Homozinniol, a New Phytotoxic Metabolite from *Alternaria solani*

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A bioassay-guided fractionation of the phytotoxic crude extract of *Alternaria solani* resulted in the isolation of two phytotoxic metabolites identified as zinniol (**1**) and a new polyketide: homozinniol (**2**). A third metabolite, 6-[(3′,3′-dimethylallyl)oxy]-4-methoxy-5-methylphthalide (**3**) was also isolated and identified. Structural assignment of the metabolites was based primarily on the analysis of their mass spectrometry and ^{1}H NMR spectroscopy data.

Keywords: *Alternaria solani; Solanum tuberosum; Lycopersicon esculentum;* phytotoxin; zinniol; homozinniol; polyketide; phthalide

INTRODUCTION

Due to their important role in plant pathogenesis, and the information that they can provide regarding the molecular basis of plant disease, phytotoxins have been the subject of extensive, multidisciplinary studies in recent years (Kohmoto and Otani, 1991). They are classified as host- and non-host-specific toxins (HSTs and non-HSTs, respectively) according to their host range and to their being considered or not primary determinants of disease (Strobel, 1982). Presently there are 14 HSTs and more than 200 non-HSTs reported in the literature (Kohmoto and Otani, 1991), both groups showing a great diversity in chemical structures (Macko, 1983). Although a number of phytotoxin-producing plant pathogenic fungi are known, the present knowledge of these metabolites has come almost entirely from the so-called "saprophytic pathogens" of the genera *Alternaria* and *Cochliobolus* (Jones and Dunkle, 1993).

A number of non-HSTs have been isolated from *Alternaria solani* (Ell. & Mart.) Jones and Grout, a pathogen recognized as the causal agent of early blight disease in potato (*Solanum tuberosum* L.) (Brian et al., 1952) and tomato (*Lycopersicon esculentum* Mill.) (Pound and Stahmann, 1951), and they include alternaric acid (Bartels-Keith and Grove, 1959; Stoessl and Stothers, 1984), the altersolanols (Yagi et al., 1993), the alterporriols (Suemitsu et al., 1990), the solanopyrones (Ichihara et al., 1983), and tentoxin (Suemitsu et al., 1992a). There is also a single report on the production of two lipid-like HSTs by cultures of *A. solani* (Matern et al., 1978); however, the chemical identity of these metabolites remains unknown.

During our search for the HSTs of *A. solani,* we have isolated and identified three structurally related metabolites identified as zinniol (**1**), the new polyketide homozinniol (**2**), and 6-[(3′,3′-dimethylallyl)oxy]-4-methoxy-5-methylphthalide (**3**). We report here on the methodology that led us to the bioassay-guided isolation and identification of metabolite **3** and phytotoxins **1** and **2**.

METHODS AND MATERIALS

^{1}H NMR spectra were recorded at 200 MHz on a Varian XL-200 or Varian GEM-200 spectrometer, using CDCl$_3$ (Aldrich Chemical Co., Milwaukee, WI) as solvent and tetramethyl-silane (TMS; Aldrich) as internal standard. EIMS were recorded at 70 eV on a Kratos MS-50TC ultrahigh-resolution mass spectrometer. GC/MS analyses were performed on a Hewlett-Packard 5890 gas chromatograph [0.5 μL of sample, Hewlett-Packard Ultra 1 column (cross-linked methyl silicone gum, 25 m long, 0.32 mm i.d., 0.52 μm fill thickness), flow rate = 1 mL/min, T_1 = 150 °C, T_2 = 300 °C, gradient = 10 °C/min] coupled to a Hewlett-Packard 5971A mass selective detector. IR spectra were recorded on a Bruker IFS-25 FT-IR, using the Spectrofile-IR Plus program and a Hewlett-Packard 7475A plotter. Flash column chromatography (flash CC; Still et al., 1978) purifications were carried out using silica gel 60 (200–400 mesh) from Aldrich. Purifications by vacuum liquid chromatography (VLC; Coll and Bowden, 1986) were performed using silica gel 60 [thin layer chromatography (TLC) grade] from Aldrich. Preparative TLC purifications were carried out on E. M. Merck (Darmstadt, Germany) silica gel 60 F$_{254}$ glass-coated plates (0.25 mm thickness). Analytical TLC was conducted on E. M. Merck aluminum-coated silica gel 60 F$_{254}$ sheets (0.20 mm thickness); the components were visualized first under UV light and then by dipping the plates in 4% phosphomolybdic acid (Sigma Chemical Co., St. Louis, MO) containing a trace of ceric sulfate (Fisher Scientific, Fair Lawn, NJ) in 5% sulfuric acid (Productos Químicos Monterrey, Monterrey, NL, Mexico), followed by drying and heating at 105 °C for 3–4 min. Solvents were of industrial grade and were glass-distilled in the laboratory prior to use.

Parent cultures of *A. solani* (Ell. & Mart.) Jones and Grout were kept in the dark, on potato dextrose agar (PDA; BIOXON, Mexico, DF, Mexico) slant tubes, at 4 °C and used to inoculate PDA Petri dishes where *A. solani* was allowed to grow for 15 days, under continuous light, at 21–22.5 °C. A spore suspension (*ca.* 5 mL) of the fungus was used to inoculate 1 L of Richard's medium and the liquid still culture grown for 35 days, under continuous light, at 21–22.5 °C. The mycelial mat was separated from the broth by gravity filtration through two layers of cheesecloth and the filtrate extracted with ethyl acetate (ratio solvent/filtrate 2:1, 1:1, 1:1). Evaporation of the solvent under reduced pressure yielded the corresponding organic crude extract (*ca.* 0.11 g/L). The mycelial mat was air-dried and extracted in a Soxhlet apparatus with ethyl acetate, producing the mycelium extract after *in vacuo* removal of the solvent. Filtrates before and after extraction, together with the corresponding organic and mycelium extracts, were evaluated for phytotoxic activity using the leaf-spot assay (Pena-Rodriguez et al., 1988) against potato and tomato leaves. The phytotoxic organic crude extract (5.70 g) was suspended in a 9:1 mixture of water/methanol (*ca.* 100 mL of solution/ 200 mg of crude extract) and the resulting suspension successively partitioned between hexane (ratio suspension/solvent 2:1, 1:1, 1:1), ethyl acetate (2:1, 1:1, 1:1), and 1-butanol (2:1) to yield the low-polarity (A, 1.30 g), medium-polarity (B, 2.20 g), and high-polarity (C, 0.56 g) fractions, respectively.

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Isolation of Zinniol (1). Successive, bioassay-guided, flash CC purifications (4 and 2 cm diameter, using hexane/acetone and dichloromethane/acetone mixtures, respectively) of fraction A (1.30 g) yielded fraction 18F (37 mg), which was further purified, by multiple elution (three times) preparative TLC (hexane/acetone 8:2) to produce **1** (3.5 mg) in pure form.

Isolation of Homozinniol Diacetate (2a). A mixture of fraction 18F (23 mg), acetic anhydride (0.8 mL), and pyridine (0.15 mL) was stirred overnight at room temperature. The reaction mixture was evaporated to dryness, under reduced pressure, by successive additions of hexane and methanol. A multiple elution (three times) preparative TLC (hexane/acetone 8:2) purification of the crude acetylated product (24 mg) yielded **1a** (4.7 mg) and **2a** (1.5 mg) as single metabolites.

Isolation of 6-[(3′,3′-Dimethylallyl)oxy]-4-methoxy-5-methylphthalide (3). A mixture of fraction A (492.6 mg), acetic anhydride (15 mL), and pyridine (5 mL) was allowed to stir overnight at room temperature. The reaction mixture was worked up in the usual manner to produce the crude acetylated product 5A (362.9 mg). Successive VLC (gradient elution, hexane/acetone) and preparative TLC (multiple elution, two times, benzene/acetone 95:5) purifications afforded **3** (3.5 mg) as a single component.

RESULTS

Isolation and Identification of Metabolites. Biological activity, i.e., the ability to cause an effect similar to that induced by the pathogen in the field, was observed only for the unextracted fungal culture filtrate and its corresponding organic crude extract when tested against potato and tomato leaves (necrosis at 0.125%). To simplify the purification process, the crude extract was partitioned between solvents of increasing polarity. Bioassay of the resulting crude fractions showed that both the lowest and highest polarity fractions possessed the strongest phytotoxic activity (necrotic center with chlorotic zone at 0.125%).

A bioassay-guided chromatographic purification of the lowest polarity fraction led to the isolation of a phytotoxic product (necrotic center with chlorotic zone at 0.100%) appearing as a single UV–vis component on TLC (R_f = 0.16, hexane/acetone 8:2) and GC (T_r = 12.31 min). The pure metabolite was identified as zinniol (**1**, Figure 1) by comparing our spectroscopic data (IR, MS, and ^{1}H NMR) with those reported in the literature (Starratt, 1968; Ichihara et al., 1985).

In an attempt to isolate additional phytotoxic metabolites present in the fraction containing **1** as the major component, this fraction was subjected to acetylation conditions and the crude acetylated product purified to yield two phytotoxic fractions (weak necrosis at 0.100%). The major, less polar (R_f = 0.56, hexane/acetone 8:2, three times), metabolite appeared as a single component by GC (T_r = 13.43 min) and was identified as zinniol diacetate (**1a**, Figure 1) by comparing our spectroscopic data (MS and ^{1}H NMR) to those reported in the literature (Starratt, 1968; Cotty et al., 1983; Ichihara et al., 1985). The minor, more polar (R_f = 0.38, hexane/acetone 8:2, three times), metabolite also appeared as a single component by GC (T_r = 13.30 min) and was identified as homozinniol diacetate (**2a**, Figure 1) by comparing its spectroscopic data with those of **1a**: EIMS, *m/z* (relative intensity) 366 (11.2) [M]$^+$, 306 (23.5), 264 (48.4), 179 (30.6), 85 (84.7), 59 (38.2), 43 (100.0); NMR δ 0.85 (3H, H-5′, $J_{5',4'}$ = 6.6 Hz), 1.35 (3H, H-6′, $J_{6',1'}$ = 7.0 Hz), 2.01 (3H, Ac-CH$_3$), 2.04 (3H, Ac-CH$_3$), 2.16 (3H, H-10), 3.67 (3H, H-9), 4.05 (1H, H-1′, m), 5.06 (2H, H-7), 5.13 (2H, H-8), 6.65 (1H, H-1).
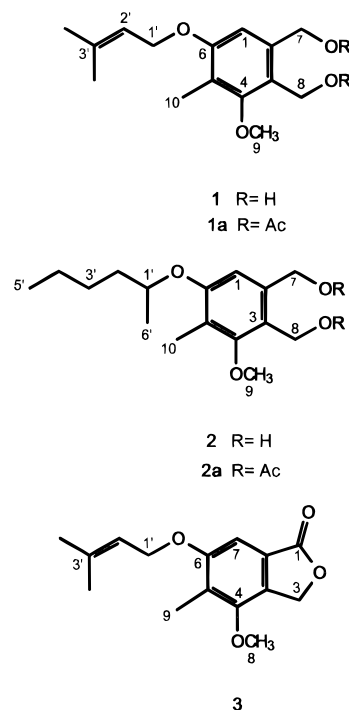

Figure 1. Metabolites of *A. solani*: zinniol (**1**), homozinniol (**2**), and 6-[(3′,3′-dimethylallyl)oxy]-4-methoxy-5-methylphthalide (**3**).

To isolate larger amounts of **1a** and **2a**, the lowest polarity fraction was acetylated. Purification of the crude acetylated product yielded a nonphytotoxic fraction containing a single, UV–vis, component on TLC (R_f = 0.76, benzene/acetone 95:5, two times). The metabolite was identified as 6-[(3′,3′-dimethylallyl)oxy]-4-methoxy-5-methylphthalide (**3**, Figure 1) by comparison of our spectroscopic data with those reported in the literature (Suemitsu et al., 1992b, 1993, 1995).

DISCUSSION

Zinniol (**1**) is a ubiquitous non-HST, first isolated from cultures of *A. zinniae* Pape, a pathogenic fungus known to cause leaf and stem blight on zinnia, sunflower, and marigold plants (White and Starratt, 1967). It has also been identified as the major phytotoxic metabolite of *Alternaria dauci* (Kuhn) Groves and Skolko, reportedly producing the same disease symptoms as those induced by the pathogen on carrot leaves (Barash et al., 1981). Production of zinniol has also been detected in culture filtrates of several *Alternaria* spp., including *A. carthami*, *A. macrospora*, *A. porri*, *A. solani*, and *A. tagetica*. It has been reported to be phytotoxic to all hosts of *Alternaria* spp., regardless of whether the corresponding pathogen produces the toxic metabolite or not, thus confirming its nonselectivity (Cotty and Mishagi, 1984).

The ^{1}H NMR of homozinniol diacetate (**2a**) showed some striking similarities to that of **1a,** namely, the presence of two acetylated hydroxymethyl groups (5.13 and 5.06 ppm), a single aromatic proton (6.65 ppm), and two methyl groups, one attached to an aromatic ring (2.16 ppm) and the other being part of an aryl methyl ether (3.67 ppm). However, the ^{1}H NMR spectrum of **2a** did not show the presence of vinylic protons or methyl groups on sp^2 carbons. Two methyl group signals, one being a triplet (0.85 ppm, *J* = 6.6 Hz) and the other appearing as a doublet (1.35 ppm, *J* = 7.0 Hz), further indicated that the arrangement of the side chain

was different from that expected for a simple, fully saturated 3′,3′-dimethylallyloxy side chain. The molecular ion peak observed at m/z 366 (11.2%) in the EIMS of the new metabolite, and the corresponding molecular formula of $C_{20}H_{30}O_6$, indicated the presence of both an additional methylene group and unsaturation site in a **1a**-related structure. That the difference of 16 mass units between the new metabolite and **1a** resided in the side chain was clearly indicated by a strong fragment ion peak at m/z 85 (84.7%) instead of the characteristic one at m/z 69 for **1a**. Finally, a one-proton multiplet at 4.05 ppm in the ^{1}H NMR of the new metabolite allowed for the correct positioning of the methyl doublet signal at C-1′. In view of the data discussed we propose structure **2a** (Figure 1) for the new metabolite and the name homozinniol for the parent metabolite **2** (Figure 1).

The phthalide **3** was first reported as one of the isomeric products obtained upon chromic acid oxidation of **1** (Starratt, 1968). It has also been reported as the sodium borohydride reduction product of zinnolide, a weak phytotoxin isolated from *A. solani* (Ichihara et al., 1985), and as a natural product from *A. porri,* the causal fungus of black spot disease in stone leek and onion (Suemitsu et al., 1992b).

CONCLUSIONS

We have identified **1**−**3** as metabolites of *A. solani*. Although **1** has already been reported as being produced by a number of *Alternaria* species, including *A. solani*, this is the first report of **3** as a metabolite of this pathogen. Furthermore, and even though there is a report of zinniol-related metabolites having a modified 3′,3′-dimethylallyloxy side chain (Stierle et al., 1993), our identification of **2** constitutes the first report of a zinniol-related phytotoxin having an additional carbon atom in the side chain. Finally, it is interesting to point out that our finding of **3** as a nontoxic metabolite from *A. solani* coincides with the report of its lack of activity when tested on lettuce and stone leek seedlings (Suemitsu et al., 1992b). This, together with the reduced phytotoxic activity observed in our assay for **1a** and **2a** when compared to that of the parent compounds **1** and **2**, is in agreement with the report that the hydroxymethyl groups are essential for the expression of phytotoxic activity (Barash et al., 1981).

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