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PHYTOTOXINS OF *BOTRYOSPHAERIA OBTUSA*[1]

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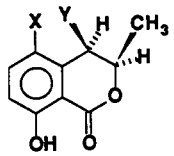
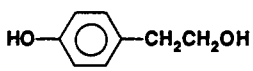
ABSTRACT.—Phytotoxins produced by the apple pathogen *Botryosphaeria obtusa* were identified as (*R*)-(−)-mellein [**1**], *cis*-(3*R*,4*R*)-(−)-4-hydroxymellein [**2**], (*R*)-5-hydroxymellein [**3**], tyrosol [**4**], and *p*-hydroxybenzaldehyde [**5**].

Botryosphearia (Physalospora) obtusa (Schw.) Shoemaker (Loculoascomycetes) is a fungal pathogen causing frogeye leaf spot on apple and black rot of apple fruit. The genera *Botryosphaeria* and *Physalospora* have not been chemically investigated previously. We report the identity of five phytotoxic metabolites **1–5** produced by *B. obtusa*. Details of the biological activity of the toxins and recovery yields of toxins from infected apple are reported elsewhere (1).

The residue from EtOAc extraction of *B. obtusa* culture filtrate was fractionated on a Si gel column. The first-eluting, major toxic component, mellein [**1**], crystallized from chromatographic fractions in a yield corresponding to 26 mg/liter of culture filtrate. After one recrystallization it had melting point, uv, ir, ^{1}H nmr, and mass spectra identical to data reported for mellein (2). Both enantiomers of mellein have been isolated previously from fungi. Although *R*-(−)-mellein is the better known fungal enantiomer (3), *S*-(+)-mellein has been isolated from an unidentified fungus (4) and from *Apiospora camptospora*,[2] *Cercospora taiwanensis* (5), *Fusarium larvarum* (6), and *Gyrostroma missouriense* (7). Therefore, it was necessary to establish the chirality of mellein isolated in this study. The cd spectrum of mellein isolated from *B. obtusa* had a negative extremum at 257 nm; a weak, positive extremum at 239 nm; and a negative extremum at 216 nm. This metabolite is thus *R*-(−)-mellein (8).



1 X=Y=H
2 X=H, Y=OH
3 X=OH, Y=H



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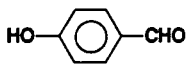

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Two phytotoxic, hydroxylated mellein derivatives were also isolated from *B. obtusa* culture filtrate. 4-Hydroxymellein was isolated in 2.5 mg/liter yield after cc and preparative tlc. Physical constants and spectral data, including a 2 Hz coupling constant between H-3 and H-4, were in agreement with those reported previously (5, 9–11) for the isomer assigned cis stereochemistry (9). The coupling constant for the trans isomer is reported to be 4 Hz (see footnote 2). The cd spectrum of 4-hydroxymellein isolated from *B. obtusa* was superimposable on the cd of *R*-(−)-mel-

[1]Part IV in the series "Phytotoxins of Plant Pathogens." For Part III, see L.M. Pena Rodriguez and W.S. Chilton, *J. Nat. Prod.*, **52**, 1170 (1989).

[2]B.F. Burroughs, cited in D.C. Aldridge, S. Galt, D. Giles, and W.B. Turner, *J. Chem. Soc. C*, 1623 (1971), footnote 8.

lein isolated from the same fungus, as expected for isomers sharing 3*R* chirality. Based on the earlier assignment of cis relative stereochemistry, this metabolite is *cis*-(3*R*,4*R*)-(−)-4-hydroxymellein [**2**].

A further hydroxylated mellein was isolated as a phytotoxin in a yield corresponding to 0.5 mg/liter of culture filtrate after Si gel chromatography and preparative tlc. Its eims contained a very strong molecular ion at *m/z* 194 (base peak) indicating one more oxygen atom than mellein. Dominant fragments were due to loss of H_2O and loss of MeCHO by retro-Diels-Alder reaction characteristic of mellein and its derivatives (11,12). The nmr signals of AB aromatic protons (doublets, 7.04 and 7.25 ppm, $J_{AB}=8$, in Me_2CO-d_6), one hydrogen-bonded phenolic OH (11.0 ppm) and one free phenolic OH (8.3 ppm) indicated that this metabolite is 5-hydroxymellein. The 5-hydroxy isomer has a cd spectrum similar to those of mellein and 4-hydroxymellein with negative extrema at 236 and 257 nm. It is, therefore (*R*)-5-hydroxymellein [**3**].

A 5-hydroxymellein of undetermined stereochemistry has been isolated previously from a stored sample of Brazilian *Virola venosa* wood (13). 5-Hydroxymellein was presumed to be a product of fungal contamination because it was not found in the freshly cut wood. Tyrosol [**4**], a frequently encountered phytotoxic fungal metabolite (14–16), was isolated from *B. obtusa* culture filtrate in a yield of 3 mg/liter. Phytotoxic *p*-hydroxybenzaldehyde [**5**] was isolated from culture filtrate of *B. obtusa* in a yield of 0.5 mg/liter. This metabolite is a predictable shikimate-tyrosine pathway product in fungi and is undoubtedly associated with the presence of tyrosol in the fungal culture filtrate. It has been isolated together with tyrosol from *Ceratocystis* spp. pathogens of lodgepole pine (14) and from a phytopathogenic *Monilia* sp. (17).

EXPERIMENTAL

ISOLATION OF TOXINS.—*B. obtusa* (ATCC #66987) was grown in shake flasks on potato dextrose broth. Culture filtrate (10 liters) was adjusted to pH 3 and extracted 3× with equal volumes of EtOAc. All biological activity resided in the 2-g residue left after evaporation of EtOAc; the concentrated H_2O phase was inactive. The entire extractable material was chromatographed on a 4 × 53 cm Si gel (E. Merck, 230–400 mesh) column eluted in 200-ml steps with C_6H_6 containing 0, 5, 10, 25, 40%, and 100% Me_2CO. The residue from four fractions showed toxicity in a leaf spot assay (18).

Concentration of the first two fractions gave crystals that were recrystallized from Me_2CO/$CHCl_3$ to give 255 mg identified as (*R*)-(−)-mellein [**1**] by comparison of mp, uv, nmr, and mass spectrum to published data (2). The residual syrup from the mother liquor of crystallization was resolved by preparative tlc (E. Merck Si gel 60F–254, 20 × 20 cm 0.5 mm thickness) using Et_2O-C_6H_6 (60:40) into two major components: 4.5 mg of *p*-hydroxybenzaldehyde [**5**] and 4.7 mg of (*R*)-5-hydroxymellein [**3**]: mp 202–210; uv λ max (MeOH) 221 nm (ε 6000), 283 (5000), 341 (1600); NaOH shift 237 sh, 330 (16,000); ^{1}H-nmr (300 MHz, Me_2CO-d_6) 1.48 (d, $J=7$, 3-Me), 2.63 (dd, $J=17$, 11, H-4a), 3.18 (dd, $J=17$, 3, H-4b), 4.74 (m, H-3), 6.69 (d, $J=9$, H-6 or -7), 7.10 (d, $J=9$, H-7 or -6), 8.35 (br, 5-OH), 10.3 (8-OH); eims *m/z* 194 (100%), 176 (72), 165 (41), 150 (37). Other zones of the tlc plate showed no significant toxicity in the leaf spot assay.

The third and fourth fractions from cc both showed the presence of two major compounds active in the leaf spot assay. The residue from the pooled fractions was chromatographed on a preparative tlc plate developed with petroleum ether-Me_2CO (80:20). Elution of the fluorescence-quenching, low mobility zone and concentration of the eluting solvent gave 30 mg of crystalline tyrosol [**4**] identified by mp, uv, ^{1}H nmr, cims, eims, and tlc comparison to a standard. Similar recovery from the fluorescent, high R_f zone gave 25 mg of colorless, non-crystalline *cis*-(3*R*,4*R*)-(−)-hydroxymellein [**2**] possessing the cd, uv, ms and ^{1}H-nmr reported for this metabolite (5, 10, 11).

Evaporation of solvent from the non-toxic fifth and sixth fractions gave a crystalline residue that was recrystallized from MeOH/hexane to give 125 mg of succinic acid.

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ERRATA

For the paper by Sekine and Brossi entitled "Expedient Synthesis of (*S*)- and (*R*)-Norcoclaurine from (*S*)- and (*R*)-Armepavine Prepared by the 1-Phenylethylurea Method," *J. Nat. Prod.*, **53**, 533 (1990), the authors have requested the following corrections:

The name of the title compound armepavine should be changed to norarmepavine. Names associated with formulas **[3]**, **[3a]**, and **[3b]** mentioned in this paper and refered to in the title are (±)-norarmepavine, (−)-norarmepavine, and (+)-norarmepavine, respectively, which were erroneously named as the corresponding armepavines.

For the paper by Suri *et al.* entitled "An *ent*-Seco-atisane Sekelton Diterpene from a Natural Source, Rhizomes of *Euphorbia acaulis*," *J. Nat. Prod.*, **53**, 470 (1990), the authors request the following corrections:

In the listing of authors T. Tsunehiro and Y. Fumiko should be T. Takano and F. Yamada, respectively.