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Isolation and Characterization of Methyl Epijasmonate from Lemon (*Citrus limon* Burm.)

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Two epimers of methyl jasmonate, methyl 2-[2(*Z*)-pentenyl]-3-oxocyclopentane-1-acetate, were isolated from lemon peel (*Citrus limon* Burm.). ^{1}H NMR, mass spectra, hydrogenation, ozonolysis, and acid-catalyzed isomerization were used to establish their identity. Gas chromatography indicated the presence of 75 μg of methyl jasmonate isomers in the peel of one lemon with more than 95% of it in the thermodynamically less stable methyl epijasmonate form.

During the study of the pheromones produced by the male oriental fruit moth [*Grapholitha molesta* (Busck.)], a lemon-like odor was recognized in specialized organs called "hairpencils", located at the base of the abdomen. Ethyl cinnamate and methyl jasmonate, and not the isoprenoid compounds commonly associated with lemons, were shown to cause this lemon-like odor (Nishida et al., 1982).

Many volatile compounds have been identified in lemons (Ranganna et al., 1983), some in the juice (Mussinan et al., 1981), but most in the oil expressed from the peel (Shaw, 1979). The major components, limonene, γ-terpinene, β-pinene, β-bisabolene, and citral, arise through isoprenoid biosynthesis. Of these, citral, a mixture of the *E* and *Z* isomers, geranial and neral, contribute much to the characteristic odor of lemon peel oil. This report described an odoriferous lipid metabolite methyl epijasmonate [methyl 3-oxo-2-[2(*Z*)-pentenyl]cyclopentane-1-acetate] (Demole, 1982; Vick and Zimmerman, 1983), which was determined to be a component of lemon peel. The presence of this compound in lemons explains why the hairpencils of the oriental fruit moth seem to have a lemon-like smell. We find the odor of methyl epijasmonate especially strong in very ripe lemons.

EXPERIMENTAL SECTION

Crude Extract of Lemon Peels. Peels from 150 lemons obtained from a local grocer were twice soaked in 2.5 L of acetone and ether (1:1) for 1-week periods. The combined extracts were concentrated at 45 °C in a rotary evaporator to an oily residue, shaken with 350 mL ether, and extracted with 150 mL of saturated NaCl. The ether extract was dried over anhydrous Na_2SO_4 and then concentrated to yield 22.2 g of a yellow oil. Approximately, 17 g of volatile components (limonene, etc.) were removed, by vacuum distillation at 0.02 mmHg and 45 °C, yielding 4.4 g of brown oily residue.

Florisil chromatography of the nonvolatile residue in a column 200 mm × 35 mm i.d. yielded five fractions successively eluted with the following solvents: 400 mL of Skelleysolve B (abbreviated SKB), followed by 400 mL of 5% ether in SKB, 400 mL of 10% ether in SKB, 800 mL of 20% ether in SKB, and then 800 mL of 30% ether in SKB. Evaporation of the solvent from the 30% ether–SKB fraction yielded 640 mg of residue with the characteristic hairpencil lemon-like smell of the Oriental fruit moth. This fraction was chromatographed on a 50 mm × 9 mm i.d. column filled with 30 g of 200-mesh silica gel deactivated with 10% water. Six fractions were obtained by successive elution with the following solvents: 45 mL of SKB, followed by 90 mL of 5% ether in SKB, 150 mL of 10% ether in SKB, 60 mL of 20% ether in SKB, and then 75 mL of 20% ether in SKB. Evaporation of the last 20% ether in SKB fraction yielded 130 mg of a yellowish oil with the characteristic hairpencil lemon-like odor.

Isolation of Methyl Jasmonate Epimers. Portions of the final 20% ether–SKB fraction were separated on a 380 mm × 10 mm i.d. column filled with 5-μm Lichrosorb SI-100 eluted with 20% ether in SKB at 25 kg/cm^2 and 2 mL/min by using an Altex 110 pump. One fraction was collected between 45 and 50 min and another between 51 and 57 min. GLC of each fraction on a 2 m × 4 mm i.d. glass column filled with 100–200-mesh Gas-Chrom Q coated with 3% OV-101 yielded two peaks in both fractions; I at an *n*-paraffin retention index of approximately

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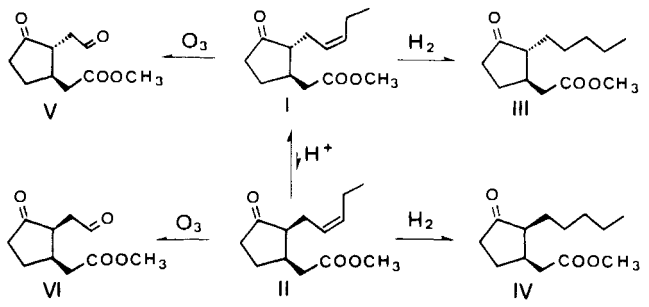

Figure 1. The reaction scheme used to estalish the identity of I as methyl jasmonate and II as methyl epijasmonate. Ozonolysis and hydrogenation of I and II yielded the epimers V, VI, III, and IV, all separable by gas chromatography on methyl silicone (OV-101).

1610 and II at approximately 1640. The oven temperature was 150 °C and the injection temperature 200 °C, a condition that was later shown to cause interconversion of these two peaks. Compound I was essentially 99% pure but II contained 5–30% of I, due to epimerization during isolation. Estimated from GLC peak areas, the total yield of I was 90 μg and the yield of II was 30 μg.

Ozonolysis was carried out by bubbling ozone gas into a 5-μg solution of I or II dissolved in 100 μL of CS_2 held in a dry ice–acetone bath for 30 s. The reaction was then treated with excess triphenylphosphine. Hydrogenation was done by bubbling hydrogen gas into a 5-μg solution of I or II in 100 μL of SKB containing catalytic amounts of platinum oxide at 0 °C and atmospheric pressure for 2 min. Isomerization of I to II was carried out as follows. A 5-μg solution of I in 100 μL of SKB was mixed with 10 μL of 1% *p*-toluenesulfonic acid in SKB and held at 55 °C for 3 h in a sealed glass tube. This muxture was washed twice with 100 μL of saturated sodium bicarbonate and twice with saturated sodium chloride. Proton magnetic resonance (^{1}H NMR) was recorded at 100 MHz with a Varian XL-100 by using Me_4Si as an internal standard. Mass spectra (MS) were measured at 70 eV on a Hewlett-Packard 5985 mass spectrometer.

RESULTS AND DISCUSSION

The two compounds I and II isolated from lemon peels seemed to be isomers. The confirmation that they are 2-epimers of methyl jasmonate was obtained by several means. First, both I and II gave essentially identical fragmentation patterns: I, MS *m/z* (rel intensity) 67 (34), 77 (34), 79 (45), 81 (30), 82 (27), 83 (100), 91 (32), 93 (42), 95 (50), 109 (45), 133 (32), 151 (72), 156 (38), 193 (21), molecular ion 224 (50); II, MS *m/z* (rel intensity) 67 (38), 77 (32), 79 (57), 81 (32), 82 (27), 83 (100), 91 (33), 93 (38), 95 (74), 109 (37), 133 (28), 151 (51), 156 (26), 193 (11), molecular ion 224 (46). These spectra are similar to that reported for methyl jasmonate (Demole and Stoll, 1962). Second, compound I and authentic methyl jasmonate showed identical retention times on both Carbowax 20M and OV-101 capillary GLC columns. This assignment was confirmed by the ^{1}H NMR spectrum of I in $CDCl_3$, which showed the presence of 20 protons assigned as follows: 5.38 (2 H, m, –CH=CH–), 3.73 (3 H, s, $-COOCH_3$), 2.9–1.3 (12 H, undissolved m), 0.96 (3 H, t, J = 7.5 Hz, $-CH_2-CH_3$). Third, the tendency of II to degenerate into I during preparative GLC suggested that II was the 2-epimer of I as described by Tanaka and Torii (1975).

Proof that II was methyl epijasmonate was provided by the reactions of I and II shown in Figure 1. Hydrogenation of I and II yielded III and IV. On OV-101 gas chromatography, each had retention indices 12 units higher than their parent compounds and both showed mass fragmentations characteristic of dihydromethyl jasmonate: III, MS *m/z* (rel intensity) 83 (100), 153 (42), 156 (54), 226 (6); IV, MS *m/z* (rel intensity) 83 (100), 153 (41), 156 (32), 226 (12). Thus, I is not a geometric isomer of II. Furthermore, the fact that ozonolysis products, V and IV, of I and II had identical mass spectra but slightly different retention times substantiates the notion that they were epimers of each other: V, MS *m/z* (rel intensity) 83 (32), 95 (20), 96 (59), 97 (100), 156 (29), 167 (20), 198 (3); VI, MS *m/z* (rel intensity) 83 (34), 95 (17), 96 (56), 97 (100), 156 (23), 167 (18), 198 (3).

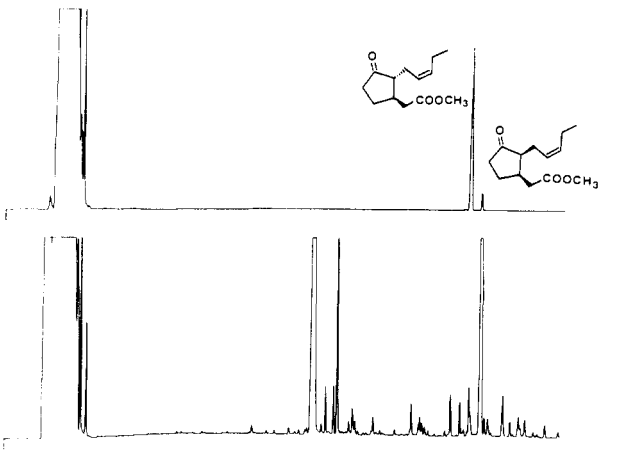

Figure 2. The FID gas chromatogram of the methyl epijasmonate isolated from lemon peel (lower chromatogram) compared to a chromatogram of standard methyl jasmonate epimers (upper chromatogram). This chromatography was done on a 25 m × 0.31 mm fused silica column coated with cross-linked-bonded methyl silicone (OV-101) 0.53 μm thick.

Finally, the treatment of I with *p*-toluenesulfonic acid yielded two compounds in a ratio of 95:5 with gas chromatographic and mass spectral properties identical with those of I and II respectively. This indicates that the isomerization occurs at position 2 (α to the carbonyl). Absolute configurations of the compounds isolated from lemons could not be determined because of the small quantities obtained. However, Nishida et al. (1984) has determined the absolute configurations of the isomers resolved from synthetic methyl jasmonate.

In order to determine the amount of I and II in lemons, the peel of one lemon was extracted with 10 mL of acetone–diethyl ether (1:9), evaporated to 1 mL, and then chromatographed on a 2 cm by 1 cm i.d. silica column with 10 mL of the following hexane solutions: pure hexane, 1% ethyl acetate (AcOEt), 12% AcOEt, and 40% AcOEt. The jasmonate-smelling 12% AcOEt fraction was chromatographed on two 30 cm × 8 mm i.d. columns filled with 5-μm silica (connected in series) by using hexane 10% in AcOEt at 3 mL/min as the mobile phase. By use of authentic standards to determine retention volumes of the methyl jasmonate epimers, the appropriate fractions were collected, combined, and analyzed by gas chromatography.

Figure 2 shows the FID gas chromatogram of the combined jasmonate-rich HPLC fractions compared with standards of methyl jasmonate epimers. Figure 3 shows the mass fragmentogram of the same sample. Both chromatograms were run with the injection port temperature at 150 °C to prevent epimerization. The peel from one lemon showed the presence of 5 μg of methyl jasmonate and 70 μg of methyl epijasmonate.

These experiments demonstrate that methyl epijasmonate is present in lemon peels. As reported by Demole and Stoll (1962), the eclipsed configuration of this epimer is

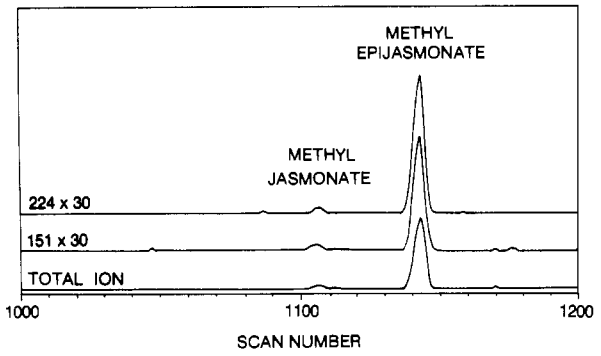

Figure 3. A mass fragmentogram of the methyl jasmonate epimers isolated from a lemon. The same gas chromatographic column described in Figure 2 was adapted to an Hitachi M-80 spectrometer and the 70-eV spectra were used to plot m/z 224 and 151.

thermodynamically less stable than the gauche arrangement of the methyl jasmonate epimer. However, methyl epijasmonate is the major form present in lemon peels and the only biologically active form present in the hairpencils of the oriental fruit moth (Baker et al., 1981; Nishida et al., 1982). Recent studies of the odor properties of synthetic methyl jasmonate stereoisomers (Acree et al., 1984) indicates that the (+)-epi isomer has the strongest odor for humans. Therefore, the original observation that an insect had a lemon-like odor is explained by the presence of methyl epijasmonate.

Registry No. I, 1211-29-6; II, 62653-86-5.

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Effect of Ascorbic Acid, Sodium Bisulfite, and Thiol Compounds on Mushroom Polyphenol Oxidase

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The effect of ascorbic acid, sodium bisulfite, glutathione (reduced), and dithiothreitol on the observed activity of mushroom polyphenol oxidase (PPO) was determined by spectrophotometry (color formation) and by polarography (O_2 uptake). By polarography, O_2 uptake began immediately on adding enzyme. Ascorbic acid and sodium bisulfite had little effect on the initial velocity while dithiothreitol and glutathione decreased the initial velocity by 35%, with no further decrease at higher concentrations. By spectrophotometry, there was an initial lag in the absorbance change followed by a slower increase in absorbance than for the control at zero time. By spectrophotometry, the I_{50} values were as follows: dithiothreitol, 0.06 mM; glutathione, 0.17 mM; sodium bisulfite, 0.20 mM; ascorbic acid, 0.24 mM. The direct effect of the reductants was determined by (1) incubation of the reductant with PPO and then measuring the remaining activity by polarography and (2) gel electrophoresis. At 0.1 mM, dithiothreitol caused a complete loss of activity after 70 min at 25 °C ($t_{1/2}$ = 8 min) while sodium bisulfite, glutathione, and ascorbic acid caused 50% inactivation after 28, 106, and 130 min, respectively, at 5 mM.

The effect of ascorbic acid, sodium bisulfite, and other reducing reagents on polyphenol oxidase (PPO) (EC 1.14.18.1) has been controversial over the years. The effects of ascorbic acid and sulfite have been most studied because of their extensive use in food processing. Early reports (Ingraham, 1956; Scharf and Dawson, 1958) indicated that ascorbic acid had no direct effect on the activity of PPO. More recently, Varoquaux and Sarris (1979) suggested that ascorbic acid neither inhibits nor activates the enzyme. However, several researchers (Baruah and Swain, 1953; Ponting, 1954; Mihály and Vámos-Vigyázó, 1976) reported inactivation of the enzyme by ascorbic acid. Duden and Siddiqui (1966) suggested a k_{cat} type of inactivation in the presence of ascorbic acid. Activation of PPO by ascorbic acid also has been reported (Krueger, 1950).

The effect of sulfite on PPO is also complex. It was shown to act as a reductant according to eq 1 (Ponting, 1960) and to react with quinones to form a colorless complex (LuValle, 1952; Schenck and Schmidt-Thomee, 1953). Direct inhibitory effect of sulfite on PPO was also shown (Embs and Markakis, 1965).

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