Toxins Produced by the Dogwood Anthracnose Fungus Discula sp.

P. Venkatasubbaiah, and William Scott Chilton

J. Nat. Prod., **1991**, 54 (5), 1293-1297• DOI:
10.1021/np50077a009 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 5, pp. 1293-1297, Sep-Oct 1991

TOXINS PRODUCED BY THE DOGWOOD ANTHRACNOSE FUNGUS *DISCULA* SP.

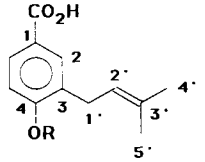
P. VENKATASUBBAIAH and WILLIAM SCOTT CHILTON*

Department of Botany, Box 7612, North Carolina State University, Raleigh, North Carolina 27695

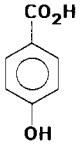
ABSTRACT.—Four phytotoxic phenols, 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoic acid [**1**], 4-hydroxybenzoic acid [**3**], (+)-6-hydroxymellein [**4**], and (−)-isosclerone [**6**], were isolated from culture filtrate of *Discula* sp., the fungus responsible for dogwood anthracnose. This is the first isolation of 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoic acid from a fungus. (+)-6-Hydroxymellein and (−)-isosclerone are enantiomers of previously reported fungal metabolites.

Anthracnose of flowering dogwood (*Cornus florida* L.) has spread throughout the eastern United States since the first report from southeastern New York in 1978 (1). The causative agent was identified as *Discula* sp. Sacc. (Coelomycetes) (2). By 1986 the disease had reached the Appalachian Mountains of Pennsylvania, Maryland, and West Virginia (3). The disease range now extends along the Appalachians into Georgia. Another focus of infection appeared around Seattle in 1976 from where it spread by 1983 into Oregon, Idaho, and British Columbia (4). The near simultaneous outbreak of the disease in the immediate vicinity of major east and west coast ports suggests a foreign origin of the pathogen (3). *Discula* sp. causes leaf necrosis and cankers that kill the tree when the canker completes girdling of the stem. A desire to control this disease of wild and ornamental dogwoods has stimulated work on the pathology of *Discula* sp.; however, no chemical examination of the anthracnose-causing *Discula* sp. or of any other member of the genus has been reported. Therefore we have investigated the identity of phytotoxic metabolites in the culture filtrate of the causative fungus.

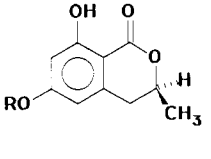
Na_2CO_3 extraction of the crude EtOAc fraction from *Discula* sp. culture filtrate gave three phytotoxic phenols, which were separated by preparative tlc. The most abundant toxin produced by *Discula* sp. was the prenylated hydroxybenzoic acid, 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoic acid [**1**]. The ^1H nmr of **1** in both Me_2CO-d_6 and $CDCl_3$ showed an unresolved singlet for the two methyl groups and an unresolved multiplet for H-2 and H-6 (Table 1). However, all signals were adequately resolved in the nmr of the acetate **2** in $CDCl_3$. The pattern of two ortho-coupled aromatic proton signals, one of which shows meta coupling with the third aromatic proton, establishes the asymmetric trisubstitution pattern of the benzene ring. All resonances of carbons bearing hydrogen were correlated directly to their attached protons by a heteronuclear COSY experiment. Quaternary aromatic carbon assignments were made on the basis of chemical shifts of model phenols and benzoic acids (5). Location of the isoprenyl group at C-3 on biogenetic grounds was supported by observation of a heteronuclear COSY interaction of H-1′ with the aromatic C-3 (134.80 ppm). The close-lying signal at 134.67 ppm was assigned to vinyl C-3′ by virtue of its strong heteronuclear interaction with the methyl protons. Long-range interaction of H-1′ with C-2 and C-4, and not



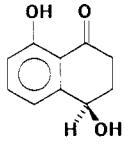
1 R=H
2 R=Ac



3



4 R=H
5 R=Ac



6

TABLE 1. ^{1}H- and ^{13}C-nmr Assignments for 4-Hydroxy-3-(3′-methyl-2′-butenyl)benzoic acid [**1**] and Its Acetate **2**.

Position	Compound		
	1	**2**	
	^{1}H nmr	^{1}H nmr	^{13}C nmr
1	—	—	127.8
2	7.90[a]	8.00, d, J = 1	133.0
3	—	—	134.80
4	—	—	153.9
5	6.86, d, J = 9	7.14, d, J = 8	123.4
6	7.90[a]	7.98, dd, J = 8, 1	130.0
1′	3.38, d, J = 7	3.29, d, J = 7	29.4
2′	5.33, m	5.24, br t, J = 7	121.5
3′	—	—	134.67
4′ or 5′	1.80[b], s	1.72, s	18.5
5′ or 4′	1.80[b], s	1.77, s	26.4
CO_2H	ca. 8.2[c], br		172.0
*Me*CO	—	2.34, s	21.6
Me*C*O	—	—	169.5

[a]Overlapping aromatic signals.
[b]Two unresolved methyl singlets.
[c]Phenolic and carboxylic OH.

with any of the remaining aromatic carbons, confirms the location of the isoprenyl group on C-3. The hrms elemental composition, eims fragmentation pattern, uv, and ir are compatible with the assignment of structure **1** to the major toxin of *Discula* sp. A second toxic component of the Na_2CO_3 fraction proved to be 4-hydroxybenzoic acid [**3**], consistent with the presence of its prenylated derivative **1**. Compound **1** has not been isolated previously; however, it occurs as an amide of an aminocoumarin glycoside in the antibiotic novobiocin (6).

A third phenol isolated by Na_2CO_3 extraction followed by chromatography proved to be (+)-6-hydroxymellein [**4**] by comparison of its ^{1}H nmr and eims fragmentation pattern to data reported (7) for (−)-6-hydroxymellein. The identification was further confirmed by acetylation, which gave (+)-6-acetoxymellein [**5**] with expected nmr and mass spectral features, including one hydrogen-bonded hydroxyl resonance at 12 ppm. The presence of the unacetylated phenolic OH was confirmed in the uv by observation of a large bathochromic shift from 264 to 307 nm immediately after addition of NaOH. The failure to obtain a diacetate is consistent with the strong hydrogen bond between the 8-hydroxyl and carbonyl at C-1 and the inertness of similarly hydrogen-bonded 5-hydroxyflavones (8) and *peri*-hydroxynaphthoquinones and anthraquinones (9). Contrary to a previous report (7), the 300 MHz nmr signals of the methylene protons at C-4 were well resolved for metabolite **4** with a geminal coupling of 17 Hz. The vicinal coulings (4 and 11 Hz) indicate the lactone ring is in a half-chair conformation with the methyl group equatorial. The neighboring methine proton signal was also well resolved with 14 of the theoretical 16 lines observable. The corresponding resonances of **5** were equally well resolved.

This is the first report of the isolation of (+)-6-hydroxymellein from a natural source. Its enantiomer (−)-6-hydroxymellein is one of the less frequently encountered fungal hydroxymelleins, although (−)-6-methoxymellein is well known as a carrot phytoalexin (10,11). Levorotatory 6-hydroxymellein was first isolated from an *Aspergil-*

lus terreus strain containing a mutation in the sulochrin biosynthetic pathway (12) and was subsequently isolated as a minor metabolite of *Gilmaniella humicola* (7).

The neutral EtOAc fraction remaining after Na_2CO_3 extraction contained a phytotoxic component that was further purified by preparative tlc to give 10 mg of crystals. Spectra for this metabolite were identical to those previously reported (13,14) for isosclerone [**6**]. However, by virtue of higher field strength we are able to resolve and assign chemical shifts to H-2a, H-2b, H-3a, and H-3b. Resolution of the H-2 methylene proton signals was sufficiently high to deduce the three coupling constants for this ABXY spin system. However, that of the C-3 methylenes was insufficient to resolve their five-spin system. Isosclerone isolated from culture filtrate of *Discula* sp. is levorotatory, $[\alpha]_D$ −68°. Its cd possesses a weak positive Cotton effect at 270 nm and a strong negative effect at 220 nm. Isosclerone of unspecified rotation has been isolated from *Scytadilium* sp. (13). The (+)-isosclerone isolated from *Sclerotinia sclerotiorum* (14) had a negative Cotton effect centered at 268 nm in the ORD. Its absolute configuration was assigned (14) on the basis of the dibenzoate chirality rule (15). Based on that assignment (−)-isosclerone from *Discula* sp. has structure **6**.

Toxicity of the purified metabolites of *Discula* sp. was tested on dogwood (Table 2)

TABLE 2. Necrotic Area (mm^2) Produced on Dogwood Leaf by 50 μg of *Discula* Metabolites and Derivatives.

Dogwood collections	Compound					
	1	**2**	**3**	**4**	**5**	**6**
NCPV 100	38	51	11	7	2	12
NCPV 101	14	23	10	14	1	5
NCPV 102	24	42	5	10	1	13

and on sorghum and weeds (Table 3) by the detached leaf spot method (16). The dogwoods tested were collected randomly as seedlings in an area of the Smoky Mountains affected by dogwood anthracnose. Some variation in response to toxins is probably due to genetic diversity of wild dogwoods. However the prenylated hydroxybenzoic acid **1** and its acetate **2** were consistently the compounds most toxic to dogwoods, sorghum, and the eight weeds tested. Acetylation of **1** did not diminish its activity. Compounds **1** and **2** were also generally the most active of the six compounds tested against bacteria and fungi, although neither was active against *Escherichia coli* at 500 μg/disk. Isosclerone and 6-hydroxymellein at 500 μg/disk showed no inhibitory activity against

TABLE 3. Necrotic Area (mm^2) Produced by 100 μg of *Discula* Metabolites and Derivatives on Weed Leaves.

Weed	Compound					
	1	**2**	**3**	**4**	**5**	**6**
Sicklepod	22	18	10	20	14	2
Prickly sida	42	36	31	40	34	18
Morning glory	28	19	15	24	13	4
Jimsonweed	31	22	12	24	18	6
Johnsongrass	46	51	22	34	24	13
Sorghum	75	73	19	40	24	7
Lambs quarters	23	8	9	14	6	5
Watercress	56	48	17	36	31	14
Ragweed	36	38	15	60	30	12

any of the seven microorganisms tested. Other workers have reported negligible antifungal activity for isosclerone (13).

EXPERIMENTAL

GENERAL PROCEDURES.—Preparative tlc was carried out on E. Merck Si gel 60 F-254 plates (0.5 mm thickness). ^{1}H-nmr spectra were measured on a GE 300 Omega spectrometer. COSY and ^{13}C-nmr spectra were measured on a GE 500 Omega spectrometer. Hrms were determined on the A.E.I. MS-902 mass spectrometer, and lrms were obtained on a Hewlett-Packard 5985-B mass spectrometer. Optical rotations were measured in a 10-cm cell on a Rudolph Autopol III polarimeter, and cd measurements were made in a 1-cm cell on a JASCO J-600 spectropolarimeter.

PREPARATION OF CULTURE FILTRATE.—Eighteen isolates of *Discula* sp. were made from diseased dogwoods in North Carolina and Georgia. Isolates were maintained on potato dextrose agar. Isolate 89-5 (ATCC 76078) was selected for large-scale fermentation based on biomass and quantity of crude toxins produced. An agar block from a 10-day-old culture growing on V-8 potato dextrose agar was inoculated into 0.5 liter of a liquid medium containing 250 ml potato dextrose broth, 75 ml V-8 juice, 175 ml H_2O, and 0.75 g $CaCO_3$. Twenty 500-ml cultures in 1-liter turbulence flasks were shaken at 100 rpm at 18° for 12 days. A temperature below 20° was necessary for good growth of the fungus.

FRACTIONATION OF TOXINS.—Culture filtrate (10 liters) was adjusted to pH 3.0 and extracted three times with equal volumes of EtOAc. Solvent was removed by rotary evaporation to give 725 mg residual syrup containing crystals. This residue was partitioned between EtOAc and 0.4% Na_2CO_3. The phenolic fraction was recovered from aqueous Na_2CO_3 by acidification and back extraction into EtOAc. After solvent evaporation the neutral fraction weighed 75 mg and the phenolic fraction 350 mg.

The neutral fraction was subjected to preparative tlc [C_6H_6-Me_2CO (60:40)] from which a high R_f, fluorescence-quenching band was eluted and evaporated to give 10 mg crystalline isosclerone [**6**].

The phenolic fraction was further resolved by preparative tlc [$CHCl_3$-MeOH (90:10)]. Elution of a high R_f, blue-fluorescent zone gave 18 mg crystalline 6-hydroxymellein [**4**]. An intermediate R_f, fluorescence-quenching zone gave 60 mg crystalline 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoic acid [**1**], and a low R_f, florescence-quenching zone gave 12 mg crystalline 4-hydroxybenzoic acid [**3**].

ACETYLATION.—Phenols **1** and **4** (10 mg) were each acetylated in 1 ml pyridine plus 1 ml Ac_2O. Solvent was removed 24 h later, and the residue was purified by preparative tlc developed with $CHCl_3$-MeOH (90:10) for **2** and with C_6H_6-Me_2CO (60:40) for **5**. Each acetate was crystallized from EtOAc/hexane.

4-HYDROXY-3-(3′-METHYL-2′-BUTENYL)BENZOIC ACID [**1**].—Mp 100°; uv λ max (MeOH) 256 nm (a_M 36,500), NaOH shift 286 nm (60,700); eims *m/z* [M]$^+$ 206 (55%), [M − Me]$^+$ 191 (16%), 164 (18%), [M − CH=CMe_2]$^+$ 151 (100%); ^{1}H nmr see Table 1.

4-ACETOXY-3-(3′-METHYL-2′-BUTENYL)BENZOIC ACID [**2**].—Mp 103°; uv λ max (MeOH) 233 nm (a_M 16,500), NaOH shift 286 (24,300); hrms [M]$^+$ 248.1041, calcd for $C_{14}H_{16}O_4$, 248.1049; eims *m/z* [M]$^+$ 248 (2%), [M − ketene]$^+$ 206 (38%), [M − Ac]$^+$ 205 (87%), 191 (35%), 151 (100%); ^{1}H nmr and ^{13}C nmr see Table 1.

(+)-6-HYDROXYMELLEIN [**4**].—Uv, nmr, and ms identical to previous reports (7,12). Observed rotations in MeOH: $\alpha_{589} = +0.069°$, $\alpha_{546} = +0.081°$, $\alpha_{435} = +.147°$. Observed rotations were not reduced to specific rotation because of the small sample available (less than 1 mg). Observed ellipticity of cd extrema in MeOH: 267 nm (+0.016°), 246 nm (−0.002°), 223 nm (+0.013°).

6-ACETOXYMELLEIN [**5**].—Mp 150°; uv λ max (MeOH) 264 nm (a_M 16,500), NaOH shift 307 (29,000); [α]D +76° (*c* = 0.05%, MeOH); hrms [M]$^+$ 236.0696, calcd for $C_{12}H_{12}O_5$, 236.0685; eims *m/z* 236 (21%), [M − ketene]$^+$ 194 (100%), 176 (20%), 150 (74%); ^{1}H nmr (300 MHz, Me_2CO-d_6) 9.5 ppm (br s, 8-OH) 6.53 (d, *J* = 2, H-5 or H-7), 6.38 (d, *J* = 2, H-7 or H-5), 4.40 (ddq, *J* = 16, 11, 3, H-3), 2.82 (dd, *J* = 16, 3, H-4b), 2.68 (dd, *J* = 16, 11, H-4a), 2.09 (s, AcO), 1.24 (d, *J* = 7, Me).

(−)-ISOSCLERONE [**6**].—Spectral data were identical to those reported previously (13) with the exception of higher resolution ^{1}H-nmr data for the methylenes: (300 MHz, $CDCl_3$) 2.21 ppm (m, H-3a), 2.36 (m, H-3b), 2.66 (ddd, *J* = 18, 8, 5, H-2a), 3.02 (ddd, *J* = 18, 8, 5, H-2b). Observed ellipticity of cd extrema of ca. 0.2 mg/ml in MeOH: 270 nm (+0.006°), 220 nm (−0.020°); [α]D −68° (*c* = 0.05%, MeOH).

BIOLOGICAL TESTING.—A random sample of wild dogwood seedlings was transplanted to the greenhouse. Dogwoods were acclimatized to greenhouse conditions for 6 months before samples were

taken for detached leaf assays of phytotoxicity. Sorghum and weeds were also grown in the greenhouse. Assays were performed as previously described (16).

Antimicrobial disk assays were performed by standard methods (17). The test microorganisms and widths in mm of the concentric inhibition zone beyond the disk containing 500 μg **1** were: *Agrobacterium tumefaciens*, 8 mm; *Corynebacterium flaccumfaciens*, 5; *Erwinia carotovora*, 5; *Esch. coli*, 0; *Aspergillus flavus*, 4; *Colletotrichum acutatum*, 9; and *Penicillium notatum*, 6. Inhibition zone widths in mm caused by 500 μg **2** were: *Ag. tumefaciens*, 6 mm; *Cor. flaccumfaciens*, 10; *Er. carotovora*, 4; *Esch. coli*, 0; *As. flavus*, 5; *Col. acutatum*, 6; *P. notatum*, 5. Inhibition zone widths in mm caused by 500 μg **3** were: *Cor. flaccumfaciens*, 9 mm; *Esch. coli*, 5; all others, 0 mm. Compounds **4**, **5**, and **6** were non-inhibitory at the 500 μg level.

ACKNOWLEDGMENTS

This work was supported by a grant from the CIBA-Geigy Corporation. We thank Dr. Larry Grand for providing isolates of *Discula* sp. and Dr. Robert Bruck for providing access to his dogwood nursery.

LITERATURE CITED

1. P.P. Pirone, "Diseases and Pests of Ornamental Plants," 5th ed., John Wiley and Sons, New York, 1978.
2. M.L. Daughtery and C.R. Hibben, *Phytopathology*, **73**, 365 (1983).
3. C.R. Hibben, *Plant Dis.*, **72**, 199 (1988).
4. D.S. Saloga and J.F. Ammirati, *Plant Dis.*, **67**, 1290 (1983).
5. L.F. Johnson and W.C. Jankowski, "Carbon-13 NMR Spectra," John Wiley and Sons, New York, 1972.
6. C.H. Shunk, C.H. Stammer, E.A. Kaczka, E. Walton, C.F. Spencer, A.N. Wilson, J.W. Richter, F.W. Holly, and K. Folkers, *J. Am. Chem. Soc.*, **78**, 1770 (1956).
7. K.K. Chexal, C. Tamm, K. Hirotsu, and J. Clardy, *Helv. Chim. Acta*, **62**, 1785 (1979).
8. V.B. Mahesh, S. Neelakantan, and T.R. Seshadri, *J. Sci. Ind. Res.*, **15B**, 287 (1956).
9. R.H. Thompson, "The Naturally Occurring Quinones," 2nd ed., Academic Press, London, pp. 256, 268.
10. E. Sondheimer, *J. Am. Chem. Soc.*, **79**, 5036 (1957).
11. P. Condon, J. Kuc, and N.H. Draudt, *Phytopathology*, **53**, 1244 (1963).
12. R.F. Curtis, P.C. Harries, C.H. Haxall, J.D. Levi, and D.M. Phillips, *J. Chem. Soc. C*, 168 (1966).
13. J.A. Findlay and D. Kwan, *Can. J. Chem.*, **51**, 3299 (1973).
14. T. Morita and H. Aoki, *Agric. Biol. Chem.*, **38**, 1501 (1974).
15. N. Harada and K. Nakanishi, *Chem. Commun.*, 310 (1970).
16. L.M. Pena-Rodriguez, N.A. Armingeon, and W.S. Chilton, *J. Nat. Prod.*, **51**, 821 (1988).
17. H.G. Cutler, R.H Cox, F.G. Crumley, and P.D. Cole, *Agric. Biol. Chem.*, **50**, 2943 (1986).

Received 31 January 1991