Phytochemistry, Vol. 25, No. 1, pp. 69–72, 1986
Printed in Great Britain

0031-9422/86 $3.00 + 0.00

PLANT PATHOTOXINS FROM *ALTERNARIA CITRI*: STEREOCHEMISTRY OF THE MAJOR AND MINOR TOXINS*

Y. KONO, J. M. GARDNER†, K. KOBAYASHI, Y. SUZUKI, S. TAKEUCHI and T. SAKURAI

Institute of Physical and Chemical Research, Wako-shi, Saitama 351, Japan; †Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, U.S.A.

(*Received* 26 *February* 1985)

Key Word Index—*Citrus jambhiri*; *C. reticulata*; rough lemon; Rangpur lime; host-specific toxin; *Alternaria citri*; α-pyrone; dihydro-α-pyrone; polyalcohol; X-ray crystallography.

Abstract—The absolute configuration of the previously characterized host-specific pathotoxins from *Alternaria citri* were elucidated by NMR, circular dichroism and X-ray crystallography. Each of the major tautomeric forms of the ACRL toxins are stereochemically analogous.

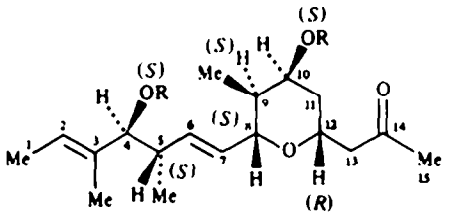
INTRODUCTION

The major and minor ACRL toxins from *Alternaria citri* have been structurally and biologically characterized in preceding reports [1, 2]. One remaining problem was to define stereochemically the toxin structures so that accurate models of the compounds could be made. The major obstacle to this goal was the difficulty in obtaining adequate crystals of the toxins. We attempted to overcome this obstacle by obtaining sufficiently large crystals from compound A, a derivative (decarboxylation product) of the major toxin, toxin I. To relate the structure of compound A to each of the other toxins, a series of ^{1}H NMR, ^{13}C NMR and circular dichroism (CD) studies were analysed along with the crystallographic data for compound A. This report presents a stereochemical analysis of each of the major tautomeric forms of the major and minor ACRL toxins.

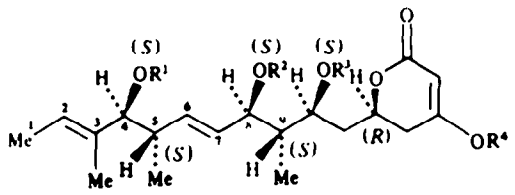
RESULTS AND DISCUSSION

Due to the difficulty of crystallizing the toxins, configurational analysis of the toxins was achieved by comparing the NMR and CD spectral data for compound A and each of the toxins and subsequently relating this data to the complete absolute configurations derived from X-ray crystallography of compound A.

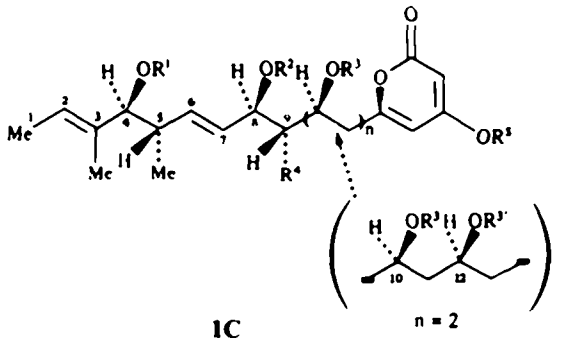
Firstly, the relative configurations for the substituents on the tetrahydropyran ring of compound A (Fig. 1A), assuming a chair form (Fig. 2A, B), were estimated by coupling constants in ^{1}H NMR spectra as follows. The methine proton signal at C-12 (δ4.27, H-12$_{ax}$) was coupled with one of the methylene proton nuclei at C-11 (δ1.53; H-11$_{ax}$) with a large coupling constant ($J_{12a,11a}$ = 12 Hz) indicating a diaxial configuration. The methine proton signal at C-10 (δ3.97, H-10$_{ax}$) was coupled with the methylene protons at C-11 (H-11e, δ1.83; H-11a, 1.53) and the methine proton at C-9 (H-9a, δ1.50) with small



1A



1B



1C

Fig. 1. Structures with absolute configurations of compound A (1A, R = H), Band I toxin (1B, R^1–R^4 = H), and the minor toxins (1C, R^1–R^5 = H).

*Part 3 of the series "*Alternaria citri* Toxins". For Part 2 see ref. [2].

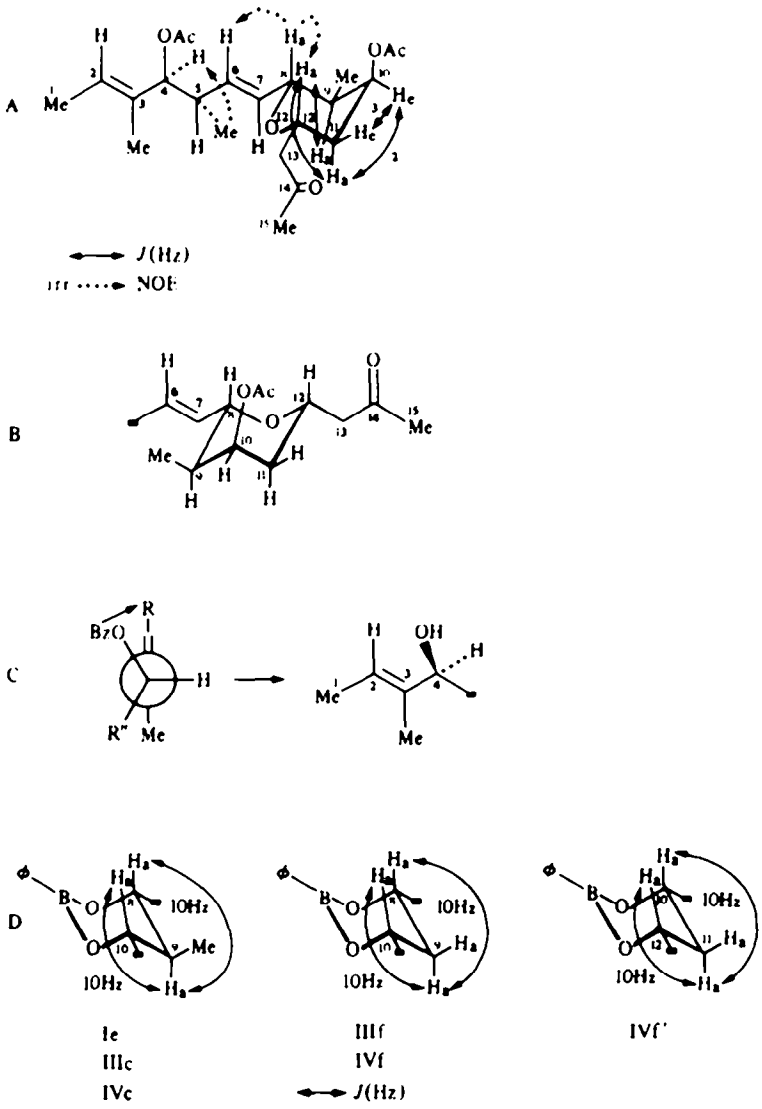


Fig. 2. Assignments of relative configurations in toxins by ^{1}H NMR and CD spectra and absolute configurations by X-ray crystallography. (A) Relative configurations in compound A and absolute configuration of the tetrahydropyran ring (chair form). (B) Alternate stereoisomer (chair form) for compound A. (C) Configurations at C-4 for both compound A and toxins. (D) Phenylboronates of toxins and proton configurations between C-8 and C-10 (C-10 and C-12 for toxin IV).

coupling constants ($J_{10e,11e}$ = 3; $J_{10e,11a}$ = 2; $J_{10e,9a}$ = 3 Hz). The small coupling constants supported a diequatorial configuration between H-10e and H-11e, and an axial–equatorial configuration between H-10e and H-11a, and between H-10e and H-9a. The methine proton at C-8 (H-8a, δ3.93) was coupled with the proton at C-9 (H-9a, δ1.50) with a large coupling constant of 10 Hz indicating a diaxial configuration. These relative configurations (Fig. 2A, B) were confirmed using differential ^{1}H–^{1}H NOE spectra of the diacetyl derivative of compound A. When the methine proton at C-8, (H-8a, δ3.82) was irradiated, NOE were observed for two methine protons at C-6 (δ5.56) and at C-12 (δ4.12) indicating *cis* configurations between C-6 H and C-8 Ha, and between C-8 Ha and C-12 Ha (Fig. 2A).

Configurations at C-1 through C-6 were analysed as follows. Irradiation of the methyl protons at C-5′ (δ0.88) gave a weak NOE for the methine proton at C-4 (δ4.95) suggesting a *trans* configuration between the methyl and hydroxyl groups on C-5 and C-4, respectively (Fig. 2A). The double bond between C-6 (H-6, δ5.56, *dd*, J = 14, 8 Hz) and C-7 (H-7, δ5.34, *dd*, J = 14, 6 Hz) was assigned a *trans* configuration due to a large coupling constant (14 Hz) (Fig. 2A). The double bond between C-2 and C-3 was also assigned a *trans* configuration by comparison with ^{13}C chemical shifts of methyl carbon nuclei on the double bond with those of authentic (*E*)- or (*Z*)-3-methyl-2-hexene [3] (observed for compound A; C-1 and C-3′; 12.5 and 10.6: for (*E*)-3-methyl-2-hexene; 13.8 and 16.1: for (*Z*)-3-methyl-2-hexene; 13.8 and 23.9) (Fig. 2A). A higher field shift for one of the two methyl signals in compound A relative to (*E*)-3-methyl-2-hexene was due to the γ-effect of the hydroxy function [4] on C-4.

The dibenzoyl derivative of compound A was prepared

to elucidate the absolute configuration at C-4 (Fig. 2C). ^{1}H NMR (400 MHz, $CDCl_3$ or CD_3OD) of the dibenzoate gave essentially the same coupling constants for each signal as those of diacetyl derivative. The CD spectrum of the dibenzoate gave a position Cotton effect at *ca* 225 nm ($\Delta\varepsilon$ = +2.45, MeOH). No Cotton effect was observed for compound A alone. Two different Cotton effects for the dibenzoate were possible. In the presence of two benzolny groups the Cotton effect due to Davydov splitting would have occurred and the wavelength would have shifted to 235 nm. However, in the presence of an allylic benzoyl group, no such splitting would be expected to occur and the wavelength shift would have been closer to 225 nm [5, 6]. The experimental results in fact showed that the latter occurred (no Cotton effect due to Davydov splitting), supporting an (*S*)-configuration at C-4 (Fig. 2C).

From the collective results, two structures with opposite absolute configurations at the ring from C-8 to C-12 were considered possible for compound A due to an unidentified absolute configuration at C-8 and C-12 (Fig. 2A, B).

In order to clarify every relative configuration of compound A, X-ray analysis was carried out. The crystal data of compound A ($C_{18}H_{30}O_4 \cdot H_2O$, M_r 328.4) showed the following dimensions: orthorhombic, $P2_12_12_1$, a = 12.449 (7), b = 26.950 (18), c = 5.839 (6) Å, Z = 4, V = 1959 (3) Å^3, D_c = 1.114 mg·m^{-3}, μ (Mokα) = 0.074 mm^{-1}, λ = 0.7073 Å. Twenty-one H atoms were found on a difference Fourier map and the remaining nine H atoms were calculated from the positions of C atoms. All atoms were refined with anisotropic thermal parameters. The final R was 0.047 and R_w was 0.046. All computations were performed with a FACOM M-380 computer using the UNICS III program system. The resulting structure for compound A is shown in Fig. 3 with the perspective view of all configurations.

Compound A, isolated from the culture filtrates, gave identical ^{1}H and ^{13}C NMR spectra with those of a decarboxylated product of Band 1 toxin. In addition, their optical rotation values were +40 (c = 0.17; MeOH) and +44 (c = 0.22; MeOH), respectively. Therefore, absolute configurations in toxin I, except those at the C-8 and C-12 were determined to be (*S*)-configurations for C-4, C-5, C-9 and C-10 (Fig. 1B) as described for compound A (Fig. 1A). The two double bonds at C-2/C-3 and C-6/C-7 were assigned as having (*E*)-configurations from results of ^{1}H and ^{13}C NMR spectra as mentioned above for compound A.

The complete stereochemical data as described above for compound A were systematically compared to ^{1}H NMR and CD data for each of the toxins or toxin

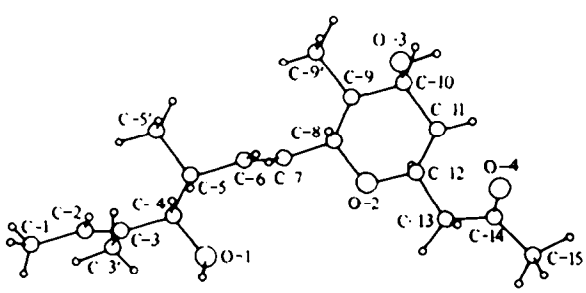


Fig. 3. Perspective view of molecular structure of compound A.

derivatives. The absolute configuration at C-12 in toxin I (CD: Ib, 246 nm, $\Delta\varepsilon$ = −7.75; Ic, 246 nm, $\Delta\varepsilon$ = −6.75 in MeOH) was determined to be in the (*R*)-configuration by comparison with the CD spectrum of pestalotin(LL-P880) (CD; 243 nm, $\Delta\varepsilon$ = −7.90) [7]. A positive Cotton effect at 225 nm ($\Delta\varepsilon$ = +3.65; 246 nm, $\Delta\varepsilon$ = −6.83 in MeOH) was observed in the CD spectrum of the monobenzoate derivative of toxin I (R = B_2 in Fig. 1B; prepared from the *n*-butylboronate of methylated toxin I) supporting the (*S*)-configuration at C-4 by comparison with that of methylated toxin I (225 nm; $\Delta\varepsilon$ = +2.18; 246 nm, $\Delta\varepsilon$ = −7.75 in MeOH). The absolute configuration at C-8 was elucidated by ^{1}H NMR spectra of the phenylboronate of toxin I (Id, Fig. 2D). In the ^{1}H NMR spectrum of Id, the C-9 proton nucleus (δ1.66; C-9 Ha) was coupled with proton nuclei at C-8 and C-10 (δ4.16; C-8 Ha: δ3.98; C-10 Ha) each with large coupling constants (10 Hz) indicating diaxial configurations. Thus, the relative configuration of hydroxyl groups on C-8 and C-10 was also assigned as *cis* (Fig. 2D). Furthermore, the absolute configuration at C-8 of toxin I was reasonably assumed to be in an (*S*)-configuration based on the (*S*)-configuration of C-10 in its decarboxylation product, compound A. From these results, the absolute configuration of toxin I was elucidated as shown in Fig. 1B.

Toxins II, III, III', IV, and IV' also contained two double bonds in the linear chain part of each molecule which was structurally identical to the C-1 through C-7 part of compound A and toxin I. These double bonds were also assigned as (*E*)-configurations from the coupling constants of proton signals on C-6 and C-7 (14–16 Hz) in the ^{1}H NMR spectrum of each methylated toxin, and chemical shifts of methyl carbon nuclei (C-1 and C-3'; δ10.4 and 12.9–13.2) in ^{13}C NMR spectra of each methylated toxin. The coupling constants of signals of methine proton nuclei on C-5 (δ2.31–2.35, *m*, $J_{4,5}$ = 9; $J_{5,6}$ = 7 Hz) and on C-8 (toxin II, 4.33, *dd*, J = 8, 8 Hz: toxin III, 4.10, *dd*, J = 8, 8: toxin III'; 4.41, *m*, J = 8, 8, 5: toxin IV, 4.03, *dd*, J = 8, 8: toxin IV', 4.42, *m*, J = 8, 7, 3) in each of the minor toxins showed almost the same coupling constants in their ^{1}H NMR spectra as those of methylated toxin I suggesting that each toxin contained the same relative configurations from C-1 to C-8 as those of methylated toxin I (H-5, 2.34, *m*, J = 9, 8, 7 Hz: H-8, 4.04, J = 8, 7) [1].

The secondary hydroxyl groups on C-8 and C-10 of toxins III, III', IV and IV' were derivatized with phenylboronic acid in pyridine. Each product was then acetylated and analysed by ^{1}H NMR by ^{1}H decoupling studies. The coupling constants ($J_{8a,9a}$ = 10 Hz) and ($J_{9a,10a}$ = 10 Hz) of methine protons on C-8 and C-10 of each of these toxins indicated diaxial relationships with the axial proton on C-9. These results indicated a *cis* configuration between the hydroxyl groups on C-8 and C-10 for toxins III, III', IV and IV' as previously observed (Fig. 2D). Furthermore, toxin IV' gave two isomers by derivatization with phenylboronic acid (IVf and IVf'). The coupling constants of methine protons on C-10 and C-12 of the minor isomer (IVf') also indicated diaxial relationships with the axial proton on C-11 (Fig. 2D) and a *cis* configuration between hydroxyl groups on C-10 and C-12. Toxins II, III, III', IV and IV' therefore contained the same relative configurations from C-8 to C-12 as those of toxin I (Fig. 1C).

The CD spectrum of the tribenzoyl derivative of toxin I gave a positive Cotton effect at 225 nm ($\Delta\varepsilon$ = +11.7;

MeOH) with no clear Cotton effect due to Davydov splitting. The higher $\Delta\varepsilon$ value (+11.7) compared with that of compound A (+2.45) was due to the effects of two allylic benzoate groups with the same configuration [C-4 (*S*)- and C-8 (*S*)-configurations]. The dibenzoyl derivative of toxin II (IId) gave a positive Cotton effect at 225 nm ($\Delta\varepsilon$ = +14.5; MeOH), again with no clear Cotton effect due to Davydov splitting at *ca* 225 nm, and negative Cotton effect at 286 nm ($\Delta\varepsilon$ = −4.50) which would be expected from interaction between benzoyl and pyrone groups. The results suggested an (*S*)-configurations at C-4 and C-8 (Fig. 1B, C) by comparison with the values for the toxin I dibenzoate. From these results, it was concluded that the absolute configurations at C-4 and C-8 of toxin II were identical to those in toxin I.

Absolute configurations of toxins III, III', IV and IV' were likewise estimated to have the same configurations as those of toxins I and II, a reasonable result in consideration of their common biosynthetic pathway. All of the ACRL-toxins exist as a mixture of tautomers, however, only the major 2-keto tautomers are presented (Fig. 1B, C). Future work will focus on chemical synthesis of the toxins and relationships between structure and biological activity.

EXPERIMENTAL

Analytical methods, toxin production and purification and derivative preparation and properties of toxins and derivatives are given in refs [1, 2]. Benzoyl derivatives of methylated toxins (1–2 mg) were prepared by treatment with CoCl (50 μl) and dry pyridine (200 μl) for 18 hr at 4° prior to separation by TLC (C_6H_6–EtOAc, 4:1, R_f 0.33 for the toxin I tribenzoate and 0.40 for and the toxin II dibenzoate). Methylated phenylboronated toxins were acetylated in the usual way and separated by TLC (C_6H_6–EtOAc, 9:1) with R_fs of 0.15, 0.15, 0.09, 0.10 and 0.04 for toxins I, III, IV, III' and IV', respectively.

For X-ray crystallography, a single crystal (0.1 × 0.2 × 1.4 mm) was used and intensities ($2\theta_{max}$ = 55) were collected on a Rigaku automated four-circle diffractometer (Rigaku AFC-4). A total of 1058 unique reflections were observed above the threshold [$/Fo/ > 3\sigma(Fo)$] and corrected for Lorentz and polarization factors. The structure was solved by direct methods using a MULTAN 78 program and refined by block diagonal least squares. Unit wt was given to all reflections. Complete X-ray data and the list of refined coordinates is on file at the Cambridge Crystallographic Data Centre.

Properties of isolated compounds are as presented previously [1, 2].

Compound A dibenzoate. EI-MS: $[M]^+$ *m/z* 518. ^{1}H NMR [400 MHz, $CDCl_3$, *J* (Hz) all assignments below are given from left to right according to Fig. 1]: δ1.62 (3H, *d*, 7), 5.63 (1H, *q*, 7), 1.67 (3H, *s*), 5.21 (1H, *d*, 7), 2.68 (1H, *m*, 8, 7, 7), 0.99 (3H, *d*, 7), 5.73 (1H, *dd*, 14, 8), 5.48 (1H, *dd*, 14, 6), 3.94 (1H, *dd*, 10, 6), 1.55 (1H, *m*, 10, 7, 3), 0.76 (3H, *d*, 7), 5.30 (1H, *m*, 3, 3, 2), 1.55 (1H, ABX_p, 14, 12, 2), 1.95 (1H, ABX_p, *m*, 14, 3, 2), 4.14 (1H, *m*, 12, 7, 6, 6), 2.30 (1H, ABX, 14, 6), 2.58 (1H, ABX, 14, 7), 2.00 (3H, *s*); 7.4 to 8.1 (phenyls).

Toxin I monobenzoate (*R* = *B2*, *Fig. 1B*). ^{1}H NMR [400 MHz, $CDCl_3$, *J* (Hz)]: δ1.64 (3H, *d*, 7), 5.64 (1H, *q*, 7), 1.70 (3H, *s*), 5.21 (1H, *d*, 9), 2.66 (1H, *m*, 9, 8, 7), 0.70 (3H, *d*, 7), 5.62 (1H, *dd*, 15, 8), 5.52 (1H, *dd*, 15, 8), 3.93 (1H, *dd*, 8, 8), 1.80 to 1.85 (1H, *m*, 8, 7, 7), 0.97 (3H, *d*, 7), 3.71 (1H, *m*, 8, 7, 4), 1.83 (1H, ABX, 15, 8, 3), 1.94 (1H, ABX, 15, 7, 4), 4.68 (1H, *m*, 10, 7, 5, 3), 2.45 (1H, ABX, 17, 10), 2.50 (1H, ABX, 17, 5), 3.73 (3H, *s*), 5.13 (1H, allylic coupling), 7.2–7.6 (5H, phenyl).

REFERENCES

1. Gardner, J. M., Kono, Y., Tatum, J. H., Suzuki, Y. and Takeuchi, S. (1985) *Phytochemistry* **24**, 2861.
2. Kono, Y., Gardner, J. M., Suzuki, Y. and Takeuchi, S. (1985) *Phytochemistry* **24**, 2869.
3. Bu'Lock, J. D. and Smith, H. G. (1960) *J. Chem. Soc.* 502.
4. Stothers, J. B. (1972) *Carbon-13 NMR Spectroscopy*. Academic Press, New York.
5. Harada, N., Iwabuchi, J., Yokota, Y., Uda, H., Ochi, M., Gonnella, N. C., Nakanishi, K., Martin, V. S. and Scharpless, K. B. (1982) *Chem. Nat. Prod. Tokyo* **25**, 39.
6. Harada, N. and Nakanishi, K. (1982) *Circular Dichromic Spectroscopy*. Tokyokagakudojin Co. Ltd.
7. Ellested, G. A., McGahren, W. J. and Kunstmann, M. P. (1972) *J. Org. Chem.* **37**, 2045.