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PHYTOTOXINS AND RELATED METABOLITES PRODUCED BY *BIPOLARIS COICIS*, THE PATHOGEN OF JOB'S TEARS

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Key Word Index—*Bipolaris coicis*; phytotoxins; radicinin; radicinol; diastereomers; *Coix lachryma-jobi*; Job's tears; Gramineae.

Abstract Four metabolites were isolated and characterized from the phytopathogenic fungus *Bipolaris coicis* H-13-3. Radicinin and its diastereomer were phytotoxic against Job's tears (*Coix lachryma-jobi*), a host of this fungus, but a diastereomer of radicinol and its epoxide were not phytotoxic. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Plant pathogens are a largely untapped reservoir of natural compounds with potential as herbicides or as herbicide leads [1]. During a screening-based search for natural herbicides or herbicide leads among the metabolites of phytopathogenic fungi, we found that an isolate (H-13-3) of *Bipolaris coicis* (Nishikado) Shoemaker from a seed of Job's tears produced phytotoxins against this plant. *B. coicis* is a phytopathogen which causes serious leaf blight on Job's tears (*Coix lachryma-jobi* L.), whose seeds have been used as medicine or health food [2]. One of the phytotoxins produced by this fungus was identified as the known phytotoxin radicinin [3, 4], and another was its stereoisomer. Two metabolites structurally related to radicinin were also isolated and identified as the stereoisomer of the fungal metabolite radicinol [5] and its epoxide. We report the characterization of these metabolites and their phytotoxicity to Job's tears and discuss the mechanism for formation of the stereoisomers.

RESULTS AND DISCUSSION

Compounds **1**, **2**, **3a** and **5** were isolated with respective yields of 6.9, 0.78, 11 and 0.11 mg l^{-1} from the culture filtrate of the fungus *B. coicis* H-13-3 grown on malt extract medium.

Compound **1**, obtained as colorless needles after crystallization from methanol, was identified as the fungal metabolite radicinin on the basis of its ^1H, ^{13}C NMR data (Tables 1 and 2), negative ion HRCIMS, mp and optical rotation [6–8]. Radicinin is a phytotoxin, and its production has been reported in *Alternaria radicina* [3, 4], *A. chrysanthemi* [9], *A. helianthi* [10] and *Cochliobolus lunata* [5].

Compound **2** was obtained as colorless needles after prep. TLC. It had the same molecular formula, $C_{12}H_{12}O_5$ (negative ion HRCIMS and ^{13}C NMR data), as radicinin, and their UV spectra were very similar. Carbon resonances were nearly superimposable (Table 2), and their ^1H resonances (Table 1) indicated the same structural units. These data led us to assign **2** the same structure as that of radicinin, compound **2** being a diastereomer of radicinin. The absolute stereochemistry of radicinin has been established as (2*S*, 3*S*) [5, 9], therefore the stereochemistry of **2** is (2*S**, 3*R**). This is the first report of the production of a diastereomer of radicinin.

Compound **3a** was obtained as an oil after prep. TLC. The ^{13}C NMR and HREIMS data for **3a** showed its molecular formula to be $C_{12}H_{14}O_5$, two more hydrogens than in radicinin. When compared with the findings for radicinin, the NMR data for **3a** (Tables 1 and 2) indicated that the α-pyrone ring and the 1-propenyl group are present in **3a**, and that **3a** has one more hydroxy methine on its dihydropyrane ring in contrast to the carbonyl carbon in radicinin. On the basis of these NMR data, **3a** has the same structure as the fungal metabolite radicinol, the natural metabolite derived from the reduction of radicinin at C-4 whose production has been reported in *Cochliobolus lunata*

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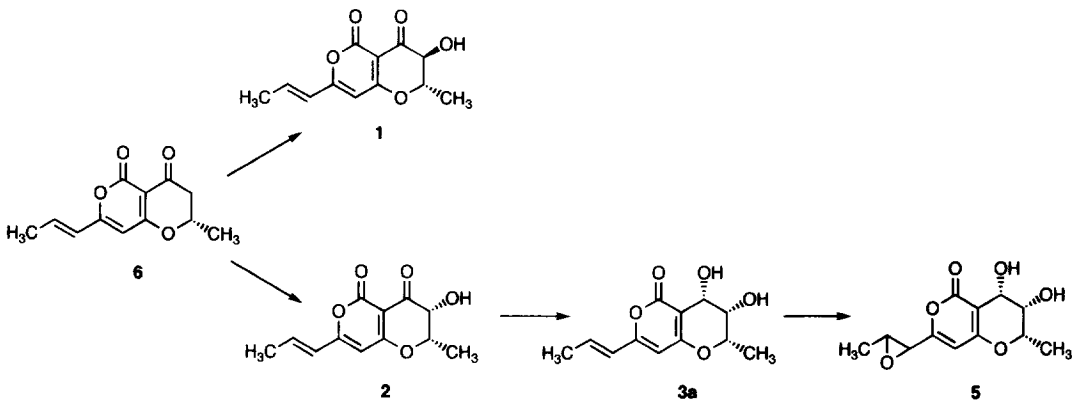


Fig. 1. Proposed biosynthetic relationships between the isolated metabolites (**1**, **2**, **3a** and **5**).

Table 1. ^{1}H NMR spectral data for compounds **1**, **2**, **3a** and **5** (270 MHz, $CDCl_3$)

C	1	2	3a	5
2	4.36 *dq* (12.4, 6.3)	5.07 *dq* (6.0, 6.5)	4.37 *dq* (1.1, 6.3)	4.37 *brq* (6.7)
3	3.98 *d* (12.4)	4.52 *d* (6.0)	3.88 *br*	3.84 *br*
4			4.60 *d* (2.8)	4.56 *d* (2.4)
8	5.84 *s*	5.83 *s*	5.79 *s*	6.05 *s*
9	6.03 *dq* (15.5, 1.8)	6.03 *dq* (15.3, 1.3)	5.97 *dq* (15.7, 1.5)	3.33 *d* (1.9)
10	6.95 *dq* (15.5, 7.0)	6.96 *dq* (15.3, 7.0)	6.72 *dq* (15.7, 6.8)	3.21 *dq* (1.9, 5.4)
11	1.95 *dq* (7.0, 1.8)	1.96 *dd* (7.0, 1.3)	1.91 *dd* (6.8, 1.5)	1.42 *d* (5.4)
12	1.64 *d* (6.3)	1.35 *d* (6.5)	1.50 *d* (6.3)	1.49 *d* (6.7)

[5] and *Alternaria chrysanthemi* [9]. Comparison of the NMR data for **3a** and for radicinol and *epi*-radicinol (C-4 epimer of radicinol) [5] showed that **3a** is not identical to either compound; rather it is a diastereomer of radicinol and *epi*-radicinol. The respective absolute stereochemistries of radicinol and *epi*-radicinol are (2*S*, 3*R*, 4*S*) and (2*S*, 3*R*, 4*R*) [5, 9], therefore **3a** must have either the configuration (2*S**, 3*S**, 4*R**), or (2*S**, 3*S**, 4*S**). This is the first report of the natural occurrence of a diastereomer of radicinol and *epi*-radicinol.

Table 2. ^{13}C NMR spectroscopic data for compounds **1**, **2**, **3a** and **5** (67.8 MHz, $CDCl_3$)

C	1*	2*	3a†	5‡
2	80.0	78.9	72.4	72.6
3	72.0	70.1	69.1	69.1
4	188.6	188.0	63.2	63.3
4a	97.9	97.6	99.5	100.4
5	156.7	156.7	165.4	164.8
7	164.3	164.5	158.5	160.4
8	98.0	98.4	99.3	99.7
8a	176.3	174.9	166.6	165.9
9	122.6	122.6	122.6	55.1
10	141.0	141.0	135.2	57.3
11	18.8	18.8	18.4	17.3
12	18.1	12.1	15.8	15.7

*Assignments are based on DEPT data and a comparison with the data reported for radicinin in DMSO-d_6 [6].

†Assignments are based on results of the DEPT, C,H-COSY and COLOC experiments.

‡Assignments are based on results of the DEPT, C,H-COSY and a comparison with the data for **3a**.

The most favoured conformations of radicinol, *epi*-radicinol and the two diastereomers, (2*S*, 3*S*, 4*R*) and (2*S*, 3*S*, 4*S*), were searched for using MOPAC/PM3 calculations. Table 3 shows the dihedral angles between H-2 and H-3 and between H-3 and H-4 in the most favoured conformations of these compounds as well as the coupling constants calculated from the equation reported by Haasnoot *et al.* [11]. For radicinol and *epi*-radicinol the calculated coupling constants agreed with the reported ones [5]. The coupling constants between H-2 and H-3 and between H-3 and H-4 (1.1 and 2.8 Hz, respectively) in the ^{1}H NMR spectrum of **3a** (Table 2) suggest that **3a** is the diastereomer (2*S**, 3*S**, 4*S**) rather than (2*S**, 3*S**, 4*R**). This was confirmed by the NOE experiment, in which irradiation of the H-2 proton caused NOE enhancement of the signal owing to the H-4 proton as well as the signal owing to the H-3 proton. In addition, **3a** easily formed acetonide **4** on treatment with dry acetone and *p*-TsOH, indicative that the orientation of the two vicinal hydroxyls is *cis*. Consequently, we concluded that the stereochemistry of **3a** is (2*S**, 3*S**, 4*S**).

The absolute stereochemistry was determined by application of the exciton chirality rule [12] to the CD spectrum of **3b**, the 3,4-bis-*p*-bromobenzoate derivative of **3a**. The conformation of **3b** is almost the same as that of **3a** as seen from the comparison of the

Table 3. The dihedral angles (degrees) in the most favoured conformations of radicinol, *epi*-radicinol and the diastereomers of radicinol, (2*S*, 3*S*, 4*R*) and (2*S*, 3*S*, 4*S*), together with the coupling constants (Hz) obtained by the method of Haasnoot *et al.* [11] based on the dihedral angles

Compound	H-2 and H-3			H-3 and H-4		
	ϕ	J_{calc}	J_{obs}	ϕ	J_{calc}	J_{obs}
Radicinol	180.0	8.3	8.0	−163.9	6.7	6.5
epi-Radicinol	−176.5	8.1	8.5	−44.3	3.6	4.0
(2*S*, 3*S*, 4*R*)	−56.0	4.1		160.8	6.3	
(2*S*, 3*S*, 4*S*)	64.6	0.2		−46.9	3.4	

coupling constants between H-2 and H-3 and those between H-3 and H-4 in the ^{1}H NMR spectra. In the CD spectrum of **3b**, the sign of the first CD Cotton effect at 254 nm caused by excitons was clearly negative, but the second CD Cotton effect was obscured by the negative broad CD peak at 211 nm. The chirality of the *p*-bromobenzoates showed a counterclockwise rotation. On the basis of these findings, the stereochemistry of **3a** was determined to be (2*S*, 3*S*, 4*S*). Furthermore, the reduction of **2** with $NaBH_4$ afforded **3a**, therefore the stereochemistry of **2** was also (2*S*, 3*R*).

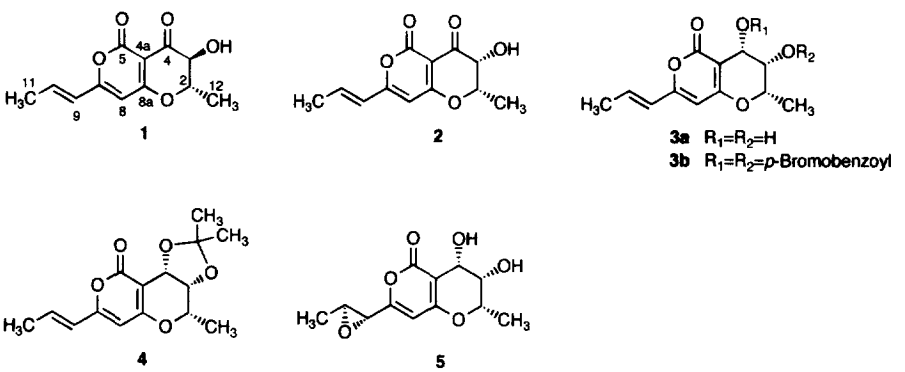
Compound **5** was obtained as an oil after prep. TLC. Its ^{13}C NMR data (Table 1) and HREI mass spectrum showed that its molecular formula is $C_{12}H_{14}O_6$, indicative that it has one more oxygen than **3a**. A comparison of the NMR data for **5** and **3a** indicated that a 3,4-dihydro-3,4-dihydroxy-2-methyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-5-one portion is present in **5** and that the compound contains a 1,2-epoxypropyl moiety instead of the 1-propenyl moiety found in **3a**. This was confirmed by conversion using the *m*CPBA of **3a** to **5**. Moreover, on the basis of this conversion the stereochemistry of C-2, 3 and 4 in **5** was determined to be (*S*, *S*, *S*). Irradiation of the methyl proton, H-11, caused NOE enhancement of the signal of the methine proton, H-9, indicative of a *trans* relation between the substituents on the epoxide ring. The absolute configurations at C-9 and 10, however, have yet to be established.

The biosyntheses of radicinin and related metabolites has been investigated [6,13–15], and they are believed to be derived from two distinct polyketides. In *B. coicis* H-13-3 (Fig. 1) it is probable that the deoxyradicinin (**6**) [7] synthesized from two distinct polyketides is subjected to non-stereospecific hydroxylation at C-3 to give radicinin (**1**) and its isomer (**2**) and that **2** is converted to **3a** by stereospecific reduction at C-4, followed by epoxidation of the side chain in **3a** to produce **5**. Non-specific hydroxylation that produces diastereomers, as in *B. coicis*, has also been reported in *Curvularia pallescens* metabolites, curvupallides A and B [16] and in *Neocosmospora vasinfecta* metabolites, neovasipyrones and neovasifuranones [17]. It is believed to be catalysed by cytochrome P-450 monooxygenases [18].

In the preliminary leaf spot bioassay of Job's tears, at 0.3 μg leaf^{-1} radicinin (**1**) caused necrotic lesions on the leaves, but not at 0.1 μg leaf^{-1}. The diastereomer of radicinin (**2**) caused necrotic lesions at 1 μg leaf^{-1}, but not at 0.3 μg leaf^{-1}. The diastereomer of radicinol (**3a**) and its epoxide (**5**) caused no lesions, even at 10 μg leaf^{-1}.

EXPERIMENTAL

General. NMR spectra were recorded in $CDCl_3$ on a JEOL JNM GX-270 FT NMR spectrometer. NMR chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0). Mass spectra were obtained with a JEOL AX-505 spectrometer. IR spectra were measured with a JASCO FT/IR-7000 spectrometer. Optical rotations were determined with a Horiba SEPA-200 high sensitive polarimeter. The CD was measured with a JASCO J-720 CD spectrometer. Daisogel IR-60 was the silica gel used for the CC. Prep. TLC was done on a



Merck Kieselgel 60 HF_{254} glass plate (20 × 20 × 0.05 cm).

Fungus. Strain H-13-3 of *Bipolaris coicis* (Nishikado) Shoemaker was isolated from a dried seed of *Coix lachryma-jobi* L. var. *ma-yuen* (Roman.) Stapf and maintained on potato dextrose agar.

Isolation of radicinin (**1**), *compounds* **2**, **3a** *and* **5**. The fungus was grown without shaking at 24° for 21 days in the dark in a 500 ml conical flask containing liquid medium (250 ml × 40) made up of glucose (30 g l^{-1}), peptone (3 g l^{-1}) and the extract from 50 g l^{-1} of malt and water. The culture filtrate was acidified to pH 2.0 with HCl, after which the metabolites in the culture filtrate were extracted with EtOAc (7 l × 3). The EtOAc extracts were dried over Na_2SO_4 and concd. The residue (2.7 g) was applied to a silica gel column (18 × 1.8 cm), and the column washed with 1500 ml of 10% Me_2CO in *n*-hexane then developed successively with 750 ml each 20, 30 and 40% Me_2CO in *n*-hexane. Each 150 ml eluate constituted one fr. Frs 10 and 11 were combined and evapd. Recrystallization of the residue (434 mg) from MeOH afforded radicinin (**1**) as needles (30 mg). The mother liquor was purified by Sephadex LH-20 column chromatography (123 × 3.2 cm, MeOH). The column was washed with 500 ml of MeOH and frs consisting of 7 ml eluates were collected. Frs 33–48 were combined and evapd. Recrystallization of the residue (293 mg) from MeOH afforded radicinin (**1**) as needles (39 mg). Compound **2** (R_f 0.38) in the mother liquor was sepd from the mixt. of compounds **3a** and **5** (R_f 0.26) by prep. TLC [Me_2CO-C_6H_6 (2:8), ×3]. The crude compound **2** was further purified with prep. TLC [EtOAc–*n*-hexane (7:3), ×3] which gave compound **2** as needles (R_f 0.63, 7.8 mg). Compounds **3a** (R_f 0.37, 107 mg) and **5** (R_f 0.25, 11 mg) were obtained pure after prep. TLC [EtOAc–*n*-hexane (7:3), ×3] of the mixture.

Radicinin (**1**). Needles (MeOH), mp 238–240°. $[\alpha]_D^{20}$ −236° ($CHCl_3$; *c* 0.1). UV λ_{max}^{EtOH} nm (log ε): 203 (4.00), 221 (4.14), 270 (3.66), 280 (3.61), 341 (4.15). IR ν_{max}^{KBr} cm^{-1}: 3462, 3098, 3034, 2990, 2924, 2918, 1760, 1659, 1605, 1522, 1456, 1435, 1379, 1325, 1228, 1172, 1056. ^1H and ^{13}C NMR spectral data: Tables 1 and 2. Negative ion CIMS (*iso*-butane, probe), 200 eV, *m/z* (rel. int.): 236$[M]^-$ (100); exact mass calcd for $C_{12}H_{12}O_5$ 236.0684, found 236.0688.

Compound **2**. Needles, mp 202–220° (decomp.). $[\alpha]_D^{20}$ −105° (EtOH; *c* 0.25). UV λ_{max}^{EtOH} nm (log ε): 203 (4.00), 220 (4.14), 269 (3.67), 280 (3.64), 338 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3420, 2928, 2858, 1742, 1655, 1605, 1531, 1458, 1438, 1381, 1263, 1170, 1118, 1044. ^1H and ^{13}C NMR spectral data: Tables 1 and 2. Negative ion CIMS (*iso*-butane, probe), 200 eV, *m/z* (rel. int.): 236 $[M]^-$ (100); exact mass calcd for $C_{12}H_{12}O_5$ 236.0684, found 236.0682.

Compound **3a**. Oil. $[\alpha]_D^{20}$ −19° (EtOH; *c* 0.85). UV λ_{max}^{EtOH} nm (log ε): 224 (4.45), 260 (3.51), 271 (3.56), 315 (3.95). IR ν_{max}^{film} cm^{-1}: 3408, 2988, 2942, 1690, 1618, 1574, 1437, 1383, 1315, 1278, 1214, 1160, 1141, 1071, 1025. ^1H and ^{13}C NMR spectral data: Tables 1 and 2. EIMS (probe), 70 eV, *m/z* (rel. int.): 238 $[M]^+$ (18), 181 (100), 152 (7), 137 (8), 111 (26); exact mass calcd for $C_{12}H_{14}O_5$ 238.0841, found 238.0845.

Compound **3b**. A soln of *p*-bromobenzoyl chloride (20 mg) in CH_2Cl_2 (2 ml) was added to **3a** (9.2 mg) in CH_2Cl_2 (2 ml) at 0°. Pyridine (1 ml) was added, and the mixt. allowed to stand at room temp. for 1 hr, after which it was poured into 0.1 M HCl (10 ml) and stirred for 1 hr. The products were extracted with EtOAc (10 ml × 3). The EtOAc extract was washed with 1 M $NaHCO_3$ (10 ml × 3) and brine (10 ml × 3) then dried over Na_2SO_4. After concn, the residue was purified by prep. TLC [EtOAc–*n*-hexane (2:8), ×2] to give **3b** as a solid (R_f 0.44, 1.5 mg). $[\alpha]_D^{20}$ −45° (EtOH; *c* 0.15). UV λ_{max}^{EtOH} nm (log ε): 204 (4.61), 223 (4.54), 249 (4.57), 318 (4.03). ^1H NMR (270 MHz, $CDCl_3$): δ 1.45 (3H, *d*, *J* = 6.8 Hz, H-12), 1.94 (3H, *dd*, *J* = 6.8, 1.5 Hz, H-11), 4.57 (1H, *dq*, *J* = 1.0, 6.8 Hz, H-2), 5.50 (1H, *dd*, *J* = 2.7, 1.0 Hz, H-3), 5.87 (1H, *s*, H-8), 6.03 (1H, *dq*, *J* = 15.5, 1.5 Hz, H-9), 6.05 (1H, *d*, *J* = 2.7 Hz, H-4), 6.79 (1H, *dq*, *J* = 15.5, 6.8 Hz, H-10), 7.57 (2H, *d*, *J* = 8.5 Hz, *p*-bromobenzoyl), 7.58 (2H, *d*, *J* = 8.5 Hz, *p*-bromobenzoyl), 7.84 (2H, *d*, *J* = 8.5 Hz, *p*-bromobenzoyl), 7.91 (2H, *d*, *J* = 8.5 Hz, *p*-bromobenzoyl). CD: $\Delta\varepsilon_{211}$ −3.2, $\Delta\varepsilon_{254}$ −6.6, $\Delta\varepsilon_{308}$ +1.2 (EtOH: *c* 3.3 × 10^{-5} M).

Compound **4**. A solution of **3a** (9.7 mg) and *p*-toluenesulfonic acid (15 mg) in dry Me_2CO (3 ml) was heated with a few pieces of molecular sieve (4A) then refluxed for 15 min. The cooled soln was filtered, and the filtrate diluted with EtOAc (20 ml). The EtOAc soln was washed with 1M $NaHCO_3$ (10 ml × 3) and brine (10 ml × 3) then dried over Na_2SO_4. After evapn, the residue was purified by prep. TLC [EtOAc–*n*-hexane (7:3), ×2] to give **4** as an oil (R_f 0.52, 2.3 mg). ^1H NMR (270 MHz, $CDCl_3$): δ 1.35 (3H, *s*, Me of isopropylidene), 1.41 (3H, *s*, Me of isopropylidene), 1.56 (3H, *d*, *J* = 6.8 Hz, H-12), 1.89 (3H, *dd*, *J* = 7.0, 1.6 Hz, H-11), 4.16 (1H, *dq*, *J* = 1.2, 6.8 Hz, H-2), 4.34 (1H, *dd*, *J* = 1.2, 6.8 Hz, H-3), 5.15 (1H, *d*, *J* = 6.8 Hz, H-4), 5.71 (1H, *s*, H-8), 5.94 (1H, *dq*, *J* = 15.9, 1.6 Hz, H-9), 6.72 (1H, *dq*, *J* = 15.9, 7.0 Hz, H-10). EIMS (probe), 70 eV, *m/z* (rel. int.): 278 $[M]^+$ (52), 263 (18), 221 (12), 203 (100), 177 (18).

Compound **5**. Oil. $[\alpha]_D^{20}$ −92° (EtOH; *c* 1.0). UV λ_{max}^{EtOH} nm (log ε): 215 (4.34), 288 (4.31). IR ν_{max}^{film} cm^{-1}: 3376, 3000, 2930, 1698, 1653, 1580, 1448, 1383, 1212, 1139, 1071, 1033. ^1H and ^{13}C NMR spectral data: Tables 1 and 2. EIMS (probe), 70 eV, *m/z* (rel. int.): 254 $[M]^+$ (28), 197 (100), 182 (10), 153 (79), 139 (13); exact mass calcd for $C_{12}H_{14}O_6$ 254.0790, found 254.0795.

3a *from* **2**. A solution of **2** (4.2 mg) and $NaBH_4$ (15 mg) in dry MeOH (5 ml) was stirred for 15 min at room temp. The reaction mixt. was diluted with brine (pH 2.0 with HCl, 15 ml), after which the products were extracted with EtOAc (10 ml × 3). The EtOAc extract was dried over Na_2SO_4 and evapd *in vacuo*. The residue was purified by prep. TLC [EtOAc–*n*-hexane (7:3), ×2] to give **3a** (R_f 0.33, 1.2 mg).

5 *from* **3a**. A solution of **3a** (20.6 mg), *m*-chloroperbenzoic acid (22 mg) and KH_2PO_4 (19 mg) in CH_2Cl_2 (5 ml) was stirred at 35° for 3 days then filtered, and the filtrate concd *in vacuo*. The residue was purified by prep. TLC [EtOAc–*n*-hexane (7 : 3)] to give **5** (R_f 0.18, 4.7 mg).

Molecular modeling procedure. Molecular modelling was done in a DEC α-150 workstation using MOPAC (Ver. 6.02), a revision of MOPAC Ver. 6.01 [19, 20], by PM3 method. The graphic display was made with a Macintosh Centris 650 using CSC Chem 3D Plus (Ver. 3.1) software.

Assay of phytotoxicity. Job's tears (*Coix lachryma-jobi* L. var. *ma-yuen* (Roman.) Stapf) was grown in vermiculite at 24° under fluorescent light. The second leaves of 2-week-old plants were detached and punctured with a needle. The sample was dissolved in 10% MeOH, and a 5-μl droplet placed on a needle-puncture wound. The lesions that developed were examined after 3 days at 24° in the dark.

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