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# AN EPOXYDON-DERIVED ESTER FROM A *PHOMA* SP. PATHOGENIC TO RHUBARB

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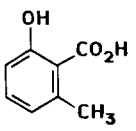
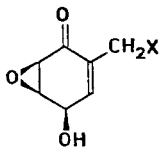
ABSTRACT.—A newly identified rhubarb pathogen, *Phoma* sp., produces in liquid culture four phytotoxins, which were identified as 6-methylsalicylic acid [**1**], epoxydon [**2**], desoxyepoxydon [**3**], and 4,5-dihydroxy-6-(6′-methylsalicyloxy)-2-hydroxymethyl-2-cyclohexen-1-one [**4**]. The ester was synthesized from 6-methysalicylic acid and epoxydon. These metabolites were generally toxic to plants, bacteria, and fungi, including the producing fungus.

A previously unreported leaf and petiole disease of rhubarb was noted recently in North Carolina. Lesions on leaves are brown, surrounded by a red margin, 2–10 mm in diameter. Most affected leaves turn yellow after 2 weeks. Lesions on petioles are larger and elongated. The same fungus was consistently isolated from diseased areas of leaf and petiole. The fungus was identified as *Phoma* sp. (Sphaeropsidales), intermediate between *Phoma exigua* Desm. and *Phoma macrostoma* Mont. in cultural characters and conidial morphology. Inoculation of healthy rhubarb with spores of *Phoma* sp. produced the disease symptoms on all plants, while there were no symptoms on plants sprayed with distilled $H_2O$. The *Phoma* sp. was recovered from 70% of the lesions 10 days after inoculation. We have investigated the phytotoxins produced by this fungus in liquid culture.

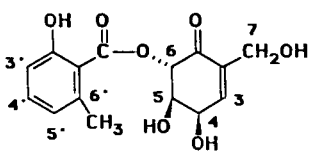
$Na_2CO_3$ partition of the crude EtOAc fraction from *Phoma* sp. culture filtrate gave three previously isolated phytotoxins (1,2) which were separated by flash cc and preparative tlc. The most abundant toxin was 6-methylsalicylic acid [**1**]. Epoxydon [**2**] was also purified from the $Na_2CO_3$ fraction, while desoxyepoxydon [**3**] was obtained from the neutral EtOAc fraction remaining after $Na_2CO_3$ extraction. The isolation of epoxydon and desoxyepoxydon from different fractions in the acid-base partition scheme was surprising in view of their close structural similarity. However, epoxydon partitions nearly equally between $H_2O$ and EtOAc.



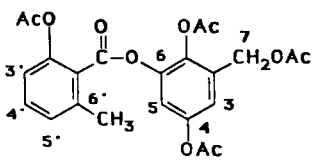
**1**



**2** X=OH
**3** X=H



**4**



**5**

A fourth compound, 4,5-dihydroxy-6-(6′-methylsalicyloxy)-2-hydroxymethyl-2-cyclohexen-1-one [**4**], isolated from the $Na_2CO_3$ fraction, had uv absorption maxima (309, 240, and 211 nm) and intensities essentially the same as those for **1**. The uv shift on addition of NaOH was the same as measured for **1**. Similarly, the dominant fragments in the eims of this new toxin were identical to those of **1**, including fragments corresponding to the molecular ion of **1**, *m/z* 152 (17%), and loss of $H_2O$ from **1**, *m/z* 134 (100%). The cims ($CH_4$) was also dominated by facile fragmentation to 6-methylsalicylic acid: *m/z* 153 (90%) and 135 (100%, loss of $H_2O$). The eims molecular ion occurred at *m/z* 308 (5%) and the cims quasi-molecular ion at *m/z* 309 (1.7%). Thus the new toxin must be an ester of 6-methylsalicylic acid.

Subtraction of the $^1$H-nmr spectrum of 6-methylsalicylic acid from that of its ester (Table 1) left six non-exchangeable protons assignable to the alcohol portion of the ester.

TABLE 1. Nmr Assignments for Ester **4** and its Acetate **5**.

| Position | Compound | | |
|---|---|---|---|
| | **4** | | **5** |
| | 1H nmr ($Me_2CO$-$d_6$) | $^{13}$C nmr ($Me_2CO$-$d_6$) | $^1$H nmr ($Me_2CO$-$d_6$) |
| 1 | — | 194.1 | — |
| 2 | — | 145.5[a] | — |
| 3 | 7.11, dt, $J$=6, <2 | 142.3 | 7.19[b,c], d, $J$=2 |
| 4 | 4.60, m | 67.2 | — |
| 5 | 4.16, dd, $J$=11, 4 | 71.2 | 7.14[b], d, $J$=2 |
| 6 | 5.97, d, $J$=11 | 77.5 | — |
| 7 | 4.21, m, 2H | 59.3 | 4.99, s, 2H |
| 1′-carboxyl | — | 171.0 | — |
| 1′ | — | 116.2 | — |
| 2′ | — | 161.3 | — |
| 3′ | 6.78[d], d, $J$=8 | 115.8 | 7.04, d, $J$=8 |
| 4′ | 7.32, t, $J$=8 | 134.4 | 7.42, t, $J$=8 |
| 5′ | 6.79[d], d, $J$=8 | 123.5 | 7.20[c], d, $J$=8 |
| 6′ | — | 141.8[a] | — |
| 6′-methyl | 2.47, s | 22.8 | 2.77, s |

[a]Arbitrary assignment of C-2 and C-6′

[b]Arbitrary assignment of meta-coupled H-3 and H-5.

[c]Resolution of H-5 and H-5′ improved by addition of $H_2O$.

[d]Arbitrary assignment of overlapping doublets H-3′ and H-5′.

An AB pattern at 4.21 ppm, which is further split by a small long-range coupling, is assignable to a $CH_2OH$ group in a chiral molecule. Two other multiplets at 4.16 and 4.60 ppm are assignable to methine hydrogens on carbons bearing oxygen atoms. A strongly deshielded vinyl hydrogen occurs at 7.11 ppm, and a one-proton doublet, not coupled to the vinyl proton, occurs at 5.97 ppm. These features of the alcoholic portion are similar to those for epoxydon [**1**] (3), except that the H-5 and H-6 epoxide resonances are shifted downfield (from 3.79 ppm to 4.16 ppm and 3.33 to 5.97 ppm, respectively) as expected for a ring-opened epoxide with acylation at C-6. The large coupling constant $J_{5,6}$=11 Hz is consistent with 5,6-trans stereochemistry as expected for epoxide opening. A 5,6-cis model is known to have a small coupling constant, $J_{5,6}$=3 Hz (4).

All $^{13}$C-nmr signals of ester **4** were correlated with their attached protons, and HMBC confirmed the complete C,H-bond connectivity of the hydroxy-

methylcyclohexenone system of ester **4**. Acetylation of ester **4** brought about aromatization of the cyclohexenone to give the tetraacetate **5**.

Ester **4** was synthesized by treatment of a THF solution of 6-methylsalicylic acid and epoxydon with $BF_3$. Synthetic ester **4** was recovered in 25% yield. The synthetic ester gave a $^1$H-nmr spectrum identical to that of the natural ester. Other, highly polar materials, possibly including products of epoxide opening by $H_2O$ in the acidic aqueous workup, were detected on tlc.

*Phoma* sp. produces 6-methylsalicylic acid and epoxydon; therefore it is conceivable that the ester might be an artifact of isolation. However, this seems unlikely because the ester was readily detectable by tlc in a separate experiment, in which fresh culture filtrate (pH 6.2) was extracted with EtOAc without pH adjustment. In addition, no ester was detectable 3 days after 6-methylsalicylic acid and epoxydon were dissolved in $H_2O$ at the pH of culture filtrate (pH 6.5), even at the artificially high concentration of 1 mg/ml (greater than 30 times the culture filtrate concentration).

All four metabolites displayed phytotoxic effects. Characteristic brownish-black necrotic lesions occurred within 48 h after placing toxins on detached leaves of rhubarb and other weed species. Rhubarb was most sensitive to culture filtrate, while the uninoculated medium itself did not cause necrosis on leaves of any of nine plants tested (Table 2). A drop of $H_2O$ containing 50 μg of a purified toxin was sufficient to cause

TABLE 2. Phytotoxicity of *Phoma* sp. Culture Filtrate (cf) and Purified Toxins on Detached Leaves of Rhubarb and Other Plants.

| Plant | Necrotic area ($mm^2$) | | | | |
|---|---|---|---|---|---|
| | cf[a] | **1**[b] | **2**[b] | **3**[b] | **4**[b] |
| Rhubarb | 21 | 55 | 27 | 42 | 18 |
| Sicklepod | 2 | 11 | 17 | 51 | 33 |
| Prickly sida | 3 | 23 | 25 | 74 | 24 |
| Jimsonweed | 13 | 48 | 31 | 73 | 9 |
| Johnson grass | 11 | 15 | 37 | 30 | 7 |
| Morning glory | 3 | 15 | 51 | 64 | 21 |
| Sorghum | 7 | 37 | 31 | 69 | 41 |
| Watercress | 6 | 17 | 41 | 34 | 44 |
| Lambsquarters | 3 | 27 | 62 | 57 | 13 |

[a]20 μl of culture filtrate concentrated 10-fold.
[b]50 μg of toxin absorbed on Si gel covered with 20 μl drop of $H_2O$.

significant necrosis on leaves of most of the plants tested. Metabolites **1**, **2**, and **3** inhibited growth of most microorganisms tested in the antibiotic disk assay (Table 3). Metabolites **1** and **3** also inhibited the growth of the producing fungus. These metabolites produced by *Phoma* sp. have broad plant and microbial toxicity and may be more generally toxic to other life forms. Although these metabolites have been isolated following a phytotoxicity screening procedure, the toxins might have an equal or more important role in microbial competition on the leaf surface.

The epoxydon group of toxins identified from *Phoma* sp. are produced by several other fungi as well, including *Ascochyta* (5), *Penicillium* (6), *Phyllosticta* (7), and *Stagnonospora* (8). The genera *Lagerstroemia* (9), *Poronia* (10), and *Scopulariopsis* (11) also produce isomers of epoxydon and desoxyepoxydon. Esters derived from epoxide opening of epoxydon have not been found previously in fungi. The 6-crotonate of the corresponding 5,6-*cis*-diol is known from *Streptomyces griseosporeus* (12). Epoxydon has been shown to be an interme-

TABLE 3. Inhibitory Effect of *Phoma* sp. Toxins Incorporated on Antibiotic Disks on Selected Microorganisms Based on in vitro Activity.

| Microorganisms | Diameter of inhibitory zone (mm) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | |
| | 0.5 mg | 1.0 mg | 0.25 mg | 0.5 mg | 0.25 mg | 0.5 mg |
| *Escherichia coli* | 0 | 0 | 18 | 25 | 35 | 44 |
| *Agrobacterium tumefaciens* | 18 | 25 | 10 | 12 | 43 | 56 |
| *Corynebacterium flaccumfaciens* | 16 | 21 | 12 | 20 | 38 | 49 |
| *Erwinia carotovora* | 0 | 0 | 9 | 17 | 31 | 45 |
| *Aspergillus flavus* | 10 | 17 | 0 | 8 | 15 | 22 |
| *Penicillium notatum* | 12 | 18 | 0 | 10 | 15 | 27 |
| *Colletotrichum acutatum* | 8 | 22 | 10 | 12 | 40 | 60 |
| *Phoma* sp. | 16 | NT[a] | NT | NT | NT | 30 |

[a]NT=not tested.

diate in the biosynthesis of the mammalian mycotoxin patulin by *Penicillium* spp. (6,13). Thus, further investigation of epoxydon-producing pathogens on food crops is warranted.

## EXPERIMENTAL

GENERAL PROCEDURES.—Mass spectra were measured on a Hewlett-Packard 5985-B mass spectrometer; cims ionizing gas was $CH_4$. $^1$H-nmr spectra were determined on a GE 300 Omega spectrometer, and COSY, HMBC, and $^{13}$C nmr were measured on a GE 500 Omega spectrometer. Uv spectra were obtained on a Perkin-Elmer Lambda 48 UV/VIS spectrophotometer. For flash cc, E. Merck Si gel 60 (230–400 mesh) was employed. Analytical tlc was carried out on E. Merck DC-Alufolien, Kieselgel 60 F-254 (0.2 mm thickness). Preparative tlc was carried out on E. Merck Si gel 60 F-254 plate (0.5 mm thickness). Optical rotation was determined in a 10 cm cell on a Rudolph Autopol III polarimeter.

PREPARATION OF CULTURE FILTRATE AND CRUDE EXTRACTS.—An agar block from a 10-day-old culture of *Phoma* sp. (IMI #346016, ATCC #66985) grown on potato dextrose agar was transferred to 500 ml potato dextrose broth. Culture flasks were incubated in a shaker at 100 rpm at 26°. After 8 days the cultures were harvested, filtered, and centrifuged. The culture filtrate (10 liters) was adjusted to pH 3 and extracted 3× with equal volume of EtOAc. The $H_2O$ phase was discarded because of its very low toxicity when bioassayed on rhubarb leaves. The toxic residue from the EtOAc phase (693 mg) was partitioned between EtOAc and 0.4% $Na_2CO_3$ to give a neutral fraction (65 mg) and a phenolic fraction (422 mg).

ISOLATION OF METABOLITES **1–4**.—The phenolic fraction (422 mg residue) obtained via $Na_2CO_3$ extraction was chromatographed on a 5×55 cm Si gel flash column. The column was first eluted with 200 ml $CHCl_3$. Fractions (10 ml) were then collected during elution with 500 ml $CHCl_3$-MeOH (9:1). Residues in fractions 1–12 were not phytotoxic. The two major components of toxic fractions 13–22 were separated by tlc [$CHCl_3$-MeOH (7:3)]. Recovery of a moderately polar metabolite gave 35 mg of brown syrup having uv, cims, $^1$H nmr, and optical rotation identical to those of (+)-epoxydon [**2**] (3). The more polar metabolite was recovered as a colorless syrup (18 mg) and identified spectroscopically as ester **4**: uv λ max (MeOH) 210 (23,000), 240 (9500), 309 (2800); NaOH shift 277 nm (14,600); eims *m/z* 308 (5%), 152 (17%), 138 (9%), 134 (100%); $^1$H and $^{13}$C nmr see Table 1. Phytotoxic fractions 28–42 contained one major compound which crystallized on concentration of solvent. Recrystallization ($Me_2CO$/$CHCl_3$) gave 80 mg of colorless needles of **1**, having melting point and spectral properties identical to those of 6-methylsalicylic acid (8).

The neutral fraction from EtOAc/$Na_2CO_3$ partition was purified by preparative tlc [$CH_2Cl_2$-$Me_2CO$ (4:1)] to give 24 mg of a brown syrup identified spectroscopically as desoxyepoxydon [**3**] (14).

ACETYLATION OF ESTER **4**.—Ten mg of **4** was acetylated in 1 ml pyridine plus 1 ml $Ac_2O$. Solvent was removed 24 h later, and the residue was purified by preparative tlc [$CH_2Cl_2$-$Me_2CO$ (95:5)] to give 8 mg of syrup of aromatized tetraacetate **5**: $^1$H nmr see Table 1; eims *m/z* [M] *m/z* (0.1%), 282 (0.3%), 240 (0.5%), 197 (0.6%), 177 (66%), 155 (1.2%), 135 (100%); cims *m/z* $[M-H]^+$ (0.1%), 399 (16%), 357 (8%), 315 (0.6%), 223 (4%), 177 (100%), 135 (14%).

SYNTHESIS OF ESTER **4**.—Epoxydon (15 mg) and 6-methylsalicylic acid (15 mg) were dissolved in 15 ml of THF. The solution was cooled to −78°, and 11 ml $BF_3$ was added. After 8 h the solution was allowed to warm to room temperature, and the reaction was quenched with $H_2O$. Organic products were extracted into $Et_2O$ and purified by preparative tlc [$CHCl_3$-$Me_2CO$-HOAc (90:10:1)]. The yield of synthetic ester **4** possessing a $^1$H-nmr spectrum identical to that of the fungal product was 7.2 mg.

ISOLATION OF PATHOGEN.—Severely spotted rhubarb leaves and petioles were collected from the field during the 1989 spring season in North Carolina. Sections of leaves and petioles were surface-sterilized with 0.5% NaOCl for 2 min. The pieces were washed with sterile distilled $H_2O$ 3 times and placed in a sterile moist chamber for 5 days. The fungus that developed from the spots on the leaves and petioles was isolated by spreading the spores on water agar plates and scooping single spores with the aid of a stereobinocular microscope. Identification as *Phoma* sp. was made by E. Punithalingam (International Mycological Institute, England). This *Phoma* sp. is intermediate between *P. exigua* Desm. and *P. macrostoma* Mont. in cultural characters and conidial morphology. Ten isolates were made from thirty pieces of leaves and petioles.

PATHOGENICITY TESTS.—Two experiments were conducted, with four plants per treatment. One-month-old rhubarb plants grown in the greenhouse at 26°, with 8–10 true leaves, were used. *Phoma* sp. spores were collected from 15-day-old cultures grown on potato dextrose agar by flushing the colonies with sterile $H_2O$. The spore suspension was washed three times with sterile $H_2O$. Spores were collected by centrifugation and diluted to 100,000 spores per ml as determined by hemocytometer. The leaves and petioles were sprayed with the spore suspension until run-off, and control plants were sprayed with distilled $H_2O$. The inoculated plants were incubated in a moist chamber for 48 h and kept in a greenhouse for 1 week. The experiment was repeated once.

PHYTOTOXICITY AND MICROBIAL TOXICITY ASSAYS.—Greenhouse-grown rhubarb, sorghum [*Sorghum bicolor* (L.) Moench] and seven weeds [prickly sida (*Sida spinosa* L.), morning glory (*Ipomoea* sp.), jimsonweed (*Datura stramonium* L.), Johnson grass (*Sorghum halepense* (L.) Pers.), watercress (*Nasturtium officinale* R. Br.), sicklepod (*Cassia obtusifolia* L.), and lambsquarters (*Chenopodium album* L.)] were used for the leaf bioassay. Phytotoxicity of isolated metabolites was determined by a detached leaf assay (15). Antimicrobial disk assays were performed by standard methods (16) on *Escherichia coli* (DH 5α), *Agrobacterium tumefaciens* (C-58), *Corynebacterium flaccumfaciens* (CF-20), *Erwinia carotovora* (Jones) (EC-14), *Aspergillus flavus* (AF-3357), *Penicillium notatum* (AT-9479), *Colletotorichum acutatum* (NC 1618), and *Phoma* sp.

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