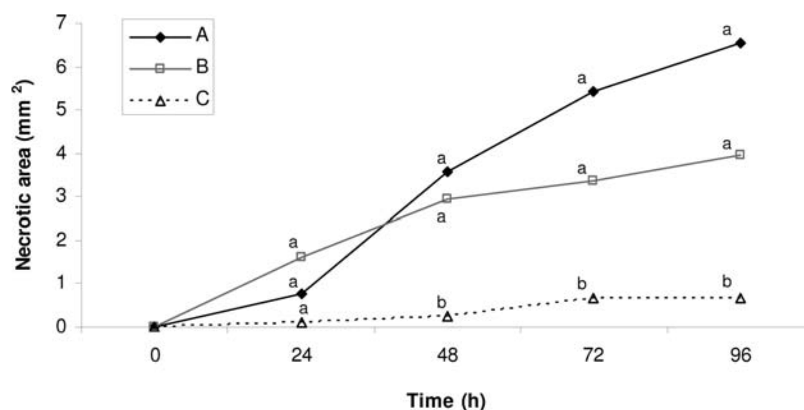

Figure 4. Necrotic area (mm^2) incited by metabolite **1** at different concentrations and at different times (h) after treatment of leaves of *V. faba* (A, 1 mg/mL; B, 0.5 mg/mL; C, 0.25 mg/mL). Values followed by the same letter at each time are not significantly different (LSD test, $p < 0.05$).

because of their already known instability. In fact, the orange pigment, observed when **3** and **4** were TLC chromatographed, probably was due to their oxidation to the corresponding naphthoquinone, 3-hydroxyjuglone, **7** (Figure 1), an artifact as also previously observed in the isolation of both 2,4,8-trihydronaphthalenones from *C. fimbriata* f. sp. *platani*.[10] This was confimed by the NMR data and ESI MS spectra of the orange pigment, which are consistent with the structure of 3-hydroxyjuglone.[11] The experimental conditions and the kinetics of this oxidation reaction in vitro were also previously investigated by HPLC, and the mechanism of oxidation both in vitro and in vivo was hypothesized.[10]

The results of bioassays of **1**, **2**, and **3**, and **4** (Figure 3) showed that all compounds were phytotoxic, although with differences between them (ANOVAs detected significant differences for factor "metabolite" at all times, $p < 0.001$). Considering the possibility that **3** and **4** might need to undergo oxidation to 3-hydroxyjuglone to become phytotoxic, as in the case of the *C. fimbriata* f. sp. *platani*/*Platanus acerifolia* pathosystem,[10] 3-hydroxyjuglone was also tested. However, **3** and **4** showed a rapid effect, being the most phytotoxic 24 h after treatment of the leaves, whereas the reaction to the other metabolites was very limited. One day later, toxicity levels of all compounds increased, although those of **1** remained low. At this time, there was no significant difference between the toxicity of **3** and **4** and that of 3-hydroxyjuglone (**7**). **2**, on the contrary, presented less phytotocity than **3** and **4**. The scenario changed at 72 h, with a dramatic increase of the phytotoxicity of **2**, overcoming the rest of the compounds, although the differences with **3** and **4** were not significant. Actually, the phytotoxicity of both **3** and **4** and 3-hydroxyjuglone (**7**) seemed to stabilize, showing minor increases. Conversely, the phytotoxicity of **1** started to rise, although at modest levels. Finally, 96 h after treatment, **2** clearly differentiated from the rest of the metabolites, which kept a moderate pace of growth in phytotoxicity.

As for the bioassay of **1** at three different concentrations (Figure 4), the analyses of variance found significant differences for factor "concentration" at all times but 24 h after treatment ($p < 0.05$). Overall, the concentration of 0.25 mg/mL presented low levels of phytotoxicity, whereas the levels of phytotoxicity of 0.5 and 1 mg/mL were higher than those of the previous bioassay, but still moderate in comparison with those reached by **2**, **3**, and **4** in that case. In summary, **2** has turned out to be the most phytotoxic metabolite, with **3** and **4** showing lower levels of toxicity, although still high; **1** presents a moderate phytotoxic effect.

In conclusion, the isolation of the new botrytone, regiolone, and both *cis*- and *trans*-3,4-dihydro-2,4,8-trihydroxynaphthalenones as phytotoxic metabolites from *B. fabae* did not come as a surprise as they are all naphthalenone pentaketides already known as fungal and plant metabolites and also as phytotoxins.[12,13] *B. cinerea*, which also infected *V. fabae*, produces isosclerone and not the related naphthalenones (**1**−**4**). Naphthalenones are also involved in the branched pathway of fungal dihydronaphthalene−melanin biosynthesis,[28−30] which are high molecular weight black pigments arranged into the layer structure between the cell wall and the cell membrane of the appressorium. Dihydronaphthalene−melanin is a necessary component of the functioning appressorium in some fungi such as *Magnaporthe grisea*.[29] This black color was also assumed by the *B. fabae* mycelium when the fungus was grown in liquid culture, and black streakings were also observed when both *P. aleophilum* and *P. chlamydospora* colonized infected grapevine wood.[21]

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 081 2539178. Fax: +39 081 2539186. E-mail: evidente@unina.it.

Funding Sources

This work was supported in part by the Spanish project AGL2008-01239 and in part by a grant from the Italian Ministry of University and Research. A. Cimmino is the recipient of a grant funded by the Spanish Juan de la Cierva program. Contribution DISSPAPA N. 246.

ACKNOWLEDGMENT

The NMR spectra were recorded in the laboratory of Chimica Biomolecolare del CNR, Pozzuoli.

REFERENCES

(1) Rubiales, D. Faba beans in sustainable agriculture. *Field Crops Res.* **2010**, *115*, 201–202.

(2) Stoddard, F. L.; Nicholas, A. H.; Rubiales, D.; Thomas, J.; Villegas, A. M. Integrated pest management in faba bean. *Field Crops Res.* **2010**, *115*, 308–318.

(3) Harrison, J. G. The biology of *Botrytis* spp. on *Vicia* beans and chocolate spot disease — a review. *Plant Pathol.* **1988**, 37, 168–201 (and references cited therein).

(4) Evidente, A.; Sparapano, L.; Andolfi, A.; Bruno, G. Two naphthalenones pentaketides from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca grapevine. *Phytopathol. Mediterr.* **2000**, *39*, 162–168.

(5) Zhang, J.; Wu, M.; Li, G.; Yang, L.; Yu, L.; Jiang, D.; Huang, H.; Zhuang, W. *Botrytis fabiopsis*, a new species causing chocolate spot of broad bean in central China. *Mycologia* **2010**, *102*, 1114–1126.

(6) Min, B. S.; Nakamura, N.; Miyashiro, H.; Kim., Y. H.; Hattori, M. Inhibition of human immunodeficient virus type 1 reverse transcriptase and ribonuclease H activities by constituents of *Juglans mandshurica*. *Chem. Pharm. Bull.* **2000**, *48*, 194–200.

(7) Liu, L.; Li, W.; Koike, K.; Zhang, S.; Nikaido, T. New α-tetralonyl glucoside from the fruit of *Juglans mandhurica*. *Chem. Pharm. Bull.* **2004**, *52*, 566–569.

(8) Talatrapa, S. K.; Karmacharya, B.; De, S. C.; Talatrapa, B. (−)-Regiolone, an α-tetralone from *Juglans regia*: structure, stereochemistry and conformation. *Phytochemistry* **1988**, *27*, 3929–3932.

(9) Ayer, W. A.; Trifonov, L. S.; Hutchison, L. J.; Chakravarty, P. Metabolites from a wood-inhabiting cup fungus, *Ursula craterium*. *Nat. Prod. Lett.* **2000**, *14*, 405–410.

(10) Bürki, N.; Michel, A.; Tabacchi, R. Naphthalenones and isocoumarins of the fungus *Ceratocystis fimbriata* f. sp. *platani*. *Phytopathol. Mediterr.* **2003**, *42*, 191–198 (and references cited therein).

(11) Tezuka, M.; Kuroyanagi, M.; Yoshihira, K.; Natori, S. Naphthoquinone derivatives from the Ebenaceae. IV. Naphthoquinone derivatives from *Dyospyros kaki* Thumb. and *D. kaki* var *sylvestris* Makino. *Chem. Pharm. Bull.* **1972**, *9*, 2029–2035 (and literature cited therein).

(12) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: London, U.K., 1983; pp 55−200.

(13) Osbourn, A. E.; Lanzotti, V. *Plant-Derived Natural Products — Synthesis, Function, and Application*; Springer: Dordrecht, The Netherlands, 2009; pp 3−50.

(14) Arima, S.; Yamada (Konda), Y.; Takeda, K.; Takayanagi, H.; Harigaya, Y. Synthesis of *cis*- and *trans*-5,8-dihydro-5,6,7,8-tetrahydro-1,4-naphthoquinone. *Chem. Pharm. Bull.* **2001**, *49*, 1340–1342 (and references cited therein).

(15) Pretsch, E.; Bühlmann, P.; Affolter, C. Structure *Determination of Organic Compounds — Tables of Spectral Data*; Springer-Verlag: Berlin, Germany, 2000; pp 161−243, 385−404.

(16) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, Germany, 1987; pp 183−280.

(17) Liu, Q.; Zhao, P.; Li, X.-C.; Jacob, M. R.; Yang, C-R; Zhang, Y.-J. New tetralone galloylglucosides from fresh pericarps of *Juglans sigillata*. *Helv. Chim. Acta* **2010**, *93*, 265–271.

(18) Kuang, K.; Li, P. *Flora of China*; Science Press: Beijing, China, 1979; Vol. 21 (in Chinese).

(19) Evidente, A.; Fiore, M.; Bruno, G.; Sparapano, L.; Motta, G. Chemical and biological characterization of sapinopyridione, a phytotoxic 3,3,6-trisubstituted-2,4-pyridione produced by *Sphaeropsis sapinea*, a toxigenic pathogen of native and exotic conifers, and its derivatives. *Phytochemistry* **2006**, *67*, 1019–1028.

(20) Tabacchi, R.; Fkyerat, A.; Poliart, C.; Dubin, G. M. Phytotoxins from fungi of esca of grapevine. *Phytopathol. Mediterr.* **2000**, *39*, 156–161.

(21) Surico, G.; Mugnai, L.; Marchi, G. Older and more recent observations on esca, a critical overview. *Phytopathol. Mediterr.* **2006**, *45*, S68–S86 (and references cited therein).

(22) Morita, T.; Aoki, H. Isosclerone, a new metabolite of *Sclerotinia sclerotorium* (Lib.) DE BARY. *Agric. Biol Chem.* **1974**, *38*, 1501–1505.

(23) Suga, T.; Hirata, T.; Utsumi, R.; Yoshioka, T. The metabolites of *Botrytis cinerea*. *J. Sci. Hiroshima Univ. Ser. A* **1984**, *48*, 75–79.

(24) Venkatasubbaiah, P.; Chilton, W. S. Phytotoxins produced by *Tiubiaka dryina*. *Mycopathologia* **1992**, *120*, 33–37.

(25) Fujimoto, Y.; Yokoyama, E.; Takahashi, T.; Uzawa, J.; Morooka, N.; Tsunoda, H.; Tarsuo, T. Studies on the metabolites of *Penicillium diversum* var. *aurem*. I. *Chem. Pharm. Bull.* **1986**, *34*, 1497–1500.

(26) Hoss, R.; Helbig, J.; Bochow, H. Function of host fungal metabolites in resistance response of banana and plantain in the Black sigatoka disease pathosystem (*Musa* spp. *Mycosphaerella fijiensis*). *J. Phytopathol.* **2000**, *148*, 387–394 (and references cited therein).

(27) Fujimoto, Y.; Satoh, M. Studies on the metabolites of *Penicillum diversum* var. *aureum*. II. Synthesis and cytotoxic activity of trihydroxytetralones. *Chem. Pharm. Bull.* **1986**, *34*, 4540–4544.

(28) Wheeeler, M. C.; Abramczyk, D.; Puckhaber, L. S.; Naruse, M.; Ebizuka, Y.; Fujii, I.; Szaniszlo, P. J. New biosynthetic step in the melanin pathway of *Wangiella* (*Exophiala*) *dermatitidis*: evidence for 2-acetyl-1,3,6,8-tetrahydronaphthalene as a novel precursor. *Eukaryotic Cell* **2008**, 1699–1711 (and references cited therein).

(29) Thompson, J. E.; Basarab, G. S.; Andersson, A.; Lindqvist, Y.; Jordan, D:B. Trihydroxynaphthalene reductase from *Magnaporthe grisea*: realization of an active center inhibitor and elucidation of the kinetic mechanism. *Biochemistry* **1997**, *36*, 1852–1860.

(30) Jacobson, E. S. Pathogenic roles for fungal melanins. *Clin. Microbiol. Rev.* **2000**, *13*, 708–717 (and references cited therein).