Identification of Fungicidal and Nematocidal Components in the Leaves of *Piper betle* (Piperaceae)

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Five propenylphenols with significant fungicidal and nematocidal activity are isolated from the chloroform extract of leaves of *Piper betle* (Piperaceae). The compounds are identified as chavicol, chavibetol, allylpyrocatechol, chavibetol acetate, and allylpyrocatechol diacetate. Implications of the ingestion of this plant by humans is discussed.

Piper betle L. (Piperaceae) is a tropical Asian vine closely related to the common pepper. It is extensively cultivated in India and the Malay peninsula where the leaves are chewed alone or with other plant materials including the areca nut, *Areca catechu* L. (Guenther, 1952; Ali and Mehta, 1970; Shulgin, 1973; Atal et al., 1975). The betel leaf itself has a spicy taste and yields an essential oil widely used as a medicine (Banger et al., 1966). One of the pharmacological effects ascribed to chewing the leaves is a sense of well-being (Chopra et al., 1958). Other biological activities described for the essential oil include antifungal, antiseptic (Banger et al., 1966), and anthelmintic effects (Chopra et al., 1950: Ali and Mehta, 1970). The roots are reported to have contraceptive activity in humans (Chopra et al., 1965).

In our evaluation of plant materials for biological activities, we discovered that chloroform extracts of the leaves of *P. betle* possessed significant fungicidal activity against the fungus *Cladosporium cucumerinum*. We have isolated five fungicidal compounds from the fresh frozen leaves and characterized them as biogenetically related propenylphenols and their acetates.

MATERIALS AND METHODS

Leaves of *P. betle* are steeped overnight in chloroform and filtered, and the solvent is evaporated in vacuo, leaving an oil. The crude oil is chromatographed over Florisil deactivated with 7% water according to the lipid class separation procedure of Carroll (1961) (Figure 1). Fungicidal activity is associated with fractions 2 (diethyl ether–hexane, 5:95 v/v), 3 (diethyl ether–hexane, 15:85 v/v), 4 (25% diethyl ether in hexane), and 5 (diethyl ether–hexane, 50:50 v/v) by the *Cladosporium* TLC bioassay (Bowers and Evans, 1984). Open column fraction 3 is preparatively chromatographed on a spinning thin-layer chromatography apparatus ("Chromatotron", Harrison Research, Palo Alto, CA). Open column fraction 3 separates into two bands on the Chromatotron during elution with diethyl ether–hexane, 25:75 v/v. The first eluting fraction from the Chromatotron separates on preparative TLC plates, 20 × 20 cm × 0.5 mm, silica gel 60 PF254 (Merck) developed in acetone–chloroform, 5:95 v/v. Three chromatographically pure fungicidal compounds are isolated (no. 3, 4, and 5, figure 2). The second band eluting from the spinning TLC is isolated as a pure component by preparative TLC (compound number 2, Figure 2).

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Florisil open column fraction 4 is preparatively separated on the Chromatotron by using a methanol–ethyl acetate–chloroform, 1:10:89 v/v/v, eluting solvent. A pure fungicidal compound is isolated and numbered 1 (Figure 2). Fungicidal isolates 2–5 are checked for purity by capillary gas chromatography on a 12-m methyl silicone column and a 12-m Carbowax column. The purity of isolate 1 is checked on thin-layer in a variety of solvents and visualized with phosphomolybdic acid.

Fungicidal activity against the fungus *Phythium ultimum* is assessed by placing a 5 mm diameter plug from the growth margin of P. *ultimum*, growing on a potato dextrose agar (PDA) Petri plate, onto the center of a PDA Petri plate prepared to contain the test compound. Chemicals are incorporated into agar at the desired concentration by dispensing the compound (dissolved in 40 μL of methanol) into 40 mL of molten PDA prior to pouring into duplicate 100 × 15 mm Petri plates. The resulting radial growth is measured, depending on rapidity of growth, 24–36 h after inoculation. The growth of *P. ultimum* on plates containing chemicals is expressed relative to *P. ultimum* growing at the same time under identical conditions except for omission of the test chemical. The ED_{50} of the compounds is measured by interpolation from regression curves of growth vs. concentration.

The fungicidal bioassay using *C. cucumerinum* is our modification of the method of Klarman and Sanford (1968) in which 0.5 mg of crude plant extract or lesser amounts of chromatographic fractions are spotted on glass-baked thin-layer plates with 0.25 mm thick silica gel G (Merck). Plates are developed for 15 cm in benzene–ethyl acetate, 85:15 v/v. A nutrient solution is prepared from "V-8" juice (Campbell Soup Co., Camden, NJ) or tomato juice by centrifugation at 10000*g* for 10 min. A 7–10-day-old Petri plate culture of *C. cucumerinum* is flooded with the supernatent, and the fungal spores are suspended by gentle scraping. Following filtration of the spore suspension through two layers of cheesecloth, the filtrate is aspirated onto the dried TLC plate at a rate of 15 mL per 20 × 20 cm TLC plate. The sprayed plate is incubated for several days at room temperature in a water-saturated atmosphere. Fungal growth is rapid, and the TLC plate is covered by dark growth within 2 days, except for regions of fungicidal activity, which appear as a contrasting white.

Nematocidal bioassays are performed by dissolving compounds to be tested in "M-9" buffer (Brenner, 1974) and observing the survival of *Caenorhabditis elegans* in these solutions.

Capillary gas chromatography is performed on a Hewlett-Packard Model 5880 with flame ionization detection and nitrogen carrier gas. Electron ionization mass spectra are obtained with a Hewlett-Packard Model 5985A GC–MS system using a 30-m OV-101 capillary column. The 60-MHz NMR spectra are recorded on a Hitachi-Perkin

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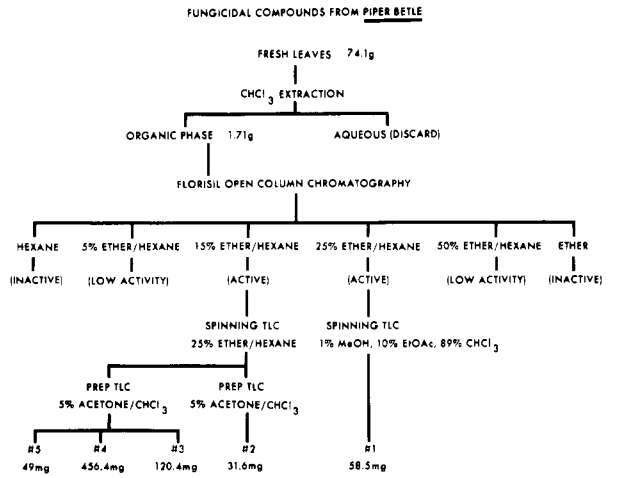

Figure 1. Isolation scheme and yields of fungicidal compounds from *P. betle*.

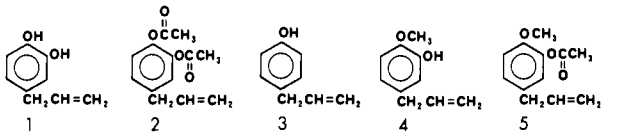

Figure 2. Structures of isolated fungicidal compounds.

Elmer R-600 FT-NMR by using $CDCl_3$ and Me_4Si as the internal standard. The 300-MHz NMR spectra were recorded on a Bruker-300 in $CDCl_3$ at the Cornell NMR Laboratory in Ithaca, NY. Infrared spectra are measured with a Perkin-Elmer 257 grating infrared spectrometer with NaCl cells.

RESULTS AND DISCUSSION

Chavicol, 4-(2-Propenyl)phenol. The 60-MHz proton NMR spectrum of isolate 3 shows a two-proton doublet at 3.4 ppm, a two-proton doublet at 5.15 ppm, and a one-proton multiplet around 5.95 ppm, indicating an allyl group on a benzene ring. The presence of four aromatic protons suggests a disubstituted ring. Mass spectra (MS) reveal a base peak at m/z 134 and a P + 1 at 135. These data suggest that this compound could be chavicol, a known constituent of *P. betle* (Ueda and Sasaki, 1951; Guenther, 1952). A chavicol standard isolated from oil of bay, *Pimenta acris*, Myrtaceae (supplied by Fritzsche—D & O), cochromatographs with isolate 3 by thin-layer chromatography (TLC) and capillary gas chromatography (cap GC) and gives identical MS and NMR spectra.

Allylpyrocatechol, 4-(2-Propenyl)-1,2-benzenediol. The 60-MHz proton NMR spectrum if isolate 1 indicates the same benzylic allyl group, but with three aromatic protons, suggesting a trisubstituted ring. On silica TLC this compound is more polar than chavicol (isolate 3), suggesting an additional polar substituent. The infrared spectrum shows a strong hydroxyl absorption. Methylation by diazomethane yields a product that is indistinguishable by capillary GC and MS from a methyleugenol standard. Infrared spectra are also superimposable. Isolate 1 is allylpyrocatechol, a known constituent of *P. betle* (Ueda and Sasaki, 1951: Guenther, 1952).

Chavibetol, 1-Methoxy-2-hydroxy-4-(2-propenyl)-benzene. The 60-MHz NMR spectrum of isolate 4 indicates an allyl group on a benzene ring, and a three-proton singlet at 3.9 ppm suggests the presence of a methoxy substituent. MS shows a molecular ion at m/z 164 and an M + 1 at 165. The second most abundant ion is m/z 149, demonstrating the characteristic loss of 15 for an aromatic methyl ether. The MS and 60-MHz NMR spectrum are similar to those of eugenol although isolate 4 did not cochromatograph with authentic eugenol on capillary GC. Methylation of isolate 4 with diazomethane yields a product that cochromatographs with methyleugenol on cap GC and gives superimposable infrared spectra. Isolate 4 is identified as chavibetol, a known compound from *P. betle* (Ueda and Sasaki, 1951; Guenther, 1952).

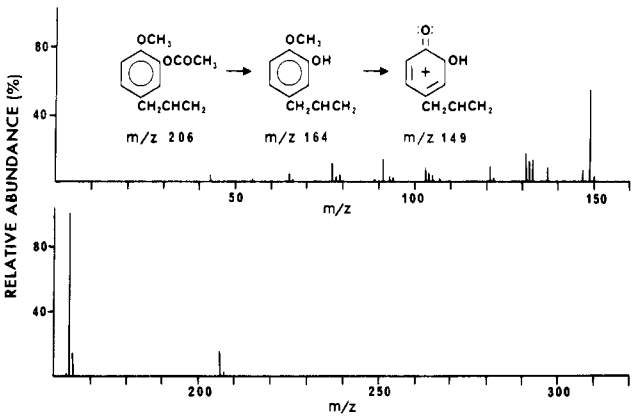

Figure 3. Mass spectra and fragmentation scheme of chavibetol acetate.

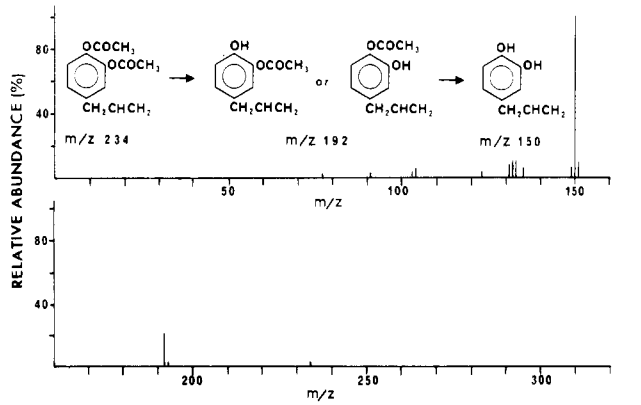

Figure 4. Mass spectra and fragmentation scheme of allylpyrocatechol diacetate.

Chavibetol Acetate, 1-Methoxy-2-acetoxy-4-(2-propenyl)benzene. The 60-MHz NMR spectrum of isolate 5 indicates an acetate by a three-proton singlet at 2.30 ppm, a methoxyl moiety by the three protons at 3.82 ppm, three aromatic protons, and an allyl group on the ring. MS gives a molecular ion at m/z 206 and a base peak at m/z 164, revealing the loss of an acetate to the phenol. The second most abundant ion at m/z 149 at 55% relative intensity shows the further loss of a methyl ether (Figure 3). Acetylation of chavibetol (isolate 4) with acetyl chloride gives a product that cochromatographs with isolate 5 on TLC in several solvent systems and on cap GC with Carbowax and methyl silicone columns. This compound is chavibetol acetate, which has not been identified previously as a natural product.

Allylpyrocatechol Diacetate, 1-Acetoxy-2-acetoxy-4-(2-propenyl)benzene. The 300-MHz NMR spectrum of isolate 2 shows a singlet at 2.89 ppm integrating to six protons. Three aromatic protons along with the familiar allyl pattern is observed. MS gives a molecular ion at m/z 234 and a peak at m/z 196, indicating the loss of an acetate. A base peak at m/z 150 reveals the loss of both acetates to allylpyrocatechol (Figure 4). Acetylation of allylpyrocatechol (isolate 1) with acetyl chloride yields a product that cochromatographs on TLC and cap GC with isolate 2 and gives superimposable infrared spectra. We assign the structure as allylpyrocatechol diacetate to this

compound, which has not been identified previously as a natural product.

From the *Cladosporium* bioassay on thin-layer plates, the minimal detectable amount is 3 μg for chavibetol acetate, 3 μg for chavibetol, 30 μg for chavicol, 10 μg for allylpyrocatechol diacetate, and 1 μg for allylpyrocatechol. The ED_{50} for allylpyrocatechol in the *Pythium* bioassay is 30.5 μg/mL (0.203 μM). By comparison, the ED_{50} for catechol in the same bioassay is 22.5 μg/mL (0.204 μM) and for eugenol is 33 μg/mL (0.201 μM).

P. betle has been reported to have anthelmintic activity (Chopra et al., 1950; Ali and Mehta, 1970), and although it takes substantial amounts (200 μg/mL each compound) of these pure isolates to produce complete mortality of the nematode *C. elegans*, the relatively high concentration of these compounds in the leaves would probably result in an effective anthelmintic dose.

Whereas antifungal activity of *P. betle* leaves and of its essential oil has been reported (Bangar et al., 1966), we have identified the active compounds and quantified their antifungal activity. These five compounds are by weight 0.97% of fresh leaves and 42% of the chloroform extract (Figure 1). Chavicol, chavibetol, and allylpyrocatechol are known natural compounds previously isolated from *P. betle* (Ueda and Sasaki, 1951); however, we think that chavibetol acetate and allylpyrocatechol diacetate are new natural compounds. The fungicidal activities of these new compounds are in the same range as the well-known natural fungicides eugenol and catechol, and their extraordinarily high level in the leaves of *P. betle* confers strong activity to the extract.

The common practice of chewing the leaves of *P. betle* may extract these allyl phenols and inhibit common opportunistic fungal pathogens such as *Candida albicans*, which can infect the oral mucosa. Since leaves of *P. betle* have been used as dressings for sores and wounds (Bangar et al., 1966), the compounds we have isolated may promote wound recovery through their antiseptic properties. It appears likely that some of the diverse biological effects of *P. betle* leaves and oil are due to these identified allylphenols and that these and similar allylphenols should be evaluated for medicinal and agricultural applications.

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Total Glycoalkaloid and Mineral Content of Potatoes Grown in Soils Amended with Sewage Sludge

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The effect of sludge-amended soil on the total glycoalkaloid (TGA) and mineral content of Katahdin potatoes was investigated. In both years TGA content of potatoes grown on sludge-amended soil was not significantly different from that of controls. The tubers grown on sludge-amended soil were significantly lower in K content and higher in Mg content than that of controls. The B, Cd, Cu, Ni, and Zn content of potatoes increased significantly when grown on sludge-amended soil whereas Al and Na decreased. No significant trend was observed for the following minerals: Co, Cr, Fe, Mn, and Pb.

Vesilind (1980) projected that approximately 10^7 dry metric tons of municipal sludge will be produced annually in the United States by 1990, and the need to find safe methods for its disposal has led New York and other industrialized states to consider its potential use as a fertilizer and soil conditioner in agriculture (Boyd et al., 1982). However, there is equal concern that trace metals such as Cd, Cu, Ni, Pb, and Zn and refractory synthetic organic compounds such as polychlorinated biphenyls generally present in sludge (Furr et al., 1976) may be toxic to crops, animals, or man and/or the concentration of toxic substances present in crops may increase sufficiently to have deleterious effects on both animals and humans.

Considerable research is under way to study the possible use of sludge as fertilizer or as a soil conditioner in agriculture. On the beneficial side, it has been shown to increase soil organic matter and moisture-holding capacity (Hansen and Hinesly, 1979). Increases in potato tuber yield were found with municipal sewage sludge fertilization rates of 112, 225, and 450 metric tons (mt) per hectare (ha) compared to the control. Since sludge was low in K, all treatments received a preplant application of K (Dowdy

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