Short Communication

Structure of the Major Component of ACRL-toxins, Host-specific Pathotoxic Compounds Produced by *Alternaria citri*

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The phytopathogenic fungus *Alternaria citri*, a causal agent of brown spot disease of rough lemon (*Citrus jambhiri* Lush.) and rangpur lime (*C. reticulata* Blanco) produces a host-specific pathotoxin complex (ACRL-toxins) that has a specific effect on leaves of susceptible varieties.¹⁻³⁾ We present the physical properties and structural assignments of the major component of ACRL-toxins (Band 1 toxin: **1a** in Fig. 1) and a non-toxic fungus product (Compound A: **IIa** in Fig. 1).

Single spore isolates of *A. citri* were grown in still culture for three weeks at 25°C using Czapek's medium supplemented with ZnSO₄ (5 to 20 mg/liter).³⁾ Toxins and Compound A were absorbed on Florisil or Amberlite XAD-7

from culture filtrates, and eluted with ethyl acetate-acetone (1:1) for the former and acetone for the latter.³⁾ Band 1 toxin and Compound A were purified further on a silica gel column, HPLC and preparative TLC (silica gel G; CHCl₃-MeOH 85:15; *R_f* 0.44 for Band 1 toxin and 0.85 for Compound A). Pure Band 1 toxin and Compound A were separated from the other minor toxins and were crystallized from *n*-hexane-acetone at -15°C.

The derivatives listed in Fig. 1 were prepared by the standard procedures and were used in structural elucidation. Band 1 toxin melted at 192~196°C. Its molecular formula was determined to be C₁₉H₃₀O₆ by HR-MS of **Id** C₂₈H₃₇O₇B (*m/z* 496.2632. Calcd. 496.2630) and by FAB-MS of the sodium salt of **Ib**, C₂₀H₃₂O₆Na (*m/z* 391). UV λ_{max}=245 nm (ε=9500, MeOH; 236 nm in 0.1N HCl/MeOH). [α]_D²⁵ of **Ib** -26° (c=0.67, MeOH).

As the Band 1 toxin was easily converted to Compound A during isolation and measurements, its enolic hydroxyl function was converted by CH₂N₂ for stabilization. Two methylated products **Ib** and **Ib'** were isolated from the reaction mixture. The ¹H-NMR spectra of **Ib** and **Ib'** were similar, and assignments of the signals were performed by ¹H-NMR with ¹H-decoupling of both the compounds. Signals of the ¹³C-NMR spectra of **Ib** and **Ib'** were also similar and their assignments were performed by selective ¹H-decoupling of **Ib**. The results are shown in Table I.

In Table I, four methyl protons (H-1, H-5', H-9' and H-3') were assigned on C-2, C-5, C-9 and C-3, respectively, from the evidence of vicinal couplings with H-2, H-5 and H-9 for

FIG. 1. Structures of Band 1 Toxin, Compound A and Their Derivatives.

TABLE I. ^1H - (400 MHz) AND ^{13}C -NMR (22.5 MHz) OF **Ib** AND **Ila** IN CDCl_3

Band 1 toxin methyl ether (Ib)			Compound A (Ila)		
	^1H (δ ppm)	^{13}C (ppm)		^1H (δ ppm)	^{13}C (ppm)
1 CH_3	1.62	12.9*	1 CH_3	1.62	12.5*
2 CH	5.47	123.1	2 CH	5.46	122.1
3 $\text{C}=\text{CH}_3$	— (1.60)	135.8 (10.3*)	3 $\text{C}=\text{CH}_3$	— (1.60)	135.8 (10.6*)
4 $\text{CH}-\text{OH}$	3.66	82.2	4 $\text{CH}-\text{OH}$	3.63	81.1
5 $\text{CH}-\text{CH}_3$	2.34 (0.86)	40.5 (17.2)	5 $\text{CH}-\text{CH}_3$	2.32 (0.87)	39.4 (16.6*)
6 CH	5.62	136.5	6 CH	5.70	135.8
7 CH	5.59	133.0	7 CH	5.48	131.2
8 $\text{CH}-\text{OH}$	4.04	78.0	8 CH	3.93	77.6
9 $\text{CH}-\text{CH}_3$	1.71 (0.82)	43.5 (12.9)	9 $\text{CH}-\text{CH}_3$	1.50 (0.89)	40.6 (13.3*)
10 $\text{CH}-\text{OH}$	3.82	72.4	10 $\text{CH}-\text{OH}$	3.97	68.4
11 CH_2	1.92, 2.02 ^a	38.8	11 CH_2	1.53, 1.83 ^a	39.4
12 CH	4.70	74.5	12 CH	4.27	67.6
13 CH_2	2.49, 2.55 ^a	32.7	13 CH_2	2.40, 2.70 ^a	49.6
14 $\text{C}-\text{OCH}_3$	— (3.74)	173.2* (55.9)	14 $\text{C}=\text{O}$	—	206.5
15 CH	5.14	90.0	15 CH_3	2.17	30.3
16 $\text{C}=\text{O}$	—	167.4*		—	—

Figures in parenthesis indicate the chemical shifts of methyl groups.

^a ABX_n type coupling. * Tentative assignment.

the first three, and of allylic coupling with H-2 for the last one, in the ^1H -NMR spectra of **Ib** and **Ib'**. The positions of the three double bonds were assigned to those between C-2/C-3, C-6/C-7 and C-14 (C-16 for **Ib'**)/C-15, by the chemical shifts of olefinic protons (H-2, H-6, H-7 and H-15) and of allylic methyl groups (H-1 and H-3') in the ^1H -NMR spectra of **Ib** and **Ib'**, and by the chemical shifts of C-2, C-3, C-6, C-7, C-14 (C-16 for **Ib'**) and C-15 in the ^{13}C -NMR spectra of **Ib** and **Ib'**. Four methine proton signals (H-4, H-8, H-10 and H-12) were considered to originate from the methine proton nuclei of oxygenated groups by their chemical shifts. The signals H-4, H-8 and H-10 were assigned to protons on C-4, C-8 and C-10 respectively, based on a comparison of their ^1H -NMR signals of **Ib** and **Ib'** with those of the acetates (**Ic** and **Ic'**), which were δ : 4.94

(H-4) and 5.14 (H-8) for both **Ic** and **Ic'**, δ : 4.98 (H-10) for **Ic** and 5.03 for **Ic'**. **Ib** forms a phenylboronate (**Id**) and this furnishes support to the above assignment of the positions of hydroxyl groups, because it indicates the 1,3-diol function on the C-8 to C-10 partial structure. The observation of a lower chemical shift of H-12 (δ 4.70 for **Ib** and 4.85 for **Ib'**) and the absence of a lower field shift of H-12 in the ^1H -NMR spectra of **Ic** and **Ic'** indicated a six-membered ring (dihydropyrone) partial structure, positioned between C-12 and C-16 of **Ib** and **Ib'**. ^1H -decoupling experiments on **Ib** and **Ib'** showed the presence of vicinal coupling between signals of the series of protons from H-4 to H-13. This evidence shows that **Ib** and **Ib'** formed an isomeric pair, with the difference of the position of the methoxy group by methylation of the hydroxyl groups caused by tauto-

merization of the dihydropyrone ring (on C-14 for dihydro- α -pyrone and C-16 for dihydro- γ -pyrone). The comparison of ^{13}C -NMR and UV spectra indicated a dihydro- α -pyrone structure for **Ib** and dihydro- γ -pyrone for **Ib'**, *i.e.* two quaternary carbon signals were observed at δ 173.2 and 167.4 for **Ib** (either one of them was attributable to a carbonyl carbon nucleus of a six-membered α,β -unsaturated lactone), and δ 174.2 and 192.8 for **Ib'** (the latter signal being attributable to a carbonyl carbon nucleus of a six-membered α,β -unsaturated ketone).⁴⁾ Thus, the structures of the methylated derivatives **Ib** and **Ib'**, and consequently that of Band 1 toxin (**Ia**), were established as shown in Fig. 1. The UV spectra of **Ib** ($\lambda_{\text{max}} = 233$ nm, $\epsilon = 6900$, MeOH) and of **Ib'** ($\lambda_{\text{max}} = 260$ nm, $\epsilon = 7400$) supported these results.^{5,6)}

Compound A was a colorless crystal of mp 79.5°C. Its molecular formula was determined to be $\text{C}_{18}\text{H}_{30}\text{O}_4$ by elemental analysis (Found: C, 69.84; H, 9.52; O, 20.64. Calcd.: C, 69.68; H, 9.68; O, 20.64) and by FD-MS of protonated **Ila** (m/z 311). UV: $\lambda_{\text{max}} = 272$ nm ($\epsilon = 180$ in MeOH). $[\alpha]_{\text{D}}^{25} + 44^\circ$ ($c = 0.22$, MeOH). Structural assignments between C-1 to C-13 of this compound (see structure **Ila** in Fig. 1) were performed by ^1H -NMR with ^1H -decoupling.

The chemical shift of H-13 (δ : 2.40 and 2.70, ABX) showed the presence of a methylketone group attached to the C-13 carbon atom, which produced characteristic signals (^1H -NMR: H-15, δ 2.17, 3H, s; ^{13}C -NMR: C-14, δ 206.5). Two secondary hydroxyl groups were assigned on C-4 and C-10, based on the lower field shifts of H-4 and H-10 in its acetate **Iib** (H-4, δ 4.95; H-10 δ 5.10). The presence of a tetrahydropyran ring was anticipated from the unsaturated degree of 4 indicated by the molecular formula and from the foregoing evidence of the presence of two double bonds and one carbonyl group. The most plausible position of the ring was between C-8 and C-12, because the signals of the protons at these carbon atoms did not shift appreciably by acetylation of the respective hydroxyl groups in **Iib** (H-8, δ 3.82; H-12, δ 4.12). The ac-

cumulated results established the structure of Compound A (Fig. 1, **Ila**). The observed chemical shifts of the proton and C-13 signals of **Ila** are summarized in Table I.

Band 1 toxin gave a decarboxylated product by treatment with a base (0.05N NaOH), which was identified with Compound A by NMR and $[\alpha]_{\text{D}}$ measurements. Facile conversion of Band 1 toxin to Compound A is consistent with the proposed structures.

The configurations of the double bond between C-6 and C-7 were assigned as *trans* in both **Ib** and **Ila**, based on their large coupling constant ($J_{6,7} = 14$ Hz). The double bond between C-2 and C-3 of Compound A was assigned the *trans* configuration, from the result of NOE measurement of H-2 and H-4. Since the ^1H - and ^{13}C -NMR chemical shifts concerning C-1 to C-4 of Band 1 toxin did not differ much from those of Compound A, the same configuration (*trans*) is also applicable to the C-2/C-3 double bond of the Band 1 toxin. The absolute configuration of the C-12 carbon atom in Band 1 toxin (CD: 246 nm, $\Delta\epsilon = -7.75$ for **Ib**) was determined as (R) by comparison with the CD spectrum of pestalotin (LL-P880 α , 243 nm, $\Delta\epsilon = -7.90$), which also has a dihydro- α -pyrone partial structure.^{5,6)}

Determination of the structure of the Band 1 toxin described above has been based on the data of its methylated products. The NMR spectrum of the Band 1 toxin was more complicated than those of the methylated products. This is probably explained on the basis of two conceivable tautomeric structures, *i.e.* the 4-keto and 2-acetal forms of the dihydropyrone ring of the structure **Ia**, which seems to be the dominant species in a $(\text{CD}_3)_2\text{CO}$ solution of Band 1 toxin, which was used for NMR studies.

Band 1 toxin showed highly specific inhibitory activity at 18 ng/ml (ED_{50}) in a ^{14}C -proline incorporation bioassay using leaf disks of susceptible varieties. Compound A was inactive at 500 $\mu\text{g}/\text{ml}$.

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REFERENCES

- 1) K. G. Pegg, *Queensl. J. Agric. Anim. Sci.*, **23**, 15 (1966).
 - 2) J. O. Whiteside, *Plant Dis. Rep.*, **60**, 326 (1976).
 - 3) K. Kohmoto, R. P. Scheffer and J. O. Whiteside, *Phytopathology*, **69**, 667 (1979).
 - 4) J. B. Stothers, "Carbon-13 NMR Spectroscopy," Academic Press, New York, 1972, pp. 279~303.
 - 5) Y. Kimura and S. Tamura, *Agric. Biol. Chem.*, **36**, 1925 (1972).
 - 6) G. A. Ellested, W. J. McGahren and M. P. Kunstmann, *J. Org. Chem.*, **37**, 2045 (1972).
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