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AN ANTIFUNGAL COMPOUND PRODUCED BY GRAPEFRUIT AND VALENCIA ORANGE AFTER WOUNDING OF THE PEEL

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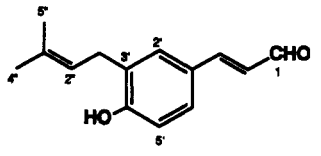
ABSTRACT.—Wounding citrus induces the production of the antifungal compound **1**, isolated from peel of injured grapefruit, *Citrus paradisa*, and injured orange, *Citrus sinensis*. Interpretation of ms, uv, and 1D and 2D nmr spectroscopic data led to the identification of the new compound as 3-[4-hydroxy,3-(3-methyl-2-butenyl)-phenyl]-2-(*E*)-propenal.

Green mold caused by *Penicillium digitatum* Sacc. is a major postharvest disease of citrus. During packing, shipping, and storage the disease may develop when fungal spores land on a fresh wound in the pericarp of an orange (*Citrus sinensus* [L.] Osbeck cv. Valencia) or a grapefruit (*Citrus paradisa* MacFaden cv. Marsh) (Rutaceae). However, if the wounded fruit is cured at high temperature (30°) and high humidity for 24 h, the tissue adjacent to the wound is filled by a material which confers resistance to green mold and the disease does not develop (1). This material characteristically reacts positively with phloroglucinol (pg)-HCl and has been described as "phenolic and ligninlike" (1) based on histochemical tests. But, unlike lignin (2), it is Mäule-negative, and it should thus be described as "wound gum" (3,4). In related work (5), several antifungal coumarins have been isolated from heat-treated pomelos.

In extracts of the wound material of *Cit. sinensus* or *Cit. paradisa* we have found several induced pg-positive compounds which have antifungal activity against *P. digitatum* and against *Cladosporium cucumerinum*. We report here the identity of the most abundant of these compounds and suggest that the positive reaction of injuries with pg-HCl should, in these species, be explained by the localization of induced antifungal compounds which are not lignins.



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RESULTS AND DISCUSSION

We report here the structure of an antifungal compound, **1**, not detectable in the peel of unwounded fruit, which was present in the tissue of injured peel after curing. The compound was isolated by a bioassay-guided (6) separation from hexane or EtOAc extracts of wounded peel of Valencia orange or of grapefruit. Preliminary separation used vlc (7); final purification was by hplc (15% EtOAc in hexanes, Maxsil, 5 μ). In addition to the bioassay, **1** could be followed by tlc as a magenta spot formed on spraying with acidic pg. No magenta spots were visible from similar treatment of peel extracts of unwounded fruit. Isolated from either orange or grapefruit, **1** had identical nmr and hplc properties.

Analysis of the ^{1}H- and ^{13}C-nmr, uv, and mass spectra of the antifungal compound led to the assignment of structure **1**. Precise mass determination indicated a molecular formula of $C_{14}H_{16}O_2$ (observed *m/z* 216.1154, Δ 0.4 mmu of calcd). It was quickly recognized from the ^{1}H- and ^{13}C-nmr and ^{1}H-^{1}H- and ^{1}H-^{13}C- COSY data (Table 1) that the compound was a phenolic cinnamaldehyde bearing an isoprene substituent. The first order coupling pattern of the aromatic proton signals was characteristic of a 1,2,4-substituted benzene. An INAPT experiment with selective irradiation of the

TABLE 1. Nmr Data for Compound **1**.[a]

Position	δ ^{1}H (multiplicity)	δ ^{13}C (multiplicity)
1	9.61 (d, 7.8)	193.8 (d)
2	6.57 (dd, 15.8, 7.8)	128.6 (d)
3	7.55 (d, 15.8)	153.1 (d)
1′		126.9 (s)
2′	7.48 (d, 2.2)	130.6 (d)
3′		127.6 (s)
4′		157.4 (s)
5′	6.92 (d, 8.2)	116.4 (d)
6′	7.42 (dd, 8.2, 2.2)	126.4 (d)
1″	3.33 (d, 7.4)	29.6 (t)
2″	5.35 (t, hep, 7.4, 1.4)	120.8 (d)
3″		136.0 (s)
4″	1.71 (m)	25.8 (q)
5″	1.71 (m)	17.9 (q)

[a] ^{1}H-nmr spectra were observed in Me_2CO-d_6 at 300 MHz; ^{13}C-nmr spectra in $CDCl_3$ at 75 MHz. Proton coupling networks were verified by ^{1}H-^{1}H-COSY. Carbon multiplicity was determined by APT. Correlation of carbons with protons was by phase-sensitive ^{1}H-^{13}C-COSY (PSCSCM) optimized for $^1J_{C\text{-}H}$=125 Hz.

isoprene methylene protons at 3.33 ppm gave rise to carbon signals at 157.4, 136.0, 130.6, 127.6, and 120.8 ppm; these are the 2- and 3-bond C-H correlations to the irradiated protons (8). Since the peaks at 136.0 ppm and 120.8 ppm were known to be due to the isoprenoid double bond (9), this defined three contiguous carbons of the benzene ring (157.4, 127.6, and 130.6 ppm) and the point of attachment of the isoprene group (127.6 ppm). The ^{13}C-nmr resonance at 157.4 ppm was characteristic of a phenolic carbon atom, and the resonance at 130.6 ppm was a CH as shown by an APT experiment. The three remaining carbon atoms were unequivocably assigned based on the sole meta coupling constant (2.2 Hz) of the proton (7.48 ppm) attached to the carbon resonating at 130.6 ppm. Only compound **1** could satisfy the required substitution pattern. The *E* configuration of the 2,3 double bond followed from the coupling constant of 15.8 Hz between H-2 and H-3.

Compound **1** is apparently a new compound. The corresponding acid has been isolated (10,11) from plants of the family Compositae; the ^{1}H-nmr spectrum of the methyl ester is comparable to that of **1**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Uv spectra were recorded using a Perkin Elmer model 202 spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were recorded on a GE NMR model QE-300 using a 5 mm dual ^{1}H/^{13}C switchable probe. All pulse sequences are standard in the QE-300 CHARM v. 13 library. INAPT spectra were optimized for long range J_{CH}=10 Hz with a 25 Hz decoupler bandwidth. Mass spectra were taken using VG 7070 EHF (low resolution) and VG ZAB IFHF (high resolution) instruments. The adsorbents used for cc vlc were Sephadex LH-20 and EM #7736 Si gel 60H. Kodak indicating Si gel plates were used for tlc, and spots were visualized with 254 nm light and with pg by spraying with 1% (w/v) pg in 70% EtOH/H_2O followed by spraying with concentrated HCl. Alternately, cc fractions could be screened with pg-HCl by addition of 2 drops 1% ethanolic pg and 1 drop 4 M HCl to a 1 ml aliquot.

BIOLOGICAL MATERIALS.—Mature grapefruit, *Cit. paradisa*, and oranges, *Cit. sinensis*, were harvested from groves in the University of California Citrus Experiment Station, Riverside, California. Experiments were initiated within 24 h of harvest. *Cl. cucumerinum* Ellis & Arth., isolate M-38, and *P. digitatum* Sacc., isolate M-6r, were grown and maintained on potato dextrose agar (PDA).

BIOASSAYS.—Samples were assayed for antifungal activity against *Cl. cucumerinum*. Inhibition zones can readily be observed when this black fungus is grown on the developed Si gel tlc plate containing antifungal compounds (6).

Since *P. digitatum* did not grow on silica plates, Kodak cellulose plates were substituted for this bioassay. The sample was spotted on the plate and dried, and the plate was sprayed with a spore suspension, air-dried, and sprayed with a growth medium containing 39 g PDA, 2 g neopeptone, 2 g yeast extract, and 1 g Tergitol XD per liter. The plate was incubated at 25° in an H_2O-saturated atmosphere for 24 h. Viable hyphae were visualized by soaking overnight in 0.04% Thiazolyl blue in 0.1 M K_2HPO_4 adjusted to pH 7. Zones of inhibition were evident where 7.5 μg of **1** had been spotted.

FRUIT TREATMENT, EXTRACTION, AND PURIFICATION.—Fruit were injured with a steel brush and rinsed with H_2O to remove peel oil. Fruit were incubated at 30° in an H_2O-saturated atmosphere for 3 days. Flavedo was removed using a produce grater. Control fruit was rinsed with H_2O but not injured or cured before use. Tissue from control and injured cured peel was separately extracted with EtOAc, chromatographed by tlc using EtOAc, and bioassayed with *Cl. cucumerinum* (6). Induced antifungal activity was associated with three spots, R_f 0.6, 0.5, and 0.45, which gave purple to magenta colors when sprayed with pg-HCl. Large scale purification of both orange and grapefruit extracts was independently performed. In the experiment with grapefruit, 1.5 kg tissue was extracted with hexane, and the residue was jar-milled overnight in EtOAc. The hexane solution was concentrated and partitioned with EtOH-H_2O (3:1). The EtOH fraction was concentrated under vacuum, and the resulting aqueous concentrate was extracted with EtOAc. This EtOAc extract was combined with a second EtOAc extract of milled tissue and reduced in volume to 50 ml. The resulting turbid solution was filtered. The filtrate was dried under vacuum, then fractionated by cc on Sephadex LH-20 using an H_2O/EtOH step gradient. Fractions were assayed with pg and by tlc bioassays. Since good correlation was found between pg screens and bioassays, later large scale purifications were guided by the quicker pg test. Fractions eluting with 55 to 70% EtOH were combined and extracted into EtOAc after the addition of H_2O. An oily liquid (2.6 g) was obtained and fractionated twice by cc on Si gel using a hexane/EtOAc stepgradient. Activity was observed for fractions eluting with 14 to 20% EtOAc. This material was concentrated, and the residue (200 mg) was further purified by hplc on a Phenomenex Maxsil-5 column (250×4.6 mm) with 15% EtOAc in hexanes, μ=1.5 ml/min, Rt 13.0 min, to afford pure compound **1** (ca. 1 mg): mp 144.5–146°; uv (MeOH) λ max 221 (ε 12096), 243 (14515), 333 (25920); eims *m/z* (%) $[M]^+$ 216 (100), 161 (72), 160 (26), 147 (43), 132 (21), 131 (29), 115 (28), 91 (21), 77 (36), 55 (26); hreims *m/z* observed 216.1154 ($C_{14}H_{16}O_2$ calcd *m/z* 216.11503); ^1H nmr (300 MHz) and ^{13}C nmr (75 MHz) see Table 1. Purified **1**, R_f 0.5 on Si gel tlc (EtOAc), produced a magenta spot with pg-HCl and was antifungal in both the *Cl. cucumerinum* and *P. digitatum* tests. An identical compound (hplc retention, ^1H- and ^{13}C-nmr spectra) was purified from wounded orange peel using similar procedures. We were not able to isolate significant amounts of the other compounds that produced color with phloroglucinol; we believe that compound **1** represents the major component of the antifungal material.

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