Studies on the Host-specific Pathotoxins Produced by
Bipolaris (Helminthosporium) maydis, Race T,
"Characterization of the Minor C₃₅-, C₄₇-
and C₄₉-Components of the Toxin Complex
and Evidence Bearing on the Stereo-
chemistry of Hydroxyl Groups"

Yoshiki KONO, Setsuo TAKEUCHI, Akira KAWARADA,
J. M. DALY* and H. W. KNOCH*

Laboratory of Phytochemistry, The Institute of Physical and Chemical Research,
Wako, Saitama 351, Japan

*Laboratory of Agricultural Biochemistry, University of Nebraska,
Lincoln, Nebraska 68583, USA

Received April 24, 1981

The host-specific pathotoxin complex isolated from the culture filtrate and mycelial mat of *Bipolaris (Helminthosporium) maydis*, race T, was reduced to hydrocarbons. The hydrocarbons were separated by molecular sieves and analyzed by GC-MS. The MS gave typical spectra of normal, odd carbon numbered hydrocarbons from C₃₅H₇₂ to C₄₉H₁₀₀. Compounds differing from hydrocarbons only in the presence of an internal tetrahydropyran ring were obtained as by-products from the toxin-complex reductions. These compounds were analyzed by CI (chemical ionization)-GC-MS and HR (high resolution)-GC-MS. The MS data supported the hypothesis that the C₃₅- to C₄₉-toxins are analogous compounds with many common partial structures. The configurations at both the C-6 and C-8 carbons of the major components appeared to be *R*.

The phytopathogenic fungus *Bipolaris (Helminthosporium) maydis*, race T, causal organism of southern corn blight, produces host-specific pathotoxins that have specific blighting effect on leaves of sensitive corn. The pathotoxins, which were obtained by charcoal adsorption from both the culture filtrates and 70% acetone extracts of mycelial mats,¹⁾ appear to be a complex of analogous compounds. Individual toxin components separated by TLC showed nearly identical biological activities.²⁾ As in previous reports, the structures of the C₄₁-toxins (Band 1- and 2-toxins)^{3,4)} and C₃₉-toxins (Band 1'- and 3-toxins)⁵⁾ are polyketopolyalcohols with straight carbon chains. Certain observations, obtained during the previous studies, suggested that polyketopolyalcohols structures with straight, odd numbered, carbon chains also are expected for the minor components. The evidence was obtained from NMR (¹³C- and

¹H-) and FD-MS of the complex, as well as from GC-MS of derivatives of toxins from which oxygen was removed. The available evidence suggested that the toxin complex might be composed of biosynthetic intermediates with various lengths of carbon chains sequentially derived from each other by one acetate unit (two carbons).

In this paper, we report confirmative results on the composition of the toxin complex by analysis of GC-MS spectra of normal hydrocarbons prepared from the toxin and separated by the molecular sieve treatment.⁶⁾ In addition, supporting evidence, based on the analysis of tetrahydropyran derivatives prepared from the toxin complex by methods reported previously, is presented. Preliminary evidence bearing on the stereochemistry of the two asymmetric carbons at C-6 and C-8 positions also is presented.

In previous ¹³C-NMR studies, each com-

FIG. 1. GC Spectrum of Hydrocarbons.

ponent of *H. maydis*, race T toxin complex did not show evidence for tertiary carbons which would be expected from a cyclic function.⁴⁾ These results suggested that each toxin was a linear carbon chain. In our previous experiments, we could not detect normal C₄₅-, C₄₇- and C₄₉-hydrocarbons of the minor toxins because of side reactions producing cyclized hydrocarbons. For example C_nH_{2n} and C_nH_{2n-2}-hydrocarbons were the main products when the toxin complex was reduced by NaBH₄ or Zn-HCl (Clemmensen reduction) suggesting cyclization during reactions. Thus, in the present studies the purification of the hydrocarbon mixture from toxins, which was prepared by the same method as in the previous report,⁴⁾ was carried out to remove complex by-products by the treatment with a molecular sieve according to the method of Mold *et al.*⁶⁾ GC-MS spectral analysis of the purified hydrocarbon mixture showed the characteristic fragmentation patterns of normal hydrocarbons between C₃₅ and C₄₉ which differed by two carbons from each other. From these results, it was clear that all of C₃₅- to C₄₉-toxins have normal carbon chains as their carbon skeletons (*n*-C₃₅H₇₂ to *n*-C₄₉H₁₀₀ with odd carbon numbers). In previous reports^{4,5)} we have discussed the analysis of important by-products in the determination of oxygen positions on the carbon chain. The by-products were cyclized derivatives containing

tetrahydropyran rings at various positions of the carbon chain originating from C₃₇- to C₄₅-toxins, but not for the C₃₅-, C₄₇- and C₄₉-toxins. In the present study, we report detection of tetrahydropyran compounds from C₃₅-, C₄₇- and C₄₉-toxins which gave (M + 1)⁺ ions at *m/z* 507, 675 and 703 by CI-GC-MS. They also were analyzed by HR-GC-MS (Fig. 2) suggesting that C₃₅-, C₄₇- and C₄₉-toxins contain many common partial structures as was the case for the C₃₇- to C₄₅-toxins.^{4,5)}

The results of the analysis of tetrahydropyran compounds from the C₃₅- to C₄₉-toxins suggested that each toxin contains the same functions between the C-1 and C-10 carbons as in the case for C₄₁- and C₃₉-toxins. In the previous report⁴⁾ we described the alkaline degradation of the oxidized toxin complex. In that instance 3-hydroxyoctanoic acid was obtained as one of the main products. The toxin complex was degraded further to obtain 3-hydroxyoctanoic acid in order to establish the configuration of the C-6 asymmetric carbon. The toxin complex was oxidized with Jones' reagent and degraded by 3% KOH in 80% ethanol-20% water. Products then were extracted with chloroform and isolated by preparative TLC using chloroform-acetic acid (98:2) as a solvent. The 3-hydroxyoctanoic acid (*R_f* 0.18~0.24) from toxins was identified by NMR and GC-MS (single peak) after

FIG. 2. Results of HR-GC-MS of Tetrahydropyran Compounds from C₃₅-, C₄₇- and C₄₉-Toxins:

esterification with diazomethane, and gave $[\alpha]_D^{25} = -16^\circ$ to -18° ($c=0.14$, in CHCl_3) for the free acid and $[\alpha]_D^{25} = -13^\circ$ to -15°

($c=0.14$, in CHCl_3) for the methyl ester. Optical rotation values of authentic 3(*R*)-hydroxyoctanoic acid and its methyl ester are

FIG. 3. Band 1-Toxin.

$[\alpha]_D = -21^\circ$ ($c=1.9$, in CHCl_3) and $[\alpha]_D = -18.4^\circ$ ($c=1$, in CHCl_3) respectively.⁷⁾ These comparative values of optical rotation suggest that the configuration at C-6 carbon is *R*, at least for the major components of the toxin complex. The stereochemical relationship of the hydroxyl groups at C-6 and C-8 was suggested to be *cis* by comparison of $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ with *cis* and *trans* 8,10-dihydroxypentadecane-6-one and their phenyl boronates synthesized by Suzuki *et al.*⁸⁾ From this result the configuration of C-8 carbon appears to be *R* as shown in Fig. 3.

EXPERIMENTAL

Gas chromatographic analysis was performed with a Shimadzu GC-4B (OV-1, 4 mm $\times$ 50 cm). Low resolution mass spectra were obtained with a Hitachi RMU-6M(G) mass spectrometer with a gas chromatographic inlet system using OV-1 column (4 mm $\times$ 50 cm). High resolution mass spectra were obtained with a JEOL JMS-D300 or with a Hitachi M-80. $^1\text{H-NMR}$ spectrum was obtained with a JEOL FX-90 Q (90 MHz). Thin layer chromatography was carried out on Merck silica gel 60 F_{254} (0.25 mm for detection, 0.5 mm for preparative). Optical rotations were measured with a Perkin-Elmer Model-141 instrument.

Isolation of normal hydrocarbons. Toxin complex (50 mg), which was precipitated from methanol, was converted to hydrocarbons with hydriodic acid-red phosphorous followed by reduction with LiAlH_4 . The products were separated by silica gel column chromatography into two fractions as described in a previous report.⁴⁾ The first eluate with *n*-hexane from the column was treated with molecular sieve according to the method of Mold *et al.*⁶⁾ as follows: The hydrocarbons (18 mg) were dissolved in 3.5 ml of distilled isooctane. This solution was shaken for 3 hr with 3 g of 5 Å molecular sieve (1/16 pellets, Wako Pure Chemical Industries, Ltd.) and allowed to remain in contact with sieve over-night. The mixture was filtered and the sieve was rinsed with fresh isooctane. The normal hydrocarbons retained in the sieve were recovered by stirring with *n*-hexane for 3 hr and allowing the suspension to remain in contact with the sieve over-night to yield 5.0 mg of product in the *n*-hexane. These hydrocarbons

gave typical fragmentation patterns for normal hydrocarbons from $\text{C}_{35}\text{H}_{72}$ to $\text{C}_{49}\text{H}_{100}$ (with odd carbon number) by GC-MS analysis.

Isolation of 3-hydroxyoctanoic acid. Toxin complex (200 mg) was dissolved in 200 ml of acetone and oxidized with 2 ml of Jones' reagent for 3 to 5 min. The upper layer of acetone solution was collected by decantation and concentrated *in vacuo*. The residue (100 mg) was dissolved in 80% ethanol-20% water containing 3% KOH (10 ml) and refluxed for 18 hr. The reacted solution was concentrated and extracted with chloroform (20 ml), then the aqueous layer was reextracted with two 20 ml portions of chloroform after acidification with dilute hydrochloric acid. The acidic extract was concentrated *in vacuo* to yield 47 mg of oil. This oily material was subjected to silica gel preparative TLC. The preparative TLC was developed by chloroform-acetic acid (98:2) as a solvent. Approximately 1/20 of the TLC plate was cut off and stained by 5% vanillin- H_2SO_4 (3-hydroxyoctanoic acid gave a typical yellowish purple color after being heated for 10 to 15 min at 120°C). The fraction from *Rf* 0.18 to 0.24 was eluted with chloroform and concentrated *in vacuo*. It gave 1.4 mg of 3-hydroxyoctanoic acid (about 6% yield). The 3-hydroxyoctanoic acid from toxin was identified with NMR and GC-MS after esterification with diazomethane, and gave $[\alpha]_D^{25} = -16^\circ$ to -18° ($c=0.14$, in CHCl_3) for the free acid and $[\alpha]_D^{25} = -13^\circ$ to -15° ($c=0.14$, in CHCl_3) for the methyl ester. In another experiment run by the above procedures the products were purified by silica gel preparative TLC (acid; CHCl_3 : AcOH 98:2, *Rf* 0.18~0.24; methyl ester; 100% CHCl_3 , *Rf* 0.27~0.33) and gave $[\alpha]_D^{25} = -16^\circ$ to -18° ($c=0.1$, in CHCl_3) for the methyl ester.

Acknowledgments. The authors thank Dr. S. Tanji, Dr. K. Sasaki and Dr. U. Miyazaki, Kaken Co. Ltd. and Mr. Y. Esumi, this Institute, for high-resolution mass spectra. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and NSF Grant No. PCM-7920685.

REFERENCES

- 1) Y. Kono and J. M. Daly, *Bioorganic Chemistry*, **8**, 391 (1979).
- 2) G. Payne, H. W. Knoche, Y. Kono and J. M. Daly, *Physiol. Plant Pathol.*, **16**, 227 (1980).
- 3) Y. Kono, S. Takeuchi, A. Kawarada, J. M. Daly and

- H. W. Knoche, *Tetrahedron Lett.*, **21**, 1537 (1980).
- 4) Y. Kono, S. Takeuchi, A. Kwarada, J. M. Daly and H. W. Knoche, *Agric. Biol. Chem.*, **44**, 2613 (1980).
- 5) Y. Kono, S. Takeuchi, A. Kwarada, J. M. Daly and H. W. Knoche, *Bioorganic Chemistry*, **10**, 206 (1981).
- 6) J. D. Mold, R. E. Means, R. K. Stevens and J. M. Ruth, *Biochemistry*, **3**, 1293 (1964).
- 7) R. U. Lemieux and J. Giguere, *Can. J. Chem.*, **29**, 678 (1951).
- 8) personal communication.
-