Tagetolone and Tagetenolone: Two Phytotoxic Polyketides from *Alternaria tagetica*

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Two new phytotoxic polyketides, tagetolone (**1**) and tagetenolone (**2**), in addition to tyrosol and *p*-hydroxybenzoic acid, have been isolated from the organic crude extract of culture filtrates from the fungal pathogen *Alternaria tagetica*. Complete characterization of all structures was carried out following a careful analysis of their spectroscopic data (IR, MS, ^{1}H and ^{13}C NMR, and 2D NMR experiments).

Keywords: *Alternaria tagetica; Tagetes erecta; phytotoxins; marigold; tagetolone; tagetenolone; tyrosol; p-hydroxybenzoic acid*

INTRODUCTION

The fungal pathogen *Alternaria tagetica* severely infects marigold (*Tagetes erecta* L.) plants, causing necrotic spots on leaves, stems, and petals and, in some cases, resulting in plant death (*1*).

As part of our search for the bioactive metabolites produced by *A. tagetica*, we have previously reported the isolation and identification of the nonhost-specific toxin zinniol and two new dimeric nonphytotoxic metabolites, *bis*-7-*O*-7″,8-*O*-8″- and *bis*-7-*O*-8″,8-*O*-7″-zinniol (*2*). Although these metabolites were obtained from a low-polarity (hexane) fraction resulting from a solvent partition of the original crude extract, testing the medium-polarity fraction (ethyl acetate) in the leaf spot assay produced necrotic damage which gave a clear indication of the presence of a second group of phytotoxic metabolites biosynthesized by *A. tagetica*. We report here the bioassay-guided purification of the medium-polarity fraction and the identification of the phytotoxic metabolites tagetolone (**1**), tagetenolone (**2**), and tyrosol, in addition to a fourth, nonphytotoxic, metabolite identified as *p*-hydroxybenzoic acid (Figure 1).

MATERIALS AND METHODS

UV spectra were determined in a DU-65 Beckman UV−vis spetrophotometer using MeOH as solvent. IR spectra were measured using a FT-IR Nicolet Magna 750 or a FT-IR Protege 460. LREIMS and HRFABMS were recorded at 70 eV on a JEOL-JMS-SX102A mass spectrometer and HREIMS was recorded at 70 eV on a JEOL-JMS-AX-505HA mass spectrometer. GC−MS analyses were performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971A mass selective detector. Following are the conditions used for GC analyses: 0.5 μL of sample; Ultra 1 column (Hewlett-Packard, cross-linked methyl silicone gum, 25 m long, 0.32 mm i.d., 0.52 μm fill thickness); flow rate, 1 mL/min; temperature programs, (a) $T_1 = 150$ °C, $T_2 = 290$ °C, (b) $T_1 = 70$ °C, $T_2 = 290$ °C, gradient = 10 °C/min. NMR spectra were recorded on Varian Unity-300, Bruker AMX-400, and Varian Unity Plus-500 spectrometers, at 300, 400, and/or 500 MHz for ^{1}H, and 75, 100, or 125 MHz for ^{13}C, using $CDCl_3$ or $(CD_3)_2CO$ (Aldrich Chemical Co., Milwakee, WI) as solvents and tetramethylsilane (TMS; Aldrich) as internal standard. Industrial-grade solvents were glass-distilled in the laboratory prior to use. Flash column (flash CC) and vacuum liquid chromatography (VLC) purifications were carried out using silica gel (200−400 mesh and TLC-grade, respectively) from Aldrich. Preparative

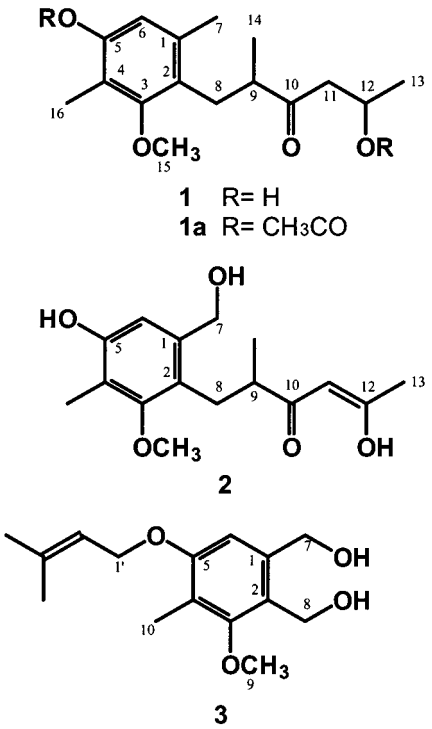

Figure 1. Metabolites of *A. tagetica*: tagetolone (**1**), tagetenolone (**2**), and zinniol (**3**).

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TLC purifications were performed on Macherey-Nagel silica gel (Germany) aluminofolium (GF_{254}, 20 × 20 mm, 0.20 mm thickness) and/or E. M. Merck (Darmstadt, Germany) glass-coated silica gel plates (GF_{254}, 20 × 20 mm, 0.25 mm thickness).

Cultures of the fungus were maintained, grown (160 L), harvested, and extracted, and the organic crude extract was partitioned, as described previously (*2*). All fractions were tested for phytotoxic activity using the leaf-spot assay on marigold leaves, following a procedure described previously (*3*).

The phytotoxic medium-polarity (ethyl acetate) fraction (35 g) was purified by VLC using a stepwise gradient elution with hexane/ethyl acetate/acetone mixtures, which resulted in fractions A to M. Leaf-spot assay evaluation of all fractions indicated that phytotoxic activity was located in fractions D, E, F, and G.

Isolation of Tagetolone (1). Successive VLC (dichloromethane/acetone mixtures in stepwise elution), and preparative TLC (dichloromethane/acetone/acetic acid, 95:5:1, 2×) bioassay-guided purifications of fraction D (2.14 g) led to the obtaining of **1** (1.7 mg/L) in pure form. TLC: R_f 0.48 dichloromethane/acetone/acetic acid, 95:5:1 (2×); 0.2 hexane/ethyl acetate 8:2 (2×). GC: 13.02 min (*a* conditions). IR ($CHCl_3$): 3602 (OH, free), 3400 (OH, bonded), 2972, 2935 (C−H), 1699 (C═O), 1257 (C−O), 1089 (C−O−C) cm^{-1}. LREIMS *m/z* (rel int. %): 280 $[M]^+$ (30), 262 $[M^+\text{-}H_2O]$ (10), 236 $[M^+\text{-}C_2H_4O]^+$ (38), 193 (10), 165 $[M^+\text{-}C_6H_{11}O_2]^+$ (100), 152 (15), 135 (90), 107 (8), 91(10), 45 (10). ^1H NMR (300 MHz, $CDCl_3$) 6.42 (1H, s, H-6), 4.18 (1H, m, H-12), 3.67 (3H, s, H-15), 2.83 (2H, m, H-8a, H-9), 2.64 (1H, dd, J = 18, 2.7 Hz, H-8b), 2.41 (1H, dd, J = 18, 8.7 Hz, H-11b), 2.20 (3H, s, H-7), 2.14 (3H, s, H-16), 1.16 (3H, d, J = 6 Hz, H-13), 1.04 (3H, d, J = 6.6 Hz, H-14) ppm.

Tagetolone Diacetate (1a). A mixture of **1** (19.5 mg), acetic anhydride (0.5 mL), and pyridine (0.2 mL) was stirred overnight at room temperature. The reaction mixture was worked up in the usual manner to produce the crude acetylated product which, after preparative TLC (hexane/ethyl acetate, 8:2, 2×) purification, yielded **1a** (14 mg) in pure form. TLC: R_f 0.5 hexane/ethyl acetate, 8:2 (2×). GC: R_t 15.01 min (*a* conditions). IR ($CHCl_3$) 3032 (Ar−H), 2982, 2931 (C−H), 1741 (COOR), 1234 (C−O), 1079 (C−O−C) cm^{-1}. HRFABMS *m/z* 364.1883 (calcd for $C_{20}H_{28}O_6$, 364.1886). LREIMS, *m/z* (rel int. %) 364 $[M]^+$ (10), 322 $[M^+ - C_2H_2O]^+$ (25), 304 $[M^+ - AcOH]^+$ (20), 286 (30), 262 $[M^+ - AcOH - C_2H_2O]^+$ (25), 245 $[M^+ - 2\ AcOH + H]^+$ (25), 193 (50), 166 (100). ^1H NMR (500 MHz, $CDCl_3$) 6.64 (1H, s, H-6), 5.26 (1H, m, H-12), 3.69 (3H, s, H-15), 2.88 (2H, m, H-8a, H-9), 2.79 (1H, dd, J = 18.5, 7 Hz, H-11a), 2.59 (1H, dd, J = 15.5, 10 Hz, H-8b), 2.42 (1H, dd, J = 16.5, 6 Hz, H-11b), 2.30 (3H, s, $C_{12}-CH_3$), 2.25 (3H, s, H-7), 2.07 (3H, s, H-16), 1.98 (3H, s, C_5-CH_3), 1.20 (3H, d, J = 6.5 Hz, H-13), 1.04 (3H, d, J = 7 Hz, H-14) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) 211.3 (C═O), 170.2 (C_5-COOR), 169.2 (C-12-COOR), 158.0 (C-3), 148.3 (C-5), 135.7 (C-1), 129.2 (C-2), 121.2 (C-4), 119.5 (C-6), 67.2 (C-12), 60.3 (C-15), 47.6 (C-11), 46.6 (C-9), 29.7 (C-8), 21.2 (C_5-CH_3), 20.8 (C_{12}-CH_3), 19.9 (C-7, C-13), 15.9 (C-14), 9.7 (C-16) ppm.

Isolation of Tagetenolone (2). Successive VLC (gradient elution, dichloromethane/acetone) and flash CC (1 cm diameter, hexane/acetone/methanol, 75:20:5) purifications of fraction G (3.61 g) afforded **2** (1.17 mg/L) as a single metabolite. TLC: R_f 0.47 hexane/acetone/methanol, 60:35:5; 0.57 dichloromethane/ethyl acetate/methanol, 80:18:2. GC: R_t 13.02 min (*a* conditions). UV (methanol) λ_{max} (log ϵ): 297 (3.1), 266 (2.0), 233 (2.8) nm. IR ($CHCl_3$): 3600 (OH, free), 3359 (OH, bonded), 3010, 2965, 2933 (C−H), 1610 (C═C), 1593 (HO−C═C−C═O), 1104 (C−O−C), 1012 (C−O) cm^{-1}. HREIMS *m/z* 294.1383 (calcd for $C_{16}H_{22}O_5$, 294.1467). LREIMS *m/z* (rel int. %) 294 $[M]^+$ (12), 276 $[M^+ - H_2O]$ (18), 259 $[M^+ - 2\ H_2O + H]^+$ (22), 240 (8), 207 (17), 192 (30), 181 $[M^+\text{-}C_6H_9O_2]^+$ (100), 165 (23), 151 (22), 136 (12), 91(8), 69 (12). ^1H NMR ($CDCl_3$, 400 MHz) 6.68 (1H, s, H-6), 5.44 (1H, s, H-11), 4.65 (1H, d, J = 12.5 Hz, H-7a), 4.54 (1H, d, J = 12.5 Hz, H-7b), 3.69 (3H, s, H-15), 2.92 (2H, dd, J = 13.3, 6.3 Hz, H-8a), 2.8 (1H, q, J = 7 Hz, H-9), 2.67 (1H, dd, J = 13.2, 7.2 Hz, H-8b), 2.16 (3H, s, H-16), 2.00 (3H, s, H-13), 1.12 (3H, d, J = 6.9 Hz, H-14). ^{13}C NMR ($CDCl_3$, 100 MHz) 199.4 (C═O), 190.8 (C-12), 158.7 (C-3), 154.0 (C-5), 138.0 (C-1), 122.6 (C-2), 117.5 (C-4), 111.4 (C-6), 99.5 (C-11), 63.0 (C-7), 60.5 (C-15), 43.6 (C-9), 30.4 (C-8), 24.8 (C-13), 17.8 (C-14), 9.4 (C-16) ppm.

Isolation of Tyrosol. Successive flash CC purifications (5, 3, and 2 cm diameter, using hexane/acetone/methanol, 75:23:2; dichoromethane/methanol, 97:3; and hexane/ethyl acetate/acetone, 60:38:2, respectively) of fraction E (2.41 g) yielded tyrosol (4-hydroxyphenylethanol, 1.13 mg/L) as a single component. TLC: R_f 0.37 dichloromethane/acetone/methanol, 75:23:2. GC: R_t 12.45 min (*b* conditions). IR, EIMS, ^1H, and ^{13}C NMR data were in agreement with those reported in the literature (*4* and *5*).

Isolation of *p*-Hydroxybenzoic Acid. Fraction F (2.39 g) was purified by flash CC (2 cm diameter, hexane/ethyl acetate/ethanol, 75:20:5) to produce *p*-hydroxybenzoic acid (0.54 mg/L) as a white solid. TLC: R_f 0.22 dichloromethane/acetone/methanol, 75:23:2. Spectroscopic data were in agreement with those reported in the literature (*6* and *7*).

RESULTS AND DISCUSSION

Bioassay-guided purification of the medium-polarity (ethyl acetate) fraction, resulting from the partition of the phytotoxic organic crude extract of *A. tagetica*, led to the isolation of four metabolites in pure form. The least polar and phytotoxic (necrotic area: 0.22 mm^2, 0.05 mg/application) metabolite **1** (Figure 1) was obtained as a yellow oil after successive VLC and preparative TLC purifications. The IR spectrum of **1** showed the presence of a characteristic ketone carbonyl band at 1699 cm^{-1}, in addition to typical absorption bands for hydroxyl groups (free and hydrogen-bonded at 3602 and 3400 cm^{-1}, respectively), aromatic double bonds (1612, 1589, and 1458 cm^{-1}), and ether groups (1089 cm^{-1}). The EIMS spectra of **1** showed a molecular ion peak at *m/z* 280, which suggested a molecular formula of $C_{16}H_{24}O_4$ and indicated five unsaturation sites in the structure**.** The ^1H NMR spectrum of **1** displayed some striking similarities with that of zinniol (**3**, Figure 1): a one-proton singlet at 6.42 ppm and two three-proton singlets at 3.67 and 2.14 ppm, confirmed the presence of the pentasubstituted aromatic ring having both a methoxyl and methyl groups as substituents. However, the ^1H NMR spectrum of **1** did not show the typical signals for the dimethylallyloxy chain or the benzylic methylene groups present in the structure of **3.** Instead, an additional three-proton singlet at 2.2 ppm in the ^1H NMR spectra of **1** indicated that a third substituent in the aromatic ring was a second methyl group. An aromatic ring and a ketone carbonyl group accounted for the five unsaturation sites implied from the molecular formula of **1**.

Treatment of **1** under acetylating conditions produced **1a** (Figure 1) whose ^1H NMR spectrum showed two new three-proton singlets at 1.98 and 2.30 ppm. Although the lower field value of the latter signal is characteristic of the methyl group of an acetylated phenolic hydroxyl group, the chemical shift of the former suggested an acetylated secondary alcohol. Presence of a secondary alcohol in the structure of **1** was confirmed by the downfield shift (to 5.26 ppm) experienced by a carbinol proton multiplet signal at 4.18 ppm in the ^1H NMR of **1**. The EIMS spectrum of **1a** showed a molecular ion peak at *m/z* 364 and significant fragments at *m/z* 322 and 262 due to the loss of a C_2H_2O and a [AcOH + C_2H_2O] unit, respectively: thus confirming the presence of two acetylated hydroxyl groups in its structure. These

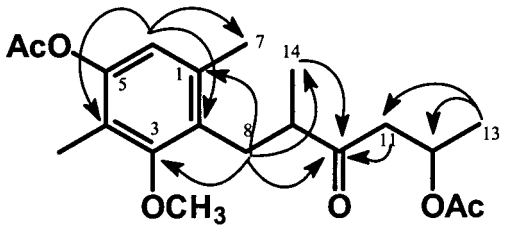

Figure 2. Selected HMBC correlations (H−C) observed for **1a**.

data supported a phenolic hydroxyl group as the fourth substituent in the aromatic ring of **1**.

The ^{13}C NMR data of **1a** showed the expected signals for all 20 carbons of the structure. They were identified as seven methyl groups (two acetyl, one aryl methyl ether, two on sp^2-carbons, and two on sp^3-carbons), two methylene groups, three methine groups (two sp^3 and one aromatic), and eight carbons not bonded to hydrogen (five aromatic, two ester carbonyls, and one ketone carbonyl). Complete characterization of **1** could be achieved by a detailed analysis of the data from ^1H-^1H COSY, ^{13}C NMR, DEPT, HMQC, and HMBC experiments of **1a**. The ^1H-^1H COSY showed the presence of two isolated spin systems: in one the oxygenated methine (m, 5.26 ppm) showed a correlation to both the methyl group at 1.2 ppm (d, J = 6.5 Hz) and the methylene protons at 2.79 and 2.42 ppm (dd, J = 18.5, 7 and 16.5, 6 Hz, respectively), and in the other a correlation between protons at 2.59 and 2.88 ppm (dd, J = 15.5, 10 Hz and m, respectively) was observed, together with that of the 2.88 ppm proton with the methyl group at 1.04 (d, 7 Hz) ppm. On the basis of these results, the isolated systems could be identified as $CH_3-CH(OH)-CH_2-$ and $-CH_2-CH-(CH_3)-$.

Long-range correlations (^2J) observed in the HMBC experiment of **1a** between the methylene protons at 2.79 and 2.42 ppm and the carbons at 211.3 (C-10) and 67.2 ppm (C-11), indicated that the $CH_3-CH(OH)-CH_2$ fragment was linked to the carbonyl carbon. Similarly, the ^2J correlations shown between a methylene proton at 2.59 ppm and carbons at 129.2 (C-2) and 46.6 (C-9) ppm, together with ^3J correlations between methylene hydrogens at C-8 with both the carbonyl carbon (211.3, C-10) and two aromatic carbons at 158 (C-3) and 135.7 (C-1) ppm, suggested that the $-CH_2-CH-(CH_3)-$ fragment was the bridge between the ketone and the aromatic ring. This allowed the identification of a 2-methyl-5-acetoxy-3-hexanone side chain as the fifth substituent in the aromatic ring. The alkyl chain justifies the presence of a base peak fragment at m/z 165 [$M^+ - C_6H_{11}O_2$] in the EIMS spectra of both **1** and **1a**, which can be explained by the partial loss of the side chain from the molecular ion.

That the arrangement of the substituents in the aromatic ring of **1** (Figure 2) was similar to that of zinniol (**3**), was again confirmed by the long-range correlations observed between the aromatic proton (6.64 ppm) and both the methyl group carbon at 19.9 ppm (C-7) and the oxygen bearing carbon at 148.3 ppm (C-5). With the methyl and methoxyl groups occupying positions as in **3** (C-4 and C-3, respectively), the alkyl residue must occupy the remaining C-5 position. On this basis, **1** can be identified as 5-hydroxy-1-(4′-hydroxy-2′-methoxy-3′,6′-dimethyl-phenyl)-2-methyl-hexan-3-one to which we have given the trivial name tagetolone.

Phytotoxin **2** (Figure 1) produced a necrotic area (0.92 mm^2) when tested (0.1 mg/application) on marigold leaves. Its IR spectrum showed absorptions for hydroxyl, phenyl, and ether groups (3359, 3010, and 1104 cm^{-1}, respectively) but no carbonyl bands. The HREIMS spectrum of **2** showed a molecular ion peak at m/z 294 $[M]^+$ and a molecular formula of $C_{16}H_{22}O_5$, indicating six unsaturation sites.

The ^1H NMR and ^{13}C NMR data of **2**, together with the results from 2D-NMR experiments (^1H-^1H COSY, DEPT, HMQC, and HMBC) showed that **2** was structurally related to **1**. The ^1H NMR spectrum of **2** showed the presence of an aromatic proton at 6.68 ppm, a methoxyl group at 3.69 ppm and, three methyl groups at 2.16, 2.00, and 1.12 ppm, in addition to proton signals at 2.92, 2.67, and 2.80 ppm, corresponding to a $-CH_2CH-$

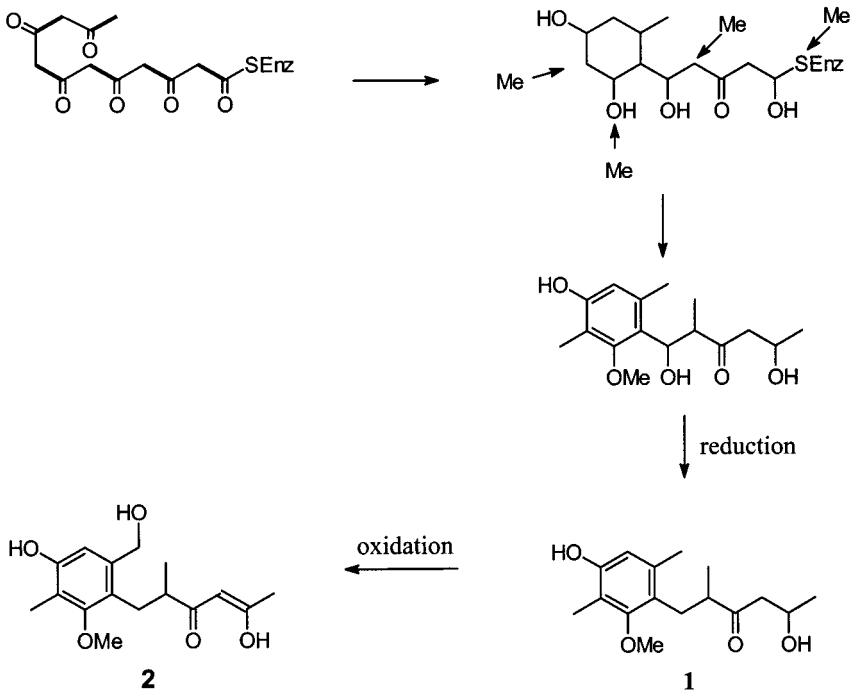

Figure 3. Possible biosynthetic origin of **1** and **2**.

(CH_3)− fragment. These data strongly suggested that **2** had a similarly substituted aromatic nucleus as **1**.

The main structural differences between **2** and **1** were given by the nature of the substituents at C-1 and C-2. The low field region of the ^{1}H NMR spectrum of **2** showed a one proton singlet at 5.44 ppm and two doublets (4.65 and 4.54 ppm, $J = 12.5$ Hz, 1H ea) of an AB quartet spin system. These signals were assigned to one vinylic proton and an oxygenated methylene, respectively. The presence of a primary alcohol in the structure of **2** was confirmed by the signal at 63.0 ppm in its ^{13}C NMR.

The C-1 location of the primary alcohol in the structure of **2** was determined by the long-range correlations, observed in the HMBC experiment, between the oxygenated methylene protons and the carbon signals at 138.0, 122.6, and 111.4 ppm (C-1, C-2, and C-6, respectively).

The ^{13}C NMR spectrum of **2** showed the expected 16 carbon signals, including two quaternary carbons at 199.4 and 190.8 ppm suggesting the presence of two ketone carbonyl groups. Although the possibility of a β-diketone was suggested by the UV spectrum of **2** (266 and 297 nm), its IR spectrum did not show an absorption in the carbonyl region. However, the absorption band at 1593 cm^{-1} indicated the presence of a 1,3-keto−enol system; β-diketones and their enol forms exist as tautomers, where the latter predominates and presents an intense absorption at 1640−1580 cm^{-1} in the IR (*8*).

Additional evidence to support the existence of a 1,3-keto−enol system in the structure of **2**, was given by the long-range correlations observed in its HMBC experiment: the vinylic proton at 5.44 ppm showed ^2J interactions with the quaternary carbon signals at 199.4 (C-10) and 190.8 (C-12) ppm. The ^2J interaction between the methyl group protons at 2 ppm and the C-12 carbon at 190.8 ppm, and a comparison of the side chain data of **2** with that of **1**, allowed for its identification and attachment to the C-2 position. On the basis of these data **2** can be described as 5-hydroxy-1-(4′-hydroxy-6′-hydroxymethyl-2′-methoxy-3′-methyl-phenyl)-2-methyl-hex-4-en-3-one, a new metabolite to which we have given the trivial name tagetenolone.

A third, highly phytotoxic metabolite (necrosis: 1.07 mm^2 at 0.1 mg/application) was obtained (1.13 mg/L) as a dark brown oil and identified as tyrosol by comparing its spectroscopic (IR, MS, ^{1}H, and ^{13}C NMR) data with those reported in the literature (*4* and *5*). Tyrosol is a primary metabolite with phytotoxic and antifeeding activities, commonly encountered in fungal cultures (*9*−*11*). A fourth, nonphytotoxic, metabolite, isolated in a yield of 0.54 mg/L, was identified as *p*-hydroxybenzoic acid by its spectroscopic data (IR, EIMS, ^{1}H, and ^{13}C NMR; *6* and *7*).

Although a number of metabolites structurally related to zinniol (**3**) have been reported, only a few lack the dimethylallyloxy side chain; these include zinnol, cichorine (*12*), silvaticol, *O*-methylsilvaticol, nidulol (*13* and *14*), and several orsellinic acid derivatives (*11*). To date, metabolites structurally related to zinniol (**3**) without one or both of the oxygenated benzylic methylenes, or where one of the oxygenated methylenes is substituted by an alkyl side chain, have not been reported. Still, it is interesting to point out that ascolitoxin, a phytotoxin produced by *Ascochyta pisi* (*15*), shows a substitution pattern of the aromatic ring very similar to that of **3**, including a benzyl-aldehyde and an oxoalkyl side chain as substituents. Similarly, the 1,3-keto−enol system present in the side chain of **2** can also be found in the chemical structure of host-specific phytotoxins belonging to the ACTG-toxins group (*16*), in alternaric acid and its derivatives (*17*), in hispolon, a yellow pigment identified from the mycelium of *Inonotus hispidus* (*18*), and in some diarylheptanoids isolated from plants of the *Alpinia* genus (*19* and *20*).

Biosynthetic studies carried out on zinniol (**3**) (*21*) established its tetraketide origin, with the initial acetate unit located at the hydroxymethyl group C-7. Taking into account the structural similarity of **1** and **2** with **3**, and the oxygenated sequence of the side chain at C-2, it can be argued that both **1** and **2** also have a polyketide origin, and that they consist of six acetate units as proposed in Figure 3. On the other hand, the presence of tyrosol and *p*-hydroxybenzoic acid, both metabolites from the shikimic acid pathway, suggest that *A. tagetica* has the capacity to use this route for the production of phytotoxic metabolites. Therefore, it is posssible that some secondary metabolites from the shikimate pathway might be reported from *A. tagetica* in the future.

This is the first report of two new phytotoxins (**1** and **2**) structurally related to zinniol which are without the dimethylallyloxy side chain and have an alkyl side chain instead of an oxygenated benzylic methylene. Even though there are reports of 1,3-keto−enol systems in some metabolites produced by several *Alternaria* species (*16* and *17*), the identification of tagetolone (**1**) and tagetenolone (**2**) constitutes the first report of an open chain 1,3-ketol and 1,3-keto−enol system, respectively. Finally, even though tyrosol was significantly more phytotoxic than **1** and **2**, its activity was weaker in comparison with that of **3** (5.18 mm^2, 0.1 mg/application).

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