

Natural Zinniol Derivatives from *Alternaria tagetica*. Isolation, Synthesis, and Structure–Activity Correlation

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Two novel phytotoxins, 8-zinniol methyl ether (**5**) and 8-zinniol acetate (**6**), in addition to 6-(3',3'-dimethylallyloxy)-4-methoxy-5-methylphthalide (**2**), 5-(3',3'-dimethylallyloxy)-7-methoxy-6-methylphthalide (**3**), and the novel metabolites 8-zinniol 2-(phenyl)ethyl ether (**4**) and 7-zinniol acetate (**7**) have been identified as natural zinniol derivatives from the organic crude extract of *Alternaria tagetica* culture filtrates. Using zinniol as the starting material, phytotoxin **5** was synthesized, together with a number of synthetic intermediates (**8–13**). Both natural and synthetic zinniol derivatives were evaluated in the leaf-spot bioassay against marigold leaves (*Tagetes erecta*).

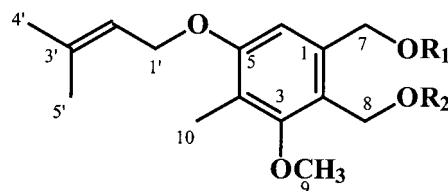
KEYWORDS: *Alternaria tagetica*; *Tagetes erecta*; marigold; phytotoxin; zinniol; zinniol derivatives; leaf-spot bioassay

INTRODUCTION

Zinniol (**1**) is a phytotoxic tetraketide first isolated from *Alternaria zimmiae* (*1*) and later detected in culture filtrates from *Phoma macdonaldii* (*2*), *Alternaria dauci* (*3*), *Alternaria solani*, *Alternaria porri*, *Alternaria carthami*, *Alternaria macrospora* (*4*), *Alternaria cichorii* (*5*), and *Alternaria tagetica* (*6*). The total synthesis of **1** has been reported following two different routes: one using a disulfonic acid as the starting material (*7*) and another using a sequence of Diels–Alder and Alder–Rickert reactions with dimethylacetylenedicarboxylate (DMAD) structure (*8*). The synthetic preparation of zinniol has confirmed both its structure and its phytotoxic activity.

The phytotoxic damage produced by zinniol in detached leaves of host plants is similar to that caused by the corresponding fungal pathogens in the field (*4*, *6*). It has been reported that the presence of the two hydroxymethyl groups in the structure of **1** is essential for the expression of its phytotoxic activity (*3*). Structure–activity studies carried out by preparing and testing zinniol derivatives can improve our knowledge of the structural requirements needed for the full expression of biological activity and our understanding of the role played by this phytotoxin in the plant–pathogen interaction.

We have previously reported the isolation of **1** as the major phytotoxic component in the organic crude extract from culture filtrates of *A. tagetica* (*9*). Here we describe a number of metabolites structurally related to zinniol (**Figure 1**), including



	R ₁	R ₂
1	H	H
4	H	CH ₂ CH ₂ C ₆ H ₅
5	H	CH ₃
6	H	OAc
7	OAc	H

Figure 1. Structure of *Alternaria tagetica* metabolites.

the isomeric phthalides **2** and **3**, 8-zinniol 2-(phenyl)ethyl ether (**4**), 8-zinniol methyl ether (**5**), 8-zinniol acetate (**6**), and 7-zinniol acetate (**7**). In addition, the synthesis of phytotoxin **5** by chemical transformation of zinniol and the biological evaluation of a number of intermediate derivatives (**8–13**) generated during the synthetic process are reported.

MATERIALS AND METHODS

General Experimental Procedures. TLC, IR, EIMS, and ¹H, ¹³C, and 2D NMR instrumentation and experimental procedures have been described previously (*9*, *10*). GC-EIMS analyses were carried out using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971A mass selective detector. Conditions for GC analyses were as follows: 0.5 μL of 0.1% sample; Ultra 1 column (Hewlett-Packard, cross-linked methyl silicone gum, 25 m × 0.32 mm i.d., 0.52

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Table 1. ^1H NMR Data (δ , m, J = Hz) for Metabolites **1**, **1a**, and **4–7** in CDCl_3 at 500 MHz

proton	1	1a ^a	4	5	6	7 ^a
6	6.68, br s	6.72, s	6.71, s	6.73, s	6.79, s	6.68, s
7	4.68, s	5.14, s	4.55, d, 6.5	4.6, br s	4.7, s	5.2, s
8	4.76, s	5.22, s	4.63, s	4.59, s	5.25, s	4.73, ABq
9	3.78, s	3.74, s	3.65, s	3.72, s	3.74, s	3.81, s
10	2.16, s	2.17, s	2.15, s	2.16, s	2.16, s	2.17, s
1'	4.52, br d, 6.5	4.54, br d, 6.6	4.53, br d, 6.5	4.54, d, 6.5	4.55, d, 6.6	4.53, d, 6.42
2'	5.49, ddd, 6.5, 1.5, 1.5	5.47, ddd, 6.6, 1.2, 1.5	5.48, ddd, 6.5, 1.5, 1.5	5.55, ddd, 6.5, 1.5, 1.5	5.49, ddd, 6.6, 1.5, 1.5	5.47, ddd, 6.3, 1.8, 1.5
3'						
4'	1.8, s	1.8, s	1.79, d, 1	1.79, s	1.78, s	1.79, d, 0.9
5'	1.74, s	1.75, s	1.73, d, 1	1.74, s	1.74, s	1.75, s
1''		2.07, s	3.8, dd, 7, 7.5	3.44, s	2.05, s	2.1, s
2''		2.05, s	2.9, dd, 7, 7			
Ar-H			7.18–7.29, m			

^a 300 MHz.

μm film thickness); flow rate, 1 mL/min (He); temperature program, $T_1 = 180^\circ\text{C}$, $T_2 = 290^\circ\text{C}$, gradient = $10^\circ\text{C}/\text{min}$; detector temperature, 300°C .

HPLC Instrumentation and Analyses. (1) A Waters Delta prep 4000 preparative chromatography system (Millipore Corp.) equipped with a Waters 486 UV detector for semipreparative separations and a Waters 996 photodiode array detector was used for analytical studies. Control of the equipment, data acquisition, and processing and management of chromatographic information were performed by the Millennium 2000 software program (Waters). Conditions for HPLC analyses were as follows: Microporasil normal phase column (Waters, $10\ \mu\text{m}$, $300 \times 3.9\ \text{mm}$ i.d.), using hexane/EtOAc 85:15 (method a) or $\text{CHCl}_3/\text{EtOAc}$ 90:10 (method b); flow rate = 1.0 mL/min; and UV detection at 260 nm.

(2) A Milton Roy CM-4000 series chromatograph was equipped with a Milton Roy SM-4000 UV detector and a Milton Roy CS-4100 integrator. Conditions for HPLC analyses were as follows: Hypersil ODS C_{18} column (Alltech, $5\ \mu\text{m}$, $250 \times 4.6\ \text{mm}$ i.d.); gradient elution using $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 10:90 to 90:10 (15 min, method c); flow rate = 1.0 mL/min; and UV detection at 254 nm.

Isolation and Purification of Metabolites 1–6. Details of the microbiological work, leaf-spot assay, and fractionation of the organic crude extract by solvent partition and VLC purification (13 fractions) have been described previously (9, 10). HPLC separations were carried out using a preparative column ($300 \times 19.5\ \text{mm}$ i.d.) packed with the same microporasil normal phase as that used for analytical studies. After VLC of the organic crude extract, purification of fraction 5 by HPLC (method a, flow = 18 mL/min) yielded metabolite **2** in pure form (0.73 mg/L of culture filtrate). Fraction 6, after successive HPLC purifications (method a, flow of 18 and 15 mL/min), produced pure **3** (0.21 mg/L) and **4** (0.17 mg/mL). A similar purification of fraction 7 (method a, flow of 15 mL/min) resulted in the isolation of **5** (0.28 mg/mL) as a single component. Finally, purification of phytotoxic fraction 9, using the same column but method b and a flow of 16 mL/min, yielded **6** (0.40 mg/L) and **7** (0.19 mg/L).

6-(3',3'-Dimethylallyloxy)-4-methoxy-5-methylphthalide (2) was obtained as a white amorphous powder: R_f 0.24 (CHCl_3) and 0.39 (hexane/EtOAc 8:2); t_R (HPLC, method a) 9.47 min; IR, EIMS, and ^1H and ^{13}C NMR spectra were identical to those reported in the literature (9–11).

5-(3',3'-Dimethylallyloxy)-7-methoxy-6-methylphthalide (3) was obtained as a colorless oil: R_f 0.1 (CHCl_3) and 0.32 (hexane/EtOAc 8:2); t_R (HPLC, method a) 13.3 min; IR, EIMS, and ^1H and ^{13}C NMR spectra were identical to those reported in the literature (10, 12).

8-Zinniol 2-(phenylethyl ether (4)) was obtained as a colorless oil: R_f 0.55 ($\text{C}_6\text{H}_6/\text{acetone}$ 95:5); t_R (HPLC, method a) 14.5 min; IR (film) ν_{max} 3445 (OH), 3020 (Ar-H), 1605 (C=C), 1214 (C-O), 1148 (C-O-C) cm^{-1} ; EIMS (70 eV), m/z 370 (M^+ , 8), 302 ($\text{M}^+ - \text{C}_5\text{H}_8$, 8), 284 ($\text{M}^+ - \text{C}_5\text{H}_8 - \text{H}_2\text{O}$, 8), 180 (100), 105 (C_8H_9 , 22), 69 (C_5H_9 , 18); ^1H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**.

8-Zinniol methyl ether (5) was obtained as a white amorphous powder: R_f 0.3 (hexane/acetone 8:2); t_R (HPLC, method a) 19.1 min,

Table 2. ^{13}C NMR Data for Metabolites **1**, **1a**, and **4–7** in CDCl_3 at 125 MHz

C no.	1	1a ^a	4	5	6	7 ^a
1	138.9	137.7	140.3	140.3	139.5	139.5
2	124.7	120.7	121.1	121.2	118.5	125.3
3	158.2	159.1	158.3	158.4	159.1	157.8
4	120.0	119.7	119.3	119.4	119.7	121.0
5	157.7	158.4	158.0	158.1	158.7	157.8
6	109.0	109.3	109.0	109.1	108.0	109.4
7	64.7	64.1	64.4	64.5	63.1	64.6
8	56.8	58.4	64.8	66.5	58.7	56.8
9	61.9	61.7	61.7	61.8	61.7	61.8
10	9.2	9.3	9.2	9.2	9.3	9.3
1'	65.3	65.3	65.3	65.3	65.4	65.4
2'	119.9	119.7	120.0	119.9	119.8	119.8
3'	137.6	134.4	137.5	137.5	137.7	137.7
4'	25.8	25.7	25.8	25.8	25.8	25.8
5'	18.2	18.2	18.2	18.2	18.3	18.2
1''		170.9	71.4	58.0	171.1	170.9
2''		21.0	36.4		21.2	21.2
		170.6	138.6 ^b			
		21.0	128.9 ^b (2)			
			128.4 ^b (2)			
			126.3 ^b			

^a 75 MHz. ^b Ar-C.

(HPLC, method c) 18.27 min; t_R (GC) 8.23 min; IR (film) ν_{max} 3433 (OH), 1604 (C=C), 1214 (C-O), 1114 (C-O-C) cm^{-1} ; EIMS (70 eV), m/z 280 (M^+ , 8), 212 ($\text{M}^+ - \text{C}_5\text{H}_8$, 36), 194 ($\text{M}^+ - \text{C}_5\text{H}_8 - \text{H}_2\text{O}$, 10), 180 (100), 69 ($\text{C}_5\text{H}_8 + \text{H}$, 25); ^1H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**.

8-Zinniol acetate (6) was obtained as a yellow oil: R_f 0.27 ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$ 9:1); t_R (HPLC, method b) 14.9 min; IR (film) ν_{max} 3435 (OH), 1739 (C=O), 1604 (C=C), 1232 (C-O), 1115 (C-O-C) cm^{-1} ; EIMS (70 eV), m/z 308 (M^+ , 7), 240 ($\text{M}^+ - \text{C}_5\text{H}_8$, 12), 222 ($\text{M}^+ - \text{C}_5\text{H}_8 - \text{H}_2\text{O}$, 5), 180 (100), 162 (180 - H_2O , 32), 69 ($\text{C}_5\text{H}_8 + \text{H}$, 42); ^1H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**.

7-Zinniol acetate (7) was obtained as a pale yellow oil: R_f 0.25 ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$ 9:1); t_R (HPLC, method b) 17.2 min; IR (film) ν_{max} 3435 (OH), 1739 (C=O), 1604 (C=C), 1232 (C-O), 1115 (C-O-C) cm^{-1} ; EIMS (70 eV), m/z 308 (M^+ , 7), 240 ($\text{M}^+ - \text{C}_5\text{H}_8$, 15), 222 ($\text{M}^+ - \text{C}_5\text{H}_8 - \text{H}_2\text{O}$, 5), 180 (100), 162 (180 - H_2O , 11), 69 ($\text{C}_5\text{H}_8 + \text{H}$, 26); ^1H NMR data, see **Table 1**; ^{13}C NMR data, see **Table 2**.

Oxidation of Zinniol (1). A solution of **1** (616.8 mg, 2.31 mmol) in acetone (485 mL) was stirred with an excess of a stock chromic acid solution (11.0 g of chromium trioxide in 10 mL of concentrated sulfuric acid diluted to 50 mL with water) at room temperature for 5 min (1). After dilution with water, the oxidation crude product was obtained by ethyl ether extraction of the aqueous suspension (three times, 2:1). The combined organic layer were washed with water and

saturated sodium chloride solution. The solvents were then removed, and the mixture of three products was filtered through a silica gel bed and separated by preparative HPLC (method a) to yield **2** (168.2 mg, 28%), **3** (176.7 mg, 29%), and **8** (117.9 mg, 18%), in pure form.

Zinniol anhydride (8) was obtained as a white amorphous powder: R_f 0.52 (CHCl₃); t_R (HPLC, method a) 4.4 min; IR (film) ν_{\max} 2939 (C–H), 1838 (anh C=O), 1772 (anh C=O), 1614 (C=C), 1214 (C–O), 1114 (C–O–C) cm⁻¹; EIMS (70 eV), m/z 276 (M⁺, 3), 208 (M⁺ – C₅H₈, 5), 164 (M⁺ – C₅H₈ – CO₂, 8), 69 (C₅H₉, 100); ¹H NMR (300 MHz, CDCl₃) δ 7.15 (1H, s, H-6), 5.47 (1H, ddd, J = 6.6, 6.6, 1.5 Hz, H-2'), 4.68 (2H, br d, J = 6.6 Hz, H-1'), 4.16 (2H, s, H-9), 2.21 (3H, s, H-10), 1.82 (3H, br d, J = 0.9 Hz, H-4'), 1.75 (3H, s, H-5'); ¹³C NMR (75 MHz, CDCl₃) δ 163.5 (C-8), 164.5 (C-7), 160.9 (C-3), 157.9 (C-5), 139.6 (C-1), 131.9 (C-3'), 118.2 (C-2'), 112.1 (C-4), 103.1 (C-6), 66.4 (C-1'), 62.3 (C-9), 25.8 (C-4'), 18.3 (C-5'), 9.6 (C-10).

Hydrolysis (13) and Methylation (14) of 6-(3',3'-Dimethylallyloxy)-4-methoxy-5-methylphthalide. A mixture of **2** (25 mg, 0.095 mmol), MeOH (10 mL, Aldrich), and an excess of K₂CO₃ (Merck) was heated under reflux for 7 h. The solvent was removed in vacuo. The residue was combined with 4 mL of dry THF (Aldrich) under nitrogen, and then a suspension of 180 mg of NaH (80% in mineral oil, 6 mmol, Aldrich), previously washed with dry THF, was added. The suspension was stirred at room temperature for 30 min, and 300 μ L of CH₃I (4.82 mmol, Baker) was added slowly. The mixture was kept at room temperature until no starting material could be detected by TLC (43 h). The reaction mixture was quenched with saturated NH₄-Cl solution, diluted with water, and extracted with EtOAc (three times, 2:1, 1:1, 1:1, v/v). The organic layer was washed with water to neutralization, then with brine, followed by treatment with Na₂SO₄ and filtration. The solvent was finally removed in vacuo, yielding **9** (12.2 mg, 42%). The remaining aqueous layer was neutralized with H₂SO₄ (2 N) and extracted with EtOAc (three times, 2:1, 1:1, 1:1, v/v). The organic layer was washed with water and brine, dried, concentrated, and filtered through a silica gel microcolumn (hexane/acetone 8:2) to yield **10** (3.7 mg, 13%).

Methyl-3-methoxy-4-methyl-5-(3',3'-dimethylallyloxy)-2-methoxymethyl benzoate (9) was obtained as a yellow oil: R_f 0.49 (hexane/acetone 8:2); t_R (GC) 8.88 min; IR (CHCl₃) ν_{\max} 3015 (Ar–H), 1730 (C=O), 1607 (C=C), 1118 (C–O–C) cm⁻¹; EIMS (70 eV), m/z 225 (M⁺ – C₅H₈ – CH₃, 9), 193 (M⁺ – C₅H₈ – H₂O, 100), 179 (3), 69 (C₅H₉, 71); ¹H NMR (300 MHz, CDCl₃) δ 7.14 (1H, s, H-6), 5.47 (1H, ddd, J = 6.6, 6.6, 1.2 Hz, H-2'), 4.71 (2H, s, H-8), 4.54 (2H, d, J = 6.6 Hz, H-1'), 3.9 (3H, s, H-1''), 3.76 (3H, s, H-9), 3.39 (3H, s, H-2''), 2.19 (3H, s, H-10), 1.79 (3H, s, H-4'), 1.75 (3H, s, H-5'); ¹³C NMR (75 MHz, CDCl₃) δ 168.1 (C-7), 158.8 (C-3), 157.3 (C-5), 137.9 (C-3'), 129.8 (C-1), 124.6 (C-2), 124.2 (C-4), 119.6 (C-2'), 109.3 (C-6), 65.7 (C-8), 65.4 (C-1'), 61.9 (C-9), 58.2 (C-2''), 52.1 (C-1''), 25.8 (C-4'), 18.3 (C-5'), 9.5 (C-10).

3-Methoxy-4-methyl-5-(3',3'-dimethylallyloxy)-2-methoxymethyl benzoic acid (10) was obtained as a white solid: R_f 0.14 (hexane/acetone 8:2); t_R (GC) 10.54 min; IR (CHCl₃) ν_{\max} 3400–2600 (COOH), 3008 (Ar–H), 1716 (C=O), 1603 (C=C), 1114 (C–O–C) cm⁻¹; EIMS (70 eV), m/z 226 (M⁺ – C₅H₈, 10), 211 (M⁺ – C₅H₈ – CH₃, 18), 193 (211 – H₂O, 100), 165 (11), 69 (C₅H₉, 61); ¹H NMR (300 MHz, CDCl₃) δ 7.23 (1H, s, H-6), 5.47 (1H, ddd, J = 6.6, 6.6, 1.2 Hz, H-2'), 4.71 (2H, s, H-8), 4.58 (2H, d, J = 6.6 Hz, H-1'), 3.74 (3H, s, H-9), 3.49 (3H, s, H-1''), 2.21 (3H, s, H-10), 1.80 (3H, br d, J = 1.2 Hz, H-4'), 1.75 (3H, s, H-5'); ¹³C NMR (75 MHz, CDCl₃) δ 168.9 (C-7), 158.3 (C-3), 158.1 (C-5), 138.2 (C-3'), 131.3 (C-1), 125.1 (C-2), 121.1 (C-4), 119.4 (C-2'), 110.7 (C-6), 66.1 (C-7), 65.3 (C-1'), 62.0 (C-9), 58.0 (C-1''), 25.8 (C-4'), 18.3 (C-5'), 9.7 (C-10).

Hydrolysis and Methylation of 5-(3',3'-Dimethylallyloxy)-7-methoxy-6-methylphthalide. A solution of **3** (21 mg, 0.08 mmol) in MeOH (10 mL) was heated under reflux with an excess of K₂CO₃ for 24 h. The reaction mixture was concentrated under vacuum, and the resulting material was combined with dry THF (4 mL) and a suspension of NaH (208 mg, 6.93 mmol, 80% in mineral oil). The mixture was stirred at room temperature for 30 min, and then CH₃I (200 μ L, 3.2 mmol) was added dropwise. Once no more starting material could be detected by TLC (44 h), the reaction mixture was quenched with a

saturated NH₄Cl solution, diluted with water, and extracted with EtOAc (three times, 2:1, 1:1, 1:1, v/v). The organic layer was washed with water and brine, dried (Na₂SO₄), and filtered. The solvent was removed under reduced pressure to produce **11** (14.1 mg, 57%). The residual aqueous layer was neutralized with H₂SO₄ (2 N) and extracted with EtOAc (three times, 2:1, 1:1, 1:1, v/v). The organic layer was washed with water and brine, dried, and concentrated to yield **12** (5 mg, 21%).

Methyl-2-methoxy-3-methyl-4-(3',3'-dimethylallyloxy)-6-methoxymethyl benzoate (11) was obtained as a pale yellow oil: R_f 0.44 (hexane/acetone 8:2); t_R (GC) 8.75 min; IR (CHCl₃) ν_{\max} 3010 (Ar–H), 1727 (C=O), 1602 (C=C), 1114 (C–O–C) cm⁻¹; EIMS (70 eV), m/z 308 (M⁺, 1), 240 (M⁺ – C₅H₈, 61), 208 (240 – CH₃O – H, 100), 193 (208 – CH₃, 66); ¹H NMR (300 MHz, CDCl₃) δ 6.71 (1H, s, H-6), 5.47 (1H, ddd, J = 6.5, 6.5, 1.5 Hz, H-2'), 4.54 (2H, d, J = 6.5 Hz, H-1'), 4.46 (1H, br s, H-7), 3.89 (3H, s, H-2''), 3.76 (3H, s, H-9), 3.56 (3H, s, H-1''), 2.14 (3H, s, H-10), 1.79 (3H, s, H-4'), 1.74 (3H, s, H-5'); ¹³C NMR (75 MHz, CDCl₃) δ 168.4 (C-8), 159.1 (C-3), 157.0 (C-5), 137.8 (C-3'), 135.6 (C-1), 119.7 (C-2'), 119.6 (C-4), 119.1 (C-2), 106.8 (C-6), 72.4 (C-7), 65.4 (C-1'), 61.9 (C-9), 58.3 (C-1''), 52.0 (C-2''), 25.8 (C-4'), 18.3 (C-5'), 9.0 (C-10).

2-Methoxy-3-methyl-4-(3',3'-dimethylallyloxy)-6-methoxymethyl benzoic acid (12) was obtained as a white solid: R_f 0.12 (hexane/acetone 8:2); t_R (GC) 9.68 min; IR (CHCl₃) ν_{\max} 3400–2600 (COOH), 3008 (Ar–H), 1715 (C=O), 1603 (C=C), 1114 (C–O–C) cm⁻¹; EIMS (70 eV), m/z 294 (M⁺, 2), 226 (M⁺ – C₅H₈, 13), 208 (226 – H₂O, 10), 193 (208 – CH₃, 100), 179 (11), 165 (12), 69 (C₅H₉, 23); ¹H NMR (300 MHz, CDCl₃) δ 7.07 (1H, s, H-6), 5.47 (1H, ddd, J = 6.6, 6.6, 1.5 Hz, H-2'), 4.81 (1H, s, H-7), 4.62 (2H, d, J = 6 Hz, H-1'), 3.86 (3H, s, H-9), 3.48 (3H, s, H-1''), 2.17 (3H, s, H-10), 1.80 (3H, br d, J = 1.2 Hz, H-4'), 1.77 (3H, s, H-5'); ¹³C NMR (75 MHz, CDCl₃) δ 166.0 (C-8), 161.0 (C-3), 158.3 (C-5), 142.1 (C-1), 138.3 (C-3'), 119.3 (C-4 and C-2'), 118.5 (C-2), 107.0 (C-6), 72.9 (C-7), 65.5 (C-1'), 62.5 (C-9), 58.6 (C-1''), 25.8 (C-4'), 18.3 (C-5'), 9.0 (C-10).

7-Zinniol Methyl Ether (13). A suspension of LiAlH₄ (26 mg, 0.68 mmol) in dry THF (5 mL), under N₂, in an ice bath, was stirred while a solution of **11** (10 mg, 0.32 mmol) in THF was added dropwise. After 5 min, the excess reagent was eliminated by successive additions of THF/water (9:1) until a white precipitate appeared. The reaction mixture was filtered, and the filtrate was extracted with ethyl acetate (three times, 2:1, 1:1, 1:1, v/v). The organic layer was washed with water and brine, dried, and evaporated to afford the crude reduction product, which was filtered through silica gel (hexane/acetone) giving **13** (4.1 mg, 46%) in pure form: R_f 0.3 (hexane/acetone 8:2); t_R (GC) 8.21 min; t_R (HPLC, method c) 19.09 min; IR (CHCl₃) ν_{\max} 3468 (OH), 3009 (Ar–H), 1606 (C=C), 1116 (C–O–C) cm⁻¹; EIMS (70 eV), m/z 212 (M⁺ – C₅H₈, 2), 194 (M⁺ – C₅H₁₁ – H₂O, 5), 180 (100), 163 (30), 69 (C₅H₉, 100); ¹H NMR (300 MHz, CDCl₃) δ 6.64 (1H, s, H-6), 5.49 (1H, ddd, J = 6.6, 6.6, 1.2 Hz, H-2'), 4.67 (2H, s, H-8), 4.52 (2H, d, J = 6 Hz, H-1'), 4.52 (2H, s, H-7), 3.81 (3H, s, H-9), 3.43 (3H, s, H-1''), 2.18 (3H, s, H-10), 1.79 (3H, br d, J = 0.9 Hz, H-4'), 1.74 (3H, s, H-5'); ¹³C NMR (75 MHz, CDCl₃) δ 158.4 (C-3), 157.3 (C-5), 137.5 (C-3'), 135.3 (C-1), 126.1 (C-2), 120.5 (C-4), 120.0 (C-2'), 109.4 (C-6), 74.4 (C-7), 65.4 (C-1'), 61.9 (C-9), 56.9 (C-8), 25.8 (C-4'), 18.2 (C-5'), 9.2 (C-10).

8-Zinniol Methyl Ether (5). A solution of **9** (4 mg, 0.013 mmol) in anhydrous THF (3 mL) was treated with LiAlH₄ (12.4 mg, 0.32 mmol) for 2 h as described above, to give **5** (1.9 mg, 49%).

Phytotoxicity Evaluation. Natural and synthetic metabolites were tested for phytotoxic activity using the leaf-spot assay (15) on marigold leaves (0.1 mg/application).

Phytotoxic metabolites (necrotic area in mm²): **1** (0.62), **5** (0.15), **6** (0.10), **10** (0.49), **12** (0.16) and **13** (0.13).

RESULTS AND DISCUSSION

Bioassay-guided VLC and preparative HPLC purifications of the phytotoxic organic crude extract from *A. taquetica* resulted in the purification and identification of six metabolites (**2–7**), two of them (**5, 6**) phytotoxic.

The isomeric phthalides 6-(3',3'-dimethylallyloxy)-4-methoxy-5-methylphthalide (**2**) and 5-(3',3'-dimethylallyloxy)-7-methoxy-6-methylphthalide (**3**) were identified by comparing their spectroscopic data with those reported in the literature (11, 12) and by direct comparison with authentic samples. Phthalide **2** has been reported previously from *A. porri* (12) and *A. solani* (16), whereas **3** has been reported only from *A. porri* (11). Both isomers were first reported as the main products resulting from the oxidation of zinniol (1).

The remaining metabolites (**4–7**) were obtained in pure form after preparative HPLC purifications of various fractions. Their IR, EIMS, and ^1H NMR spectra showed close similarities to zinniol (1, 9). These included a single aromatic proton, confirming the presence of a pentasubstituted aromatic ring, two methyl groups, one attached to the aromatic ring and the other as part of an aryl methyl ether, and signals corresponding to the dimethylallyloxy side chain. The presence of the characteristic base peak fragment at m/z 180 in the MS spectra of metabolites **4–7**, and their lower polarity on TLC when compared to **1**, suggested that these derivatives had one or both of the benzylic alcohols substituted. The nature and location of the substituents on each metabolite were established following careful analysis of their MS, ^1H NMR (Table 1), and ^{13}C NMR (Table 2) data, in addition to the results from HMQC and HMBC experiments.

The EIMS spectrum of **4** showed a molecular ion peak at m/z 370 which corresponded to a difference of 104 units with respect to that of zinniol. Additionally, the ^1H NMR spectrum of **4** showed five additional protons in the aromatic region, which appeared as multiple signal (centered at δ 7.23) typical of a monosubstituted aromatic ring; the spectrum also showed two doublets of doublets at δ 3.80 and 2.90 assigned to the protons of two methylene groups, respectively, one of which (δ 3.80) showed the typical chemical shift for a methylene-bearing oxygen. The presence of a second aromatic ring in the structure of **4** was confirmed by 12 sp^2 carbons signals in its ^{13}C NMR spectrum (6 methine and 6 quaternary, Table 1), whereas the two new methylene carbon signals appeared at δ 71.4 and 36.4. These data were constant with the presence of an oxygenated 2-(phenyl)ethyl fragment in the structure of **4**, and this fragment can readily explain the 104 amu molecular weight difference from zinniol.

To establish the correct location of the 2-(phenyl)ethyl fragment in structure **4**, it was necessary to first establish the correct assignment of the various methylene groups in its structure. The HMQC experiment of **4** showed a direct correlation between the methylene protons at δ 4.63, 4.55, 3.80, and 2.90 and the carbon signals at δ 64.8, 64.4, 71.4, and 36.4, respectively. A long-range interaction observed in the HMBC experiment of **4**, between the aromatic proton at δ 6.71 and the carbon signal at δ 64.4, allowed the latter to be assigned to C-7 and the carbon signal at δ 64.8 to C-8. Similarly, long-range interactions between the methylene protons at δ 4.63 (H-8) and the carbon signals at δ 140.3 (C-1), 121.1 (C-2), and 158.3 (C-3), and, through oxygen, with δ 71.4 (C-1''), indicated that the phenylethyl residue was located on C-8 via an ether linkage. This was supported by interactions observed between the methylene protons at δ 3.80 and the carbon signals at δ 36.4 (C-2'') and 138.6 (C-3''), and, through oxygen, with δ 64.8 (C-8). On the basis of these data, the new metabolite **4** was identified as 8-zinniol 2-(phenyl)ethyl ether.

Phytotoxic metabolite **5** was obtained as single component on TLC. A parent ion peak at m/z 280 in its EIMS suggested a zinniol structure with an additional methylene moiety. A three-

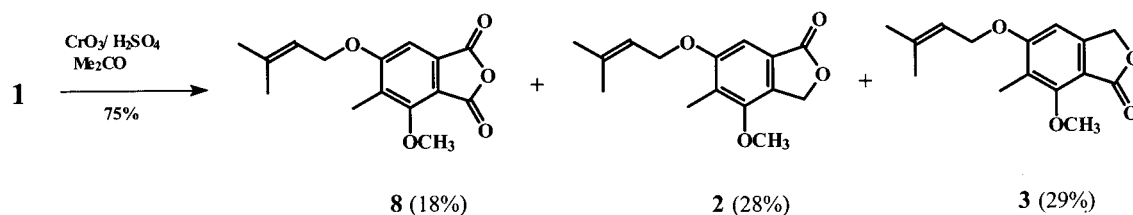
proton singlet at δ 3.44 in the ^1H NMR, together with a carbon signal at δ 58.0 in the ^{13}C NMR, confirmed the presence of an additional methoxyl group in the structure of **5**. Whereas the chemical shift for C-7 (δ 64.5) in the ^{13}C NMR of **5** was very similar to that of zinniol (δ 64.7), the corresponding C-8 signal in the spectrum of **5** appeared downfield (δ 66.5) relative to that of zinniol (δ 56.8), suggesting that the new methoxyl group was located at the C-8 position in the structure of **5**. This was confirmed by the long-range, 3J interaction through oxygen observed in the HMBC of **5** between the methyl group protons at δ 3.44 and the carbon signal at δ 66.5 (C-8). Phytotoxic metabolite **5** was thus identified as 8-zinniol methyl ether.

The IR spectra of metabolites **6** and **7** were identical, both presenting an intense absorption at 1739 cm^{-1} characteristic for an ester carbonyl group. As expected, their EIMS spectra also showed the same parent ion peak at m/z 308, where the difference of 42 units from zinniol suggested an acetylated structure. This was supported by a fragment at m/z 240 ($\text{M}^+ - \text{C}_5\text{H}_8$), originating from the loss of the dimethylallyloxy chain side, which after losing the elements of acetic acid, produced the characteristic base peak fragment at m/z 180. Further support for an acetylated zinniol structure came from the three proton methyl singlets at δ 2.16 in the ^1H NMR spectra of **6** and **7** and carbon signals at δ 171.1 and 170.9 in the ^{13}C NMR spectra. This suggested that metabolites **6** and **7** were isomeric monoacetylated derivatives of **1**. Although the HMQC experiment of metabolite **6** showed a direct correlation of the methylene protons at δ 5.25 and 4.70 with the carbon signals at δ 58.7 and 63.1, respectively, the HMQC experiment of **7** showed a direct correlation of the protons at δ 5.20 and 4.73 with the carbon signals at δ 64.6 and 56.8. A comparison of the chemical shift for both benzylic methylenes in the ^{13}C NMR spectra of **6** and **7** with those previously described for **1**, **4**, and **5** (Table 2) made it possible to identify **6** as 8-zinniol acetate and **7** as the isomeric 7-zinniol acetate. This assignment was further supported by the long-range interactions observed in the HMBC experiment of **6** between the methylene protons at δ 4.70 and the aromatic carbon at δ 108.0 (C-6) and between the methylene protons at δ 5.25 and carbon signals at δ 118.5 (C-2) and 158.7 (C-3) and the carbonyl carbon at δ 171.1 (carbonyl acetate). To rule out the possibility of **6** and **7** being formed during the isolation process, zinniol (**1**) was subjected to the same processes involved in working up the culture filtrate of *A. tagetica*, resulting only in the recovery of starting material. Additionally, treatment of **1** with acetic anhydride, at room temperature in the absence of pyridine, produced a mixture of both monoacetylated and diacetylated derivatives together with the starting material. The monoacetylated derivatives were identical on TLC to the natural derivatives **6** and **7**. It was noted that only the monoacetate **6** induced damage in marigold leaves when tested in the leaf-spot assay.

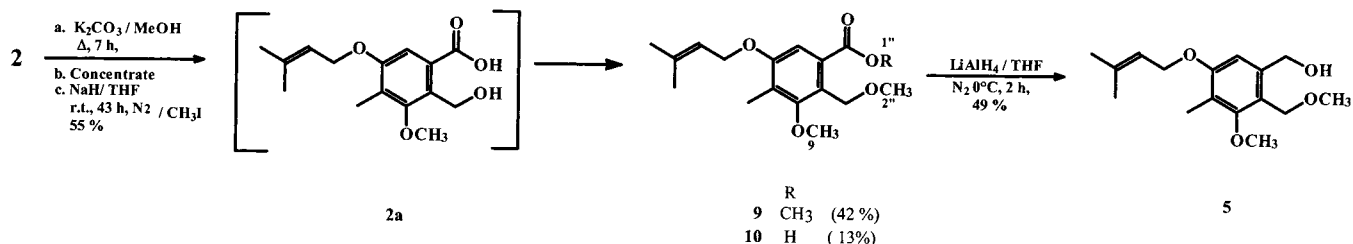
To date, a number of metabolites structurally related to zinniol have been reported. These include structures where the benzylic alcohols have been modified to form lactone (17–19) or lactam rings (5, 20) or to produce dimeric structures (9). These have no phytotoxicity to the host. However, this is the first report of natural zinniol derivatives having most of the original zinniol structure intact.

To confirm its proposed structure, metabolite **5** was prepared in the laboratory using zinniol as the starting material. Jones oxidation of **1** afforded the expected mixture of isomeric phthalides **2** and **3** together with a third product identified as zinniol anhydride (**8**, Scheme 1) (1, 8). The identity of **8** was established by the intense bands observed at 1838 and 1772

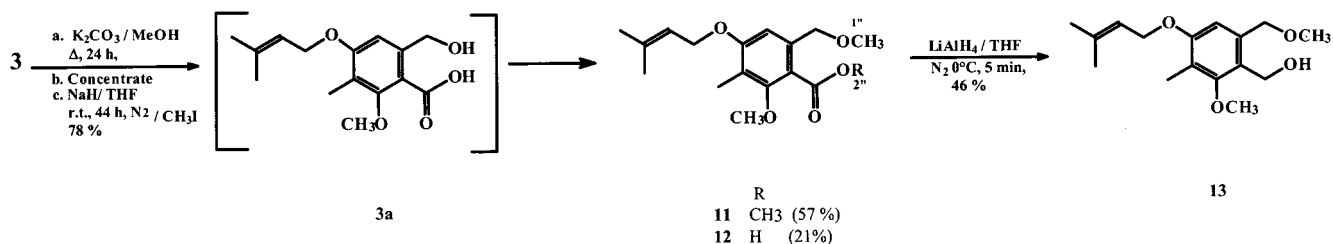
Scheme 1. Oxidation of Zinniol



Scheme 2. Synthesis of 8-Zinniol Methyl Ether (5)



Scheme 3. Synthesis of 7-Zinniol Methyl Ether (13)



cm^{-1} in its IR spectrum and by the presence of two quaternary carbon signals at δ 164.5 and 163.5 in its ^{13}C NMR spectrum, in addition to the absence of both proton and carbon signals characteristic of the benzylic methylene groups. The anhydride **8** has been reported as an intermediate during the synthesis of phthalides **2** and **3** (8). However, this is the first report of **8** being obtained as an oxidation of zinniol product.

Treatment of phthalide **2** under alkaline conditions led to the formation of a more polar product, the expected hydroxy acid (**2a**) (13). However, after workup of the reaction mixture, only starting material could be recovered, suggesting a relactonization of the hydroxy acid. To prevent this reversible reaction, once hydrolysis was shown to be complete by TLC, the solvent was removed under vacuum and the residue methylated. The reaction product contained a mixture of the ester **9** and its acid **10** (Scheme 2) (14). Similar treatment of **3** (Scheme 3) yielded the corresponding ester **11** and acid **12**. In this case, the hydrolysis product **3a** proved to be more stable than **2a**, because it could be recovered and analyzed by TLC and IR, showing the characteristic absorptions at 1715 and 1760 cm^{-1} due to the hydroxy acid and cyclization product mixture.

Reduction of **9** using LiAlH_4 produced a zinniol methyl ether, which was identical by TLC and HPLC to the natural metabolite **5** (Scheme 2). Similarly, reduction of **11** afforded 7-zinniol methyl ether (**13**) as the main component (Scheme 3). ^1H and ^{13}C NMR data for the isomeric methyl ether **13** were very similar to those of **5**; significant differences were observed only in their ^{13}C NMR spectra, where the chemical shift values of C-1, C-2, C-7, and C-8 in **13** are δ 135.3, 126.1, 74.4, and 56.9, respectively, and δ 140.3, 121.2, 64.5, and 66.5 in **5**.

The phytotoxic activity of all products (**1–13**), both natural and synthetic, was evaluated in the leaf-spot assay. Although zinniol (**1**) caused the strongest leaf necrotic lesions, neither its diacetylated derivative (**1a**) nor the isomeric phthalides **2** and

3 nor zinniol anhydride (**8**) showed significant activity. As expected, lower phytotoxic activity was detected with the monosubstituted derivatives **5** and **13**, thus confirming previous reports that both benzylic alcohols in zinniol are essential for the expression of phytotoxicity (3). However, the latter statement was seriously challenged when the monosubstituted derivatives **4** and **7** did not damage marigold leaves, and both carboxylated methyl ether derivatives **10** and **12** showed significant phytotoxic activity. This activity was readily lost upon esterification to produce **9** and **11**. These results indicate that there might be a number of factors controlling the expression of phytotoxic activity in these molecules, including the type, size, and position of the various functional groups.

In summary, we have reported the isolation and identification of six metabolites from the culture filtrates of *A. tagetica*. All metabolites were structurally related to zinniol, and four of them were identified as new natural products (**4–7**), whereas five new compounds (**9–13**) were synthetically prepared. Five of the products tested, both natural and synthetic derivatives (**5**, **6**, **10**, **12**, and **13**), showed phytotoxicity on marigold leaves. All derivatives were less phytotoxic than zinniol (**1**) with the exception of the acid derivative **10**, which showed a similar activity. The synthetic preparation of 8-zinniol methyl ether (**5**) and its isomer 7-zinniol methyl ether (**13**), using zinniol as starting material, confirmed the structure for the new natural product **5**.

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