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PLANT PATHOTOXINS FROM *ALTERNARIA CITRI*: THE MAJOR TOXIN SPECIFIC FOR ROUGH LEMON PLANTS*

J. M. GARDNER†, Y. KONO‡, J. H. TATUM§, Y. SUZUKI‡ and S. TAKEUCHI‡

University of Florida, Citrus Research and Education Center, Lake Alfred, FL 33850, U.S.A.; ‡The Institute of Physical and Chemical Research, Wako-shi, Saitama 351, Japan; §USDA Citrus and Subtropical Products Laboratory, Winter Haven, FL 33883, U.S.A.

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Key Word Index—*Citrus jambhiri*; *Citrus limonia*; Rutaceae; rough lemon; Rangpur lime; *Alternaria alternata* pv. *citri*; brown spot disease; dihydro-α-pyrone; polyalcohol; host-specific toxin.

Abstract—A pathotype of the fungus *Alternaria citri* that attacks rough lemon plants produces several toxins in culture which specifically damage rough lemon and Rangpur lime plants. The major toxin produced, Toxin I, was by far the most potent compound (ED_{50} = 30 ng/ml). Five other minor toxins were active at ED_{50} levels greater than 1 μg/ml. On the basis of mass, ^{1}H and ^{13}C NMR spectra and decoupling studies of Toxin I and derivative, Toxin I is a 19 carbon polyalcohol with an α-dihydropyrone ring. The γ-dihydropyrone tautomer was less predominant. Culture filtrates of *A. citri* also contained a biologically inactive, partially analogous, component possessing a tetrahydropyran ring. It probably arises from decarboxylation of Toxin I. Toxin I was highly specific and did not affect nonhost plants at 10 000 times the concentrations affecting rough lemon.

INTRODUCTION

Two distinct pathogenic types of the fungus *Alternaria citri* Ellis and Pierce have been previously shown to produce, in culture, toxins which affect only citrus plants that they attack [1–3]. One pathotype attacks rough lemon (*Citrus jambhiri* Lush.) and Rangpur lime (*C. limonia* Osbeck); the other is pathogenic to tangerine cultivars. Although at least 12 distinct host-specific toxins have been identified [4], the *A. citri* toxins (ACRL and ACTG toxins) present a unique situation in that closely related strains of a fungus produce toxins of different host specificity. Partial purification and general characteristics of these host-specific toxins from *A. citri* were described by Kohmoto *et al.* [3].

In this report we describe the separation of several toxins from *A. citri* attacking rough lemon (ACRL toxins), and characterize the major and most active toxin (Toxin I) and one of its inactive derivatives (compound A). An abstract and a preliminary note on the structure of ACRL Toxin I have been presented [5, 6]. Structural and other data for Toxins II, III, IV and IV′ and related analogs are presented in the following paper.

RESULTS AND DISCUSSION

Alternaria citri was grown in still culture for about 3 weeks at 25° using Czapek's medium (Difco) supplemented with 5–20 mg/ml of Zn^{2+} as zinc sulphate [3]. Twenty litres of culture filtrate were filtered and acidified to pH 3.6 with acetic acid and toxins absorbed either on Amberlite XAD-7 or Florisil (50–100 mesh, 50 g/l). Toxic fractions were eluted from XAD-7 with acetone, and from Florisil with ethyl acetate–acetone (1:1) yielding 100 ml of crude extract at *ca* 500 mg/ml in ethyl acetate after concentration *in vacuo*.

Toxic activity, estimated by necrosis, ion leakage, and reduction of proline incorporation (Fig. 1) by rough

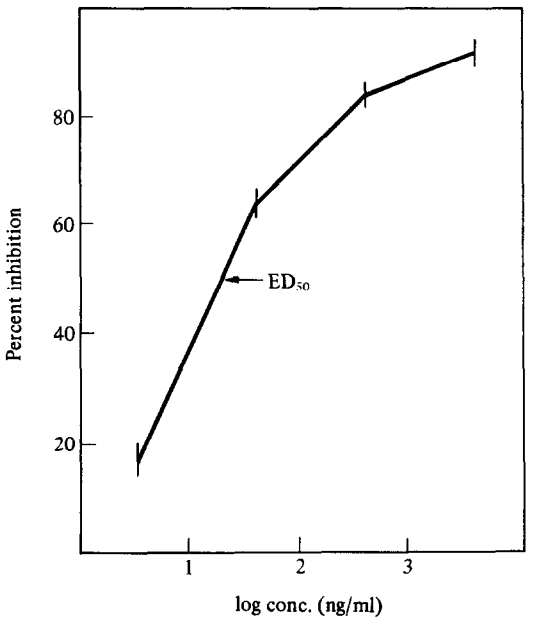

Fig. 1. Bioassay of ACRL Toxin I by inhibition of proline incorporation. See Experimental for details of bioassay procedure; tissue was preincubated with toxins for 2 hr followed by incorporation of ^{14}C-proline (0.4 mM; 655 dpm/nmole proline) for 3 hr. The toxin concentration at 50% inhibition (EC_{50}) is indicated by arrow.

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†Present address: Crop Science Laboratory, Allied Corp., P. O. Box 6, Solvay, NY 13209, U.S.A.

lemon leaves, was recovered in the aqueous phase after extractions with 0.5 M sodium bicarbonate (1:1) (pH 8.3) and was partitioned into fresh ethyl acetate after adjusting the pH to 3.6. Some minor toxic activity and an inactive relative of the toxins (compound A) were retained in the original ethyl acetate soluble crude preparation after extraction with sodium bicarbonate.

The major toxic fraction was concentrated *in vacuo* to 100 ml (*ca* 200 mg/ml), applied to a silicic acid (or in some cases, silica gel) column in ethyl acetate–dichloromethane (5:1) and eluted with a gradient of 0–20% acetone in ethyl acetate. Several toxic fractions were obtained and then applied to silica gel TLC plates (Analtech 1, 0.5 and 0.25 mm) which were developed with chloroform–methanol (85:15) and eluted with acetone. Compound A, also present in the toxin extracts, eluted first. Subsequent column fractions contained toxic components (detected by vanillin–H_2SO_4 and/or by UV) with R_f values of 0.44 (Toxin I) and 0.30 (Toxin II). Later fractions had at least four components at R_f 0.24 and 0.22 (Toxins III, III'), and at R_f 0.17 and 0.13 (Toxins IV, IV'). Toxin I was purified by repeated TLC or in some cases by normal phase (CN) HPLC (see Experimental); when concentrated it was a light orange oil from which small amounts of a colorless precipitate and, in a few instances, needle-like crystals (mp 192–196°) were separated (in hexane–acetone at −15°). The final yield of Toxin I was *ca* 20 mg/l of culture filtrate and 40 mg for compound A. The five minor TLC bands were re-purified several times by TLC with approximate yields of 5 (Toxin II), 2.5 (III), 0.5 (III'), 1 (IV) and 0.5 (IV') mg/l of culture filtrate.

Inhibition of incorporation of proline into protein was used as a sensitive assay for toxin (see Experimental). ACRL Toxin I showed a 50% inhibition at 18 to 30 ng/ml (Fig. 1). The dilution endpoint (min. detectable activity) was estimated to be about 1 ng/ml. As shown in Table 1, Toxin I was specifically active on the fungus-susceptible cultivars of rough lemon and Rangpur lime. There was one notable exception. Cleopatra mandarin (*C. reshni* Hort.), which was resistant to the fungus, was susceptible to toxin in the proline incorporation assay but much less susceptible in leaf bioassays. All other citrus varieties tested, representing ten species, were not affected by Toxin I at 10000 times the concentration affecting rough lemon. In contrast to Toxin I, the toxin present in minor amounts were much less active ($ED_{50} > 1$ μg/ml). ACRL toxins were degraded by heat. Toxin I (59 μg/ml in MES–Tris solution of pH 6.5) decreased in activity 10 to 20% after heating at 75° for 45 min and about 50% at 95° after 75 min treatment.

Compound A was fractionated by the same general procedures as the major toxic fraction except that it extracted into ethyl acetate at alkaline pH (pH 8.3) from sodium bicarbonate solutions. It was a major culture filtrate component (compound A) occurring at *ca* 40–50 mg/l (of crude filtrate) with an R_f 0.85 in chloroform–methanol (85:15). After purification, colorless needles (mp 79.5°) were obtained from *n*-hexane–acetone solutions. Preliminary spectral data indicated functional groups similar to those of the active toxins, but compound A was inactive and did not compete with Toxin I when presented simulatneously at high concentration (100 μg/ml). Nevertheless, its high yield and stability was useful in the final structural elucidation of the toxin components.

Elemental analysis of compound A gave C, 69.84; H, 9.52; O, 20.64 for the suggested empirical formula $C_{18}H_{30}O_4$ (calc. C, 69.68; H, 9.68; O, 20.64%). High resolution EI-MS gave a large $[M-OH]^+$ band at m/z 293.2086 (calc. 293.2115). FD-MS yielded an $[M+H]^+$ ion at m/z 311, CI-MS gave an m/z 309 peak $[M-H]^+$, while FAB-MS of its Na^+, K^+ and NH_4^+ salts gave the expected parent ions at m/z 333, 349 and 328, respectively.

Table 1. Specificity of Toxin I against citrus cultivars

Cultivar	Inhibition (%)*	Host susceptibility to fungus
Rough lemon (RL)	75.9	++
RL Nelspruit 8166	61.4	+
Rangpur lime	58.5	++
Cleopatra mandarin†	56.0	–
Sweet orange (Valencia)	0(−20.9)‡	–
Sweet orange (Pineapple)	0(−1.1)	–
Sour orange	0(−8.9)	–
Dancy tangerine	0(−21.2)	–
Duncan grapefruit	0(1.9)	–
Grape (Chardonnay)§	0(−4.7)	–

*The proline incorporation bioassay was performed as follows: Leaf discs were exposed to 1.8 μg/ml Toxin I for 1.5 hr and subsequently incubated with ^{14}C-Pro (0.4 mM, 375 dpm/nM) for 2 hr. Background incorporation (with cycloheximide) was not estimated; i.e. 75.9% is probably underestimated by at least 15–20%.

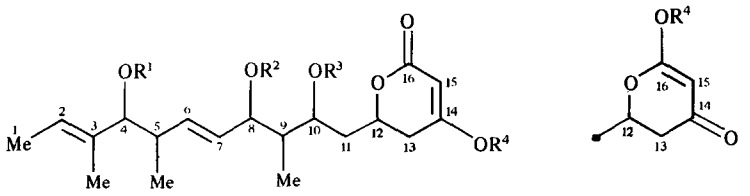
†Cleopatra mandarin is susceptible to ACRL toxin in the proline incorporation bioassay but not in leaf and cutting bioassays at this toxin concentration. This may be related to permeability factors.

‡Minus values indicate stimulation.

§Non-citrus cultivar.

Molecular ions were not found in EI- or CI-MS spectra of Toxin I. A prominent and characteristic ion, however, was at m/z 293, which also was a prominent ion in CI-MS of compound A. GC-MS of Toxin I revealed that Toxin I gave a major peak with the same retention time and cracking pattern as compound A, suggesting ahat compound A isolated from culture filtrates was identical to the breakdown product of Toxin I. In addition to apparent instability in mass spectral analysis, Toxin I exhibited several isomeric forms with HPLC procedures and this was explained by NMR spectroscopic evidence of keto-enol tautomerism. Compound A did not form tautomers.

To stabilize Toxin I, several derivatives were prepared (Fig. 2). Reaction with diazomethane yielded a major product (Fig. 2, Ib) with an R_f of 0.42 upon TLC with chloroform–methanol (95:5) and a minor one at R_f 0.24 (Fig. 2, Ib'). ^{1}H NMR of both methylated derivatives were essentially the same, except for minor changes in some chemical shifts, indicating they were structural isomers. Acetyl and phenylboronate derivatives of Toxin Ib were prepared (Ic, Id and Ie, Fig. 2) and the results of MS analysis of these derivatives are summarized in Table 2. These results are consistent with an empirical formula of $C_{19}H_{30}O_6$ for Toxin I and suggested that compound A differs from it by the absence of a single carboxyl group. This was confirmed by treating toxin with 0.05 M sodium hydroxide or 0.5 M hydrochloric acid in methanol for



		R^1	R^2	R^3	R^4	Isomer
(Toxin I)	Ia ($C_{19}H_{30}O_6$)	H	H	H	H	Ia′
	Ib ($C_{20}H_{32}O_6$)	H	H	H	Me	Ib′
	Ic ($C_{26}H_{38}O_9$)	Ac	Ac	Ac	Me	Ic′
	Id ($C_{26}H_{35}O_6B$)	H	B – Ph*		Me	
	Ie ($C_{28}H_{37}O_7B$)	Ac	B – Ph*		Me	

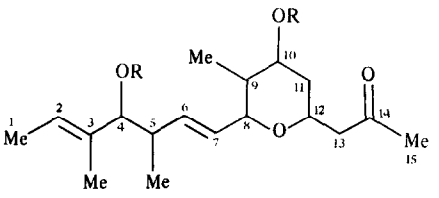
* phenylboronate

Fig. 2. Structures of Toxin I and its derivatives.

Table 2. Molecular ions of the derivatives of Toxin I shown in Fig. 1

Compound	Formula	Molecular ion	
		Expected	Observed*
Toxin I (Ia)	$C_{19}H_{30}O_6$	354	337[2] $[M-OH]^{+\cdot}$
Ib	$C_{20}H_{32}O_6$	368	369[2]
Ib	$C_{20}H_{32}O_6Na$	391	391[3,4]
Ic	$C_{26}H_{38}O_9$	494	494[1], 495[3]
Ie	$C_{28}H_{37}O_7B$	496.2632	496.2630[1]

*MS method: [1]EI-MS; [2]CI-MS; [3]FAB-MS; [4]FD-MS.



Compound A R = H
Diacetate R = Ac

Fig. 3. Structures of compound A and its acetate derivative.

12 hr at 25° or heating Toxin I at 100° for 90 min. The major product had the same R_f values, ^1H and ^{13}C NMR spectra, MS-fragmentation patterns and $[\alpha]_D$ values as compound A.

The structural differences between compound A and Toxin I, however, do not arise from the simple loss of CO_2. The formation of Ib (methylation of a enolic hydroxyl) and its conversion to Ic (three acetyls) indicates a total of four hydroxyl groups in Toxin I, two of which have a 1:3 relationship since phenylboronate derivatives can be prepared (Fig. 2: derivatives Id and Ie). Acetylation of compound A, however, yielded only a diacetate (Fig. 3) and it did not react to form a phenylboronate. Confirmation of a carbonyl and four hydroxyl groups in Toxin I was obtained from fragments in CI-MS of the silyl ethers formed after preliminary reaction with methoxyamine (MOX): *m/z* 426, 498, 570 (1, 2 and 3 TMSi), 599 (3 TMSi + MOX), 657 (4 TMSi + MOX).

The 400 MHz ^1H NMR spectra for Toxin I, compound A and methyl and acetyl derivatives, summarized in Table 3, showed many common features. Complete characterizations of Toxin I, its methyl, acetyl and phenylboronate derivatives and compound A are given in the Experimental. A description of the substituents in each derivative is given in Fig. 2. In toxin I (Ia) and derivatives Ib and Ic, chemical shifts at about δ0.85 (*d*), 0.89 (*d*), 1.58 (*d*) and 1.57 (*s*) can be assigned to four methyl groups, three of which are common to compound A. In the latter, there is also a signal at δ2.17 which can be assigned to a methyl in a methyl ketone function.

Proton signals at δ3.66, 4.04 and 3.82 in Ib were shifted to δ4.94, 5.14 and 4.98 after acetylation to Ic indicating that Ia and Ib contained three methine protons on three carbon atoms bearing hydroxyl groups. Olefinic protons were observed at about δ5.40 (*q*), 5.66 (*dd*), and 5.53 (*dd*) in Ia, Ib and Ic. The signal at about δ5.40 was coupled to the methyl group signal at about δ1.58 and showed allylic coupling (< 1 Hz) to the methine proton at about δ3.65, suggesting the structure indicated by C-1 to C-4 in Table 3 for Toxin I and its derivatives. Similar considerations led to the assignments indicated for C-5 to C-11 in Toxin I and its derivatives.

The arrangements of C-12 to C-16 were deduced from changes in spectra when Toxin I was converted to its monomethyl derivative Ib; a new singlet appeared at δ3.74 (OMe) and a multiplet, integrating for 2H at δ2.52 (C-13), was converted to 1H singlets at 2.49 and 2.55 ppm. The olefinic singlet at 5.14 (C-15) showed weak allylic coupling (1 Hz) to one of the methylene protons (δ2.49) at C-13. The UV and IR spectra of toxins Ib and Ib′ (UV λ_{max} = 233 nm, ε = 6900, IR ν_{max} 1720 and 1620 cm^{-1} for Ib; UV λ_{max} = 260 nm, ε = 7400, IR λ_{max} 1660 and 1573 cm^{-1} for Ib′) strongly indicated an unsaturated lactone for Ib and an acetal function for Ib′ [7, 8]. In

Table 3. ^{1}H NMR of Toxin I, compound A and their derivatives (400 MHz)

		Toxin I			Compound A	
		Me_2CO-d_6 R^{1-4}=H	$CDCl_3$ R^4=Me		$CDCl_3$	
		Ia	Ib	Ic	R=H	R=Ac
			R^{1-3}=H	R^{1-3}=Ac		
1	CH_3	1.58 *d*	1.63	1.60	1.62 *d*	1.60
2	CH	5.40 *q*	5.47	5.51	5.46 *q*	5.52
3,3′	C–CH_3	1.57 *s*	1.60	1.57	1.60 *s*	1.56
4	CH–OR^1	3.65 *d*	3.66	4.94	3.63 *d*	4.95
5	CH	2.31 *m*	2.34	2.46	2.32 *m*	2.47
5′	CH_3	0.85 *d*	0.86	0.88	0.87 *d*	0.88
6	CH	5.66 *dd*	5.62	5.60	5.70 *dd*	5.56
7	CH	5.53 *dd*	5.59	5.30	5.48 *dd*	5.34
8	CH–OR^2	4.06 *dd*	4.04	5.14	3.93 *dd*	3.82
9	CH	1.72 *m*	1.71	2.11	1.50 *m*	1.53
9′	CH_3	0.89 *d*	0.82	0.91	0.89 *d*	0.75
10	CH–OR^3	3.86 *m*	3.82	4.98	3.97 *m*	5.10
11	CH_2	1.79-	1.92†	1.89†	1.53†	1.56†
		1.89 *m*	2.02†	2.11†	1.83†	1.85†
12	CH	4.96 *m*	4.70	4.39	4.27 *m*	4.12
13	CH_2	2.52 *m*	2.49†	2.39†	2.40†	2.37†
O		(2H)	2.55†	2.48†	2.70†	2.65†
14	C–OR^4	–	−3.74	−3.73	—	—
15	CH	5.06 *s*	5.14	5.12	2.17 *s*	2.17*
16	C=O	—	—	—	—	—
				Ac = 1.99, 2.06, 2.07	Ac = 1.99*, 2.11*	

*Tentative assignments.
†ABX_n type coupling.

addition, the relatively low chemical shift for the methine proton at C-12 (δ4.70 for Ib and 4.85 for Ib′) indicated that C-12 was probably an oxygen-linked carbon atom in the pyrone ring. Acetylation also indicated a ring oxygen since there was no lower field shift of the C-12 proton in the acetate derivatives (4.39 for Ic and 4.57 for Ic′, i.e. slight upfield shifts). Fragmentation of Ia in CI-MS supported a splitting of the six-membered ring function (*m/z* 96) from the straight chain (C-1 to C-11) leaving a prominent [M + H − 96] fragment at *m/z* 259. The single carbonyl (C-16) in Toxin I also had a much higher shift in ^{13}C NMR spectra (168.2 for Ia and 167.4 for Ib) than the single carbonyl in compound A (206.5) indicating an oxygen-linked carbonyl, as would be expected for a lactone. The resulting proposed structure for Toxin I (Fig. 2) also explains the occurrence of tautomeric forms of the toxin, i.e. the structures 4-methoxy-5,6-dihydro-α-pyrone (Ib) and 2-methoxy-5,6-dihydro-γ-pyrone (Ib′), or the 4-keto and 2-acetal forms for Ia and Ia′, respectively.

For compound A, only two hydroxyls were acetylated and the associated methine protons were assigned to C-4 and C-10 (Fig. 3). Lower field shifts of 1.1–1.3 ppm were observed at these methine protons after acetylation (δ3.63–4.95 for C-4 and δ3.97–5.10 for C-10). The methines at oxygen-linked carbon atoms C-8 and C-12, however, were not affected appreciably by acetylation (δ3.93–3.82 for C-8 and δ4.27–4.12 for C-12). These results, in view of the molecular formula having an unsaturation number of 4, indicated a tetrahydropyran ring function between C-8 and C-12. The chemical shifts of the methylene protons at C-13 (δ2.40 and 2.70, ABX type coupling) indicated that the methyl ketone group (3H, *s*, δ2.17) was attached to C-13. ^{13}C NMR shifts (Table 4) and selective decoupling results supported these observations. From all of the results, the structure of compound A was established as shown in Fig. 3. Facile conversion of Toxin I to compound A via decarboxylation and rearrangement of the pyrone ring function was also consistent with the proposed structures of both of these compounds.

With respect to biological activity, the monomethyl ethers Ib and Ib′ were inactive, indicating that the 2′- and 4′-enolic hydroxyl functions on the dihydropyrone rings were essential for biological activity. The following article on the minor ACRL toxins [9] also points out additional structural effects on biological activity of the toxins, particularly relating to saturation in the ring (dihydropyrone vs. pyrone) and chain length differences. Further

Table 4. ^{13}C NMR of Toxin I, compound A and their derivatives (22.5 MHz)

Solvent	Toxin I Me_2CO-d_6 R^{1-4} R^{1-3}=H	Toxin I (CDCl$_3$) R^4=Me	Compound A	Compound A (CDCl$_3$) R=H	Compound A (CDCl$_3$) R=Ac
1 CH_3	13.1*	12.9*	CH_3	12.5*	12.7*
2 CH	122.0	123.1	CH	122.1	124.0
3 C	138.1	135.8	C	135.8	132.5
3′ CH_3	11.1*	10.3*	CH_3	10.6*	11.4*
4 CH–OR^1	82.4	82.2	CH–OR	81.1	82.3, 20.8
5 CH	41.2	40.5	CH	39.4	39.1
5′ CH_3	17.7*	17.2*	CH_3	16.6*	16.7*
6 CH	136.2	136.5	CH	135.8	135.8
7 CH	133.5	133.0	CH	131.2	129.7
8 CH–OR^2	77.1	78.0	CH	77.6	78.8
9 CH	45.1	43.5	CH	40.6	38.2
9′ CH_3	12.4	12.9	CH_3	13.3*	13.4*
10 CH–OR^3	71.1	72.4	O CH–OR	68.4	71.0
11 CH_2	39.4	38.8	CH_2	39.4	36.5
12 CH	73.4	74.5	CH	67.6	68.4
13 CH_2	34.0	32.7	CH_2	49.6	49.6
O					
14 C–OR^4	170.1*	173.2*	C=O	206.5	206.2
		55.9			
15 CH	94.4	90.0	CH_3	30.3	29.8
16 C=O	168.2*	167.4*		—	—

*Tentative assignments.

studies are necessary to determine whether tautomerism affects biological activity of the toxins.

EXPERIMENTAL

Analytical methods. Elemental analysis was performed by Galbraith Labs, Knoxville, TN. Standard analytical methods were carried out with the following instruments: Low- and high-resolution MS: Hitachi RMU-6M (G), M-80 or a Kratos MS-25 and MS-30; FD-MS, M-80; FAB-MS, MS-30; CI-MS, a Finnigan Model 4021. ^1H and ^{13}C NMR spectra: JEOL FX-90Q (90 MHz, 22.5 MHz), a JEOL GX-400 (400 MHz) and a Bruker 90 (270 MHz, 67.5 MHz). NMR assignments were made by $^1H/^1H$ and $^1H/^{13}C$ selective decoupling methods. ORD: Perkin–Elmer Model-241 MC polarimeter. TLC: Merck silica gel 60 F_{254} (0.24 or 0.5 mm) and Analtech silica gel G F_{254} (0.5 or 1 mm). HPLC: Perkin–Elmer Series 2 with LC-75 detector with autocontrol. GC-MS: 1% OV-17 on Supelcoport (2 mM, 6 ft), inj. temp. 250°, 30 ml/min He, column temp. 150° (held for 5 min), increased to 250° at 5°/min. Retention time for compound A was *ca* 13 min.

Isolations of Toxin I and compound A. Single spore isolates of *Alternaria citri* (Ellis and Pierce) were isolated from leaf lesions infected with *A. citri*. The fungus was isolated on corn meal agar (CMA) containing 50 to 100 μg/ml chloramphenicol. Spores, which were produced on 3% CMA agar, were selected and isolated by serial dilution with liquid agar (1.8%). The isolates retained the toxin-producing ability (> 1:1000, endpoint of dilution of the culture) for at least 1 year in a refrigerator. *Alternaria citri* may also be considered to be a pathotype of *Alternaria alternata* (*A. alternata* pathotype citri).

Toxin was produced by single spore isolates and purified as described. Compound A and all of the toxins were detected by 5% vanillin–H_2SO_4 (and UV 254 for Toxin I) on silica gel TLC plates. R_f values on silica gel TLC developed with various solvents are given in Table 5.

HPLC separations on normal phase columns were performed as follows: Waters Radial Pak CN, 8 × 120 mm: flow rate, 3 ml/min: UV detector at 254 nm: solvent, *n*-C_6H_{14}–*iso*-PrOH–EtOAc (92:4:4): with a gradient elution with *iso*-PrOH 4 to 35%, 1%/min. Compound A eluted at about 4% *iso*-PrOH and toxins (Toxin I isomers followed by minor toxins) were eluted in many peaks from 15 to 35% of *iso*-PrOH. Using a solvent system of CH_2Cl_2–0 to 15% *iso*-PrOH (UV detector at 224 nm), compound A eluted first with 100% CH_2Cl_2 and Toxin I followed by minor toxins were eluted in many peaks between 6 and 15% *iso*-PrOH. Analysis of the peak fractions on TLC indicated that many of these peaks probably separated as tautomers. Most of the toxin peaks had a λ_{max} of either 240 to 245 nm (α-dihydropyrones) or 285 to 290 nm (γ-dihydropyrones) as indicated by direct stop-flow scanning. Reverse phase (C-18) columns were less useful for distinct separation of toxins and their tautomers.

Methods of bioassay. Biological activities of isolated compounds were determined using the methods of leaf puncture bioassay and electrolyte leakage bioassay, essentially the same manner as described by Kohomoto *et al.* [3]. In addition to these

Table 5. R_f values of Toxin I and compound A on silica gel TLC

Solvent system	R_f* Toxin I	R_f* Compound A
C_6H_6–Me_2CO–HOAc (120:80:1)	0.31	0.58
C_6H_6–Me_2CO–HOAc (100:100:1)	0.43	0.75
$CHCl_3$–MeOH (9:1)	0.18	—
$CHCl_3$–MeOH (85:15)	0.44	0.85
$CHCl_3$–$MeNO_2$–MeOH (180:80:30)	0.43	0.80
CH_2Cl_2–Me_2CO–HOAc (120:80:0.4)	0.34	0.69
CH_2Cl_2–Me_2CO–HOAc (80:120:0.1)	0.75	0.90
CH_2Cl_2–EtOAc–HOAc (5:3:1)	0.06	0.41
CH_2Cl_2–C_2H_6O–EtOAc–HOAc (10:10:5:0.5)	0.47	0.85
50% MeOH–0.4 M NaCl†	0.53	—
60% MeOH–2 mM K_2HPO_4 (pH 7.0)†	—	0.51

*Silica gel G F_{254} except when noted.
†ODS C-18 (Analtech, Inc).

bioassays, incorporation of ^{14}C-amino acids (proline in most cases) into EtOH-insoluble fractions of leaf discs was the most sensitive and quantitative bioassay method. Inhibition of incorporation of ^{14}C-labelled proline into protein was determined as follows: 10 discs of 0.6 cm diam. obtained from young leaf tissue were incubated (+/− toxin) in scintillation vials with constant rapid shaking in 0.95 ml of 0.025 M MES–Tris (Morpholino ethanesulphonic acid soln adjusted to pH 6.5 with Tris base). After 1–4 hr, 0.05 ml of 0.4 mM ^{14}C-proline (100 to 500 cpm/nmole) was added and the vials were incubated another 120–180 min. In some experiments, to correct for background incorporation of ^{14}C-proline, cycloheximide (50 g/ml) was used to estimate the 100% inhibition level for cytoplasmic protein synthesis. Discs were washed briefly with buffer, then soaked for 12 hr in 15 ml of 95% EtOH and rinsed with 5 ml of the same solvent. Scintillation fluid was added to the bleached discs. Ten to 30 nMols of proline was incorporated into control discs. In this bioassay, Toxin I showed 50% inhibition (ED_{50}) at 18–30 ng/ml (Fig. 1). Compound A was inactive at 500 μg/ml and did not compete with Toxin I when presented simultaneously.

Preparation of derivatives. Samples (2–3 mg) to be acetylated were dissolved in dry C_5H_5N (0.5 ml) and Ac_2O (0.3 ml) was added at 0°. The solns were kept at 4° overnight, then concd *in vacuo*. Samples to be methylated were dissolved in Me_2CO (1 ml) and fresh CH_2N_2 in Et_2O (5–10 ml) was added followed by addition of 0.2–0.3 ml of 0.1 N HCl, and kept at 4° for overnight. The reaction mixture was concd *in vacuo* and separated by prep. TLC (silica gel; $CHCl_3$–MeOH, 95:5; R_f 0.42 for Ib and 0.23 for Ib'). To prepare phenylboronates, toxins (1–2 mg) were dissolved in dry C_5H_5N (0.5 ml) and phenylboronic acid (3–5 mg) was added at 0° and the soln was stirred at 4° for 5 hr. Samples were purified by prep. TLC (silica gel; C_6H_6–EtOAc, 4:1). The isolated phenylboronate (Id) was acetylated and purified by prep. TLC (silica gel; C_6H_6–EtOAc, 85:15; R_f 0.13) to yield Ie.

Properties of toxins and derivatives. All NMR assignments are made from left to right hand side for structures given in Figs 2 and 3.

Compound A. $C_{18}H_{30}O_4$. $[\alpha]_D = +44$ (*c* 0.22, in MeOH). Elemental analysis (obs. C; 69.84, H; 9.52, O; 20.64; calc. C; 69.68, H; 9.68, O; 20.64). HR-GC-MS: $[M]^+ - H_2O]^+$ ($C_{18}H_{28}O_3$); obs. 292.2076, calc. 292.2040 HR-MS: $[M - OH]^{+\cdot}$ ($C_{18}H_{29}O_3$); obs. 293.2086, calc. 293.2115. FAB-MS: $[M + Na]^{+\cdot}$, *m/z* 333; $[M + K]^{+\cdot}$ *m/z* 349. CI-MS (isobutane): $[M - H]^{+\cdot}$, *m/z* 309. FD-MS $[M + H]^{+\cdot}$, *m/z* 311. UV $\lambda_{max}^{MeOH} = 272$ nm ($\varepsilon = 180$). IR ν_{max} cm^{-1} (KBr): 3500, 3440, 3300, 2980, 2930, 1708, 1660, 1640, 1453, 1420, 1370, 1100, 1072, 1045, 1012, 1003, 976, 936, 895, 834, 827, 800 and 740. ^{1}H NMR [400 MHz, $CDCl_3$, *J* (Hz)]: δ 1.62 (3H, *d*, 7 Hz), 5.46 (1H, *q*, 7), 1.60 (3H, *s*), 3.63 (1H, *d*, 7), 2.32 (1H, *m*, 7), 0.87 (3H, *d*, 7), 5.70 (1H, *dd*, 14, 8), 5.48 (1H, *dd*, 14, 6), 3.93 (1H, *dd*, 10, 6), 1.53 (1H, *m*, 10, 7, 3), 0.89 (3H, *d*, 7), 3.97 (1H, *m*, 3, 3, 2), 1.53 (1H, ABX_n, 14, 12, 2), 1.83 (1H, ABX_n, 14, 3, 2), 4.27 (1H, *m*, 12, 7, 6, 6), 2.40 (1H, ABX, 14, 6), 2.70 (1H, ABX, 14, 7) and 2.17 (3H, *s*). ^{13}C NMR (22.5 MHz, $CDCl_3$): see Table 4.

Diacetyl derivative of compound A. $C_{22}H_{34}O_6$. Mp 79.50. HR-MS: $[M - HOAc]^{+\cdot}$; obs. *m/z* 334.2160, calc. *m/z* 334.2143. FD-MS $[M + H]^{+\cdot}$, *m/z* 395. EI-MS $[M]^{+\cdot}$, *m/z* 394. UV λ_{max} 280 nm ($\varepsilon = 170$, MeOH); $[\alpha]_D + 43$ (*c* = 0.35, MeOH). ^{1}H NMR [400 MHz, $CDCl_3$, *J* (Hz)]: δ 1.60 (3H, *s*), 5.52 (1H, *q*, 7 Hz), 1.56 (3H, *s*), 4.95 (1H, *d*, 7), 2.47 (1H, *m*, 8, 7, 7), 0.88 (3H, *d*, 7), 5.56 (1H, *dd*, 14, 8), 5.34 (1H, *dd*, 14, 6), 3.82 (1H, *dd*, 10, 6), 1.53 (1H, *m*, 10, 7, 3), 0.75 (3H, *d*, 7), 5.10 (1H, *m*, 3, 3, 2), 1.56 (1H, ABX_n, 14, 12, 2), 1.85 (1H, ABX_n), 4.12 (1H, *m*, 12, 7, 6, 6), 2.37 (1H, ABX, 14, 6), 2.65 (1H, ABX, 14, 7), 2.11 (3H, *s*), 2.17 (3H, *s*) and 2.19 (3H, *s*). ^{13}C NMR (22.5 MHz, $CDCl_3$, ppm): see Table 4.

Toxin I (Ia). Molecular formula $C_{19}H_{30}O_6$. Mp 192–196°. FAB-MS (glycerol/NaCl): $[M + Na]^{+\cdot}$, *m/z* 377. FD-MS [sodium salt + Na]$^{+\cdot}$, *m/z* 399. $UV_{max} = 245$ nm ($\varepsilon = 9500$, MeOH; 236 nm, MeOH + HCl). ^{1}H NMR [400 MHz, Me_2CO-d_6, *J* (Hz)]: δ 1.58 (3H, *d*, 7 Hz), 5.40 (1H, *q*, 7), 1.57 (3H, *s*), 3.65 (1H, *d*, 9), 2.31 (1H, *m*, 9, 8, 7) 0.85 (3H, *d*, 7), 5.66 (1H, *dd*, 15, 8), 5.53 (1H, *dd*, 15, 8), 4.06 (1H, *dd*, 8, 7), 1.72 (1H, *m*, 7, 7, 7), 0.83 (3H, *d*, 7), 3.86 (1H, *m*, 8, 7, 7), 1.79–1.84 (2H, *m*), 4.96 (1H, *m*), 2.52 (2H, *m*), [5.06 (< 1H, *s* enol form) and 3.56 (< 2H, *s* keto form)]. ^{13}C NMR (22.5 MHz, Me_2CO-d_6): δ 13.1 (*q*), 122.0 (*d*), 138.1 (*s*), 11.1 (*q*), 82.4 (*d*), 41.2 (*d*), 17.7 (*q*), 136.2 (*d*), 133.5 (*d*), 77.1 (*d*), 45.1 (*d*), 12.4 (*q*), 71.1 (*d*), 39.4 (*t*), 73.4 (*d*), 43.7 (*t*: keto form), 50.7 (*t*: keto form), 34.0 (*t*: enol form), 170.1 (*s*: enol form), 94.4 (*d*: enol form) and 168.2 (*s*: enol form).

Toxin derivative Ib (4-methoxy-5,6-dihydro-α-pyrone group). $C_{20}H_{32}O_6$. CI-MS $[M + H]^{+\cdot}$, *m/z* 369. FAB-MS (glycerol/NaCl): $[M + Na]^{+\cdot}$, *m/z* 391. IR ν_{max}^{film} cm^{-1}: 3360, 2910, 2850, 1720, 1695, 1620, 1595, 1565, 1450, 1395, 1380, 1230, 1220, 1045, 1020, 980, 928 and 825. UV λ_{max}^{MeOH} 233 nm ($\varepsilon = 6900$). $[\alpha]_D$ −26 (*c* 0.67, MeOH). ^{1}H NMR [400 MHz, $CDCl_3$; *J* (Hz)]: δ 1.63 (3H, *d*, 7 Hz), 5.47 (1H, *q*, 7), 1.60 (3H, *s*), 3.66 (1H, *d*, 9), 2.34 (1H, *m*, 9, 8, 7), 0.86 (3H, *d*, 7), 5.62 (1H, *dd*, 14, 8), 5.59 (1H, *dd*, 14,

8), 4.04 (1H, *dd*, 8, 7), 1.71 (1H, *m*, 7, 7, 7), 0.82 (3H, *d*, 7), 3.82 (1H, *m*, 8, 7, 7), 1.92 (1H, ABX_n, 14, 7, 3), 2.02 (1H, ABX_n, 14, 8, 7), 4.70 (1H, *m*, 10, 7, 5, 3), 2.49 (1H, ABX, 18, 5), 2.55 (1H, ABX, 18, 10), 5.14 (1H, allylic coupling) and 3.74 (3H, *s*, OMe).

Toxin derivative Ic (acetate of Ib). $C_{26}H_{38}O_9$, M_r 494. FD-MS $[M+H]^{+\cdot}$, *m/z* 495. EI-MS $[M]^{+\cdot}$, *m/z* 494. UV λ_{max} 233 nm (ε = 7000, MeOH). ^{1}H NMR [400 MHz, $CDCl_3$; *J* (Hz)]: δ1.60 (3H, *d*, 7 Hz), 5.51 (1H, *q*, 7), 1.57 (3H, *s*), 4.94 (1H, *d*, 9), 2.46 (1H, *m*, 9, 8, 7), 0.88 (3H, *d*, 7), 5.60 (1H, *dd*, 15, 8), 5.30 (1H, *dd*, 15, 8), 5.14 (1H, *t*, 8, 8), 2.11 (1H, *m*, 8, 7, 7). 0.91 (3H, *d*, 7), 4.98 (1H, *m*, 8, 7, 3), 2.11 (1H, ABX_n, 15, 10), 1.89 (1H, ABX_n, 15, 8, 3), 4.39 (1H, *m*, 10, 7, 5, 3), 2.39 (1H, ABX, 17, 10), 2.48 (1H, ABX, 17, 5), 3.73 (3H, *s*, OMe), 5.12 (1H, allylic coupling), 1.99 (3H, *s*), 2.06 (3H, *s*) and 2.07 (3H, *s*).

Toxin derivative Ib' (2-methoxy-5,6-dihydro-γ-pyrone group). $C_{20}H_{30}O_6$. IR ν_{max} cm^{-1}(film): 3380, 2910, 2850, 1660, 1573, 1453, 1400, 1265, 1251, 1075, 1020 and 780. UV λ_{max}^{MeOH} 260 nm (ε = 7400). ^{1}H NMR [400 MHz, $CDCl_3$: *J* (Hz)]: δ1.62 (3H, *d*, 7 Hz), 5.48 (1H, *q*, 7), 1.60 (3H, *s*), 3.67 (1H, *d*, 9), 2.35 (1H, *m*, 9, 8, 7), 0.86 (3H, *d*, 7), 5.63 (1H, *dd*, 14, 8), 5.58 (1H, *dd*, 14, 7), 4.06 (1H, *dd*, 8, 7), 1.71 (1H, *m*, 8, 7, 7), 0.82 (3H, *d*, 7), 3.79 (1H, *m*, 9, 7, 7), 2.02 (1H, ABX_n, 14, 9, 7), 1.92 (1H, ABX_n, 14, 7, 4), 4.85 (1H, *m*, 10, 7, 5, 4), 2.47 (1H, ABX, 18, 5), 2.58 (1H, ABX, 18, 10), 4.85 (1H, allylic coupling) and 3.82 (3H, *s*).

Toxin derivative Ic' (acetate of Ib'). $C_{26}H_{38}O_9$, M_r 494. FD-MS $[M+H]^{+\cdot}$, *m/z* 495. EI-MS $[M]^{+\cdot}$ *m/z* 494. ^{1}H NMR [400 MHz, $CDCl_3$; *J* (Hz)]: δ1.60 (3H, *d*, 7 Hz), 5.41 (1H, *q*, 7), 1.57 (3H, *s*), 4.94 (1H, *d*, 9), 2.48 (1H, *m*, 9, 8, 7), 0.87 (3H, *d*, 7), 5.60 (1H, *dd*, 15, 8), 5.36 (1H, *dd*, 15, 8), 5.14 (1H, *dd*, 8, 8), 2.10 (1H, *m*, 8, 7, 7), 0.90 (3H, *d*, 7), 5.03 (1H, *m*, 8, 7, 7), 1.94 (1H, ABX_n, 15, 7, 7), 2.10 (1H, ABX_n, 15, 8, 4), 4.57 (1H, *m*, 10, 7, 5, 4), 2.39 (1H, ABX, 18, 10), 2.46 (1H, ABX, 18, 5), 4.83 (1H, allylic coupling), 3.77 (3H, *s*), 1.99 (3H, *s*), 2.06 (3H, *s*) and 2.07 (3H, *s*).

Toxin Id (phenylboronate of Ib). $C_{26}H_{35}O_6B$. ^{1}H NMR [400 MHz, $CDCl_3$; *J* (Hz)]: δ1.65 (3H, *d*, 7), 5.50 (1H, *q*, 7), 1.64 (3H, *s*), 3.71 (1H, *d*, 8), 2.41 (1H, *m*, 9, 8, 7), 0.93 (3H, *d*, 7), 5.76 (1H, *dd*, 15, 9), 5.61 (1H, *dd*, 15, 7), 4.16 (1H, *dd*, 10, 7), 1.66 (1H, *m*, 10, 10, 7), 0.93 (3H, *d*, 7), 3.98 (1H, *m*, 10, 8, 3), 2.10 (1H, ABX_n, 15, 8, 5), 2.19 (1H, ABX_n, 15, 7, 3), 4.86 (1H, *m*, 11, 7, 5, 4), 2.55 (1H, ABX, 18, 11), 2.75 (1H, ABX, 18, 4), 5.19 (1H, allylic coupling) and 3.77 (3H, *s*, OMe), 7.2–8.0 (5H, phenyl).

Toxin derivative Ie (acetate of Id). $C_{28}H_{37}O_7B$, M_r 496. HR-MS: obs. 496.2632, calc. 496.2630. EI-MS $[M]^{+\cdot}$, *m/z* 496. ^{1}H NMR [400 MHz, $CDCl_3$; *J* (Hz)]: δ1.64 (3H, *d*, 7 Hz), 5.56 (1H, *q*, 7), 1.63 (3H, *s*), 5.01 (1H, *d*, 9), 2.55 (1H, *m*, 9, 8, 7), 0.93 (3H, *d*, 7), 5.67 (1H, *dd*, 15, 9), 5.47 (1H, *dd*, 15, 7), 4.09 (1H, *dd*, 10, 7), 1.65 (1H, *m*, 10, 10, 7), 0.95 (3H, *d*, 7), 3.96 (1H, *m*, 10, 8, 3), 2.09 (1H, ABX_n, 15, 8, 5), 2.18 (1H, ABX_n, 15, 7, 3), 4.87 (1H, *m*, 11, 7, 5, 4), 2.55 (1H, ABX, 18, 11), 2.72 (1H, ABX, 18, 4), 5.19 (1H, allylic coupling), 3.77 (3H, *s*) and 2.00 (3H, *s*); 7.2 to 8.0 (5H, phenyl).

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