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A MULTICHEMICAL DEFENSE MECHANISM OF BITTER OLIVE *Olea europaea* (OLEACEAE) Is Oleuropein a Phytoalexin Precursor?

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Abstract—*Olea europaea* (Oleaceae) is resistant in nature to insect and microbe attack. Two types of chemical protection were found in the foliage. One type is the bitter *seco*-iridoid glycosides oleuropein (1) and ligstroside (2); The other is a physical barrier of crystalline oleanolic acid (4) that coats the leaf surface. The *seco*-iridoid glycosides were isolated using two different countercurrent chromatographies: rotation locular countercurrent chromatography (RLCC) and droplet countercurrent chromatography (DCCC). The dimethyl ester (III) was shown to be an artifact. This is the first isolation of ligstroside from *O. europaea.* In an antimicrobial test by the paper disk method against *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Escherichia coli*, compounds I, II, and III inhibited a growth of *B. subtilis* at pH 7. Similar tests under the influence of β-glucosidase suggest an aglycone of oleuropein, either the hemiacetal (i) or the possible enal-aldehyde (ii), could be the active intermediate. This intermediate could be produced rapidly in response to microorganism invasion. Oleuropein producing such a postinfection active intermediate could be referred to as a phytoalexin precursor.

Key Words—*Olea europaea* (Oleaceae), oleuropein, ligstroside, oleanolic acid, rotation locular countercurrent chromatography (RLCC), droplet countercurrent chromatography (DCCC), chemical barrier, multichemical defense mechanism, enal-aldehyde active intermediate, phytoalexin precursor.

INTRODUCTION

Host-plant resistance to attack by insect and microbe is mediated by chemical agents. An investigation of these agents is an important part in understanding evolutionary and ecological aspects of plant-microbe relationships, and may also

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have some practical use. We have investigated a number of plants that are resistant to insect and microbe attack in order to identify the agents responsible for this resistance (Kubo et al., 1984). Recently our investigation has centered on the bitter olive, *O. europaea* (Oleaceae).

This paper describes the isolation and characterization of two classes of chemicals from *O. europaea* that contribute to its resistance to microbe attack and proposes the structural moiety responsible for the observed antimicrobial activity.

O. europaea is known to be relatively immune to microbe and insect attack. At least some of this immunity may be attributed to a high concentration of *seco*-iridoid glycosides (I and II), which occur predominantly in the Oleaceae family (Asaka et al., 1972). The resistance can also be attributed to a large amount of oleanolic acid (IV) on the foliage that acts as a physical barrier. Although oleanolic acid itself does not exhibit antimicrobial activity, it covers almost all of the leaf. This is undoubtedly important in limiting the penetration of microbe into the plant tissue since the microbe cannot germinate and grow unless sufficient moisture is available within the leaf phillosphore.

METHODS AND MATERIALS

Materials. Fresh fruits of *O. europaea* (2.1 kg) were collected on the UC Berkeley campus. After extraction for more than six months with methanol, the methanol was removed and 162.4 g of extract was obtained.

DCCC Separation for I. The DCCC separation was performed on a model DCCC-300-G2 (Tokyo Rikakikai Co., Tokyo, Japan). The DCCC solvent system of $CHCl_3$–CH_3OH–H_2O (13:7:4, v/v) was chosen by prescreening the main compounds of an initial methanol extract on a TLC plate (Macherey, Nagel, and Co., Duren, G.F.R., poligram Sil G/UV 254). The upper phase was chosen as the mobile phase in our DCCC system. The crude methanol extract (1.0 g) was dissolved in a (1:1, v/v) mixture of the mobile and stationary phases and injected into the DCCC apparatus using a 10-ml sample coil. The eluate was collected in 2.2-ml fractions. Fractions were monitored by TLC (Sil G/UV 254) developed with the organic layer of this solvent system. Visualization was accomplished by UV (Chromato-UVE Cabinet, model CC-60, Ultra violet products, Inc., California) and a vanillin–sulfuric acid–ethanol (3 g:1.5 ml:100 ml) spray reagent. The DCCC chromatogram is shown in Figure 1.

DCCC Separation for II and III. The mixture sample (87 mg), collected in fractions 49–72 (111 mg) of the DCCC separation for I, was reinjected using C_6H_6–$CHCl_3$–CH_3OH–H_2O (5:5:7:2, v/v) solvent system in the ascending method. The eluents were collected in 2.2 ml fraction. The DCCC chromatogram is shown in Figure 2.

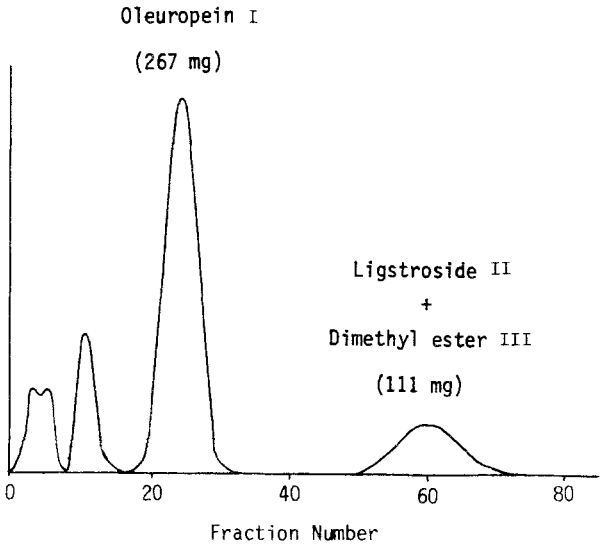


FIG. 1. DCCC of the methanol extract of *O. europeae* (1.0 g) with $CHCl_3$–CH_3OH–H_2O (13:7:4, v/v) by the ascending method; 2.2 ml/fraction; 5 days.

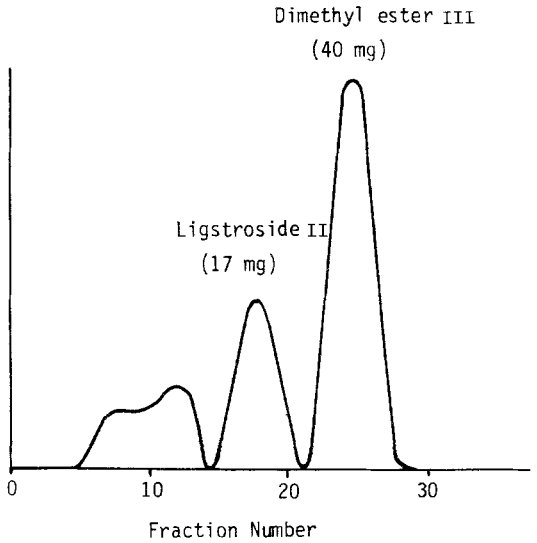
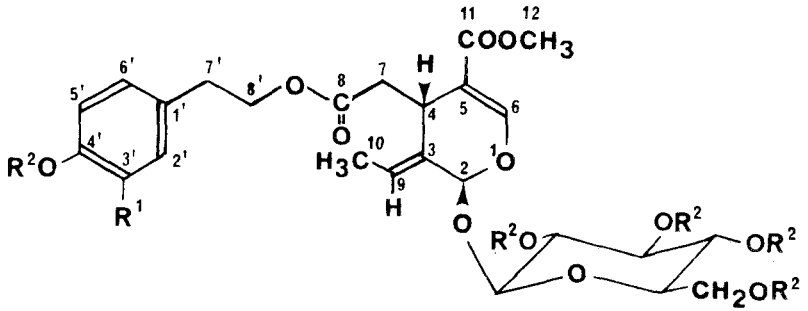


FIG. 2. DCCC of the mixture (87 mg) of IV and V with C_6H_6–$CHCl_3$–CH_3OH–H_2O (5:5:7:2, v/v) by the ascending method; 2.2 ml per fraction.

Oleuropein (I). Amorphous (Scheme 1). $[\alpha]_D^{28}$ −146.2° (c = 0.44, MeOH). UV (EtOH) 228.5 nm (ε = 18000), 278.0 nm (ε = 3300). IR (Nujol) 3100–3500 (br OH), 1680–1725 (br COO), 1620, 1520 (arom.) cm^{-1}. [^{1}H]NMR (DMSO-d_6) δ 1.65 (3H, d, *J* = 6 Hz, 10-H), 2.41 (1H, dd, *J* = 14 Hz, 9 Hz, 7-H_a), 2.63 (1H, dd, *J* = 14 Hz, 4 Hz, 7-H_b), 2.69 (2H, t *J* = 6 Hz, 7′-H), 3.65 (3H, s, 12-H), 3.86 (1H, dd, *J* = 9 Hz, 4 Hz, 4-H), 3.95–4.18 (2H, m, 8′-H), 4.66 (1H, d, *J* = 8 Hz, anomeric-H), 5.87 (1H, s, 2-H), 5.97 (1H, q, *J* =6 Hz, 9-H), 6.48 (1H, dd, *J* = 8 Hz, 2 Hz, 6′-H), 6.61 (1H, d, *J* = 2 Hz, 2′-H), 6.65 (1H, d, *J* = 8 Hz, 5′-H), 7.53 (1H, s, 6-H), 8.73 (1H, d, *J* = 8 Hz, OH). [^{13}C]NMR (DMSO-d_6) δ 12.9 (q), 30.1 (d), 33.7 (t), 39.9 (t), 51.2 (q), 61.1 (t), 65.0 (t), 69.9 (d), 73.3 (d), 76.5 (d), 77.3 (d), 92.9 (d), 99.0 (d), 107.7 (s), 115.5 (d), 116.1 (d), 119.5 (d), 123.0 (d), 128.4 (s), 129.1 (s), 143.7 (s), 145.0 (s), 153.4 (d), 166.2 (s), 170.0 (s). SI-MS *m/z*: 541 (M^+1), 427, 423, 361, 225, 137.

Hexaacetyloleuropein (Ia). Acetylation of I with acetic anhydride and pyridine gave Ia (Scheme 1). Amorphous. $[\alpha]_D^{25}$ −109.7° (c = 0.06, $CHCl_3$). UV (EtOH) 224.0 nm (ε = 12000). IR ($CHCl_3$) no OH, 3010, 735 (arom.), 1746, 1210 (COO) cm^{-1}. [^{1}H]NMR ($CDCl_3$) δ 1.70 (3H, d, *J* = 8 Hz, 10-H), 2.05, 2.31 (12H, 6H each, both s, OAc × 6), 2.43 (1H, dd, *J* = 14 Hz, 8 Hz, 7-H_a), 2.77 (1H, dd, *J* = 14 Hz, 4 Hz, 7-H_b), 2.81 (2H, t, *J* = 7 Hz, 7′-H), 3.74 (3H, s, 12-H), 3.96 (1H, dd, *J* = 8 Hz, 4 Hz, 4-H), 5.70 (1H, s, 2-H), 6.00 (1H, q, *J* = 8 Hz, 9-H), 7.05-7.10 (3H, m, 2′-, 5′-, 6′-H), 7.45 (1H, s, 6-H). [^{13}C]NMR ($CDCl_3$) δ 13.5 (q), 20.6 (q, C × 6), 30.3 (d), 34.4 (t), 40.0 (t), 51.4 (q), 61.8 (t), 64.5 (t), 68.4 (d), 70.8 (d), 72.3 (d), 72.6 (d), 93.8 (d), 97.2 (d),



I : R^1 = OH R^2 = H

Ia : R^1 = OAc R^2 = Ac

II : R^1 = H R^2 = H

IIa : R^1 = H R^2 = Ac

SCHEME 1.

108.8 (s), 123.4 (d), 123.8 (d), 124.9 (d), 127.0 (d), 128.2 (s), 136.6 (s), 140.8 (s), 142.0 (s), 153.1 (d), 166.8 (s), 168.1 (s), 168.2 (s), 169.3 (s), 169.4 (s), 170.1 (s), 170.5 (s), 171.0 (s).

Ligstroside (II). Amorphous (Scheme 1). $[\alpha]_D^{28}$ −131.4° (c = 0.30, MeOH). UV (EtOH) 223.0 nm (ϵ = 18000), 273.0 nm (ϵ = 1600). IR (Nujol) 3200–3500 (br OH,), 1685–1730 (br COO), 1625, 1518 (arom.) cm^{-1}. [^{1}H]NMR (DMSO-d_6) δ 1.61 (3H, dd, J = 8 Hz, 2 Hz, 10-H), 2.40 (1H, dd, J = 14 Hz, 10 Hz, 7-H_a), 2.63 (1H, dd, J = 14 Hz, 4 Hz, 7-H_b), 2.74 (2H, t, J = 8 Hz, 7′-H), 3.64 (3H, s, 12-H), 3.84 (1H, dd, J = 10 Hz, 4 Hz, 4-H), 4.05 (1H, dd, J = 12 Hz, 8 Hz, 8′-H_a), 4.15 (1H, dd, J = 12 Hz, 8 Hz, 8′-H_b), 4.65 (1H, d, J = 8 Hz, anomeric-H), 5.86 (1H, s, 2-H), 5.96 (1H, q, J = 8 Hz, 9-H), 6.68 (2H, d, J = 8 Hz, 2′-, 6′-H), 7.02 (2H, d, J = 8 Hz, 3′-, 5′-H), 7.52 (1H, s, 6-H), 9.24 (1H, s, OH). [^{13}C]NMR (DMSO-d_6) δ 12.8 (q), 30.1 (d), 33.4 (t), 39.8 (t), 51.1 (q), 61.1 (t), 64.9 (t), 69.9 (d), 73.2 (d), 76.5 (d), 77.3 (d), 92.9 (d), 99.0 (d), 107.6 (s), 115.1 (d, C × 2), 122.9 (d), 127.7 (s), 129.1 (s), 129.7 (d, C × 2), 153.3 (d), 155.8 (s), 166.1 (s), 170.5 (s). SI-MS *m/z*: 525 (M^+1), 427, 363, 331, 233, 225, 207, 121.

Pentaacetylligstroside (IIa). Acetylation of II with acetic anhydride and pyridine gave IIa. (Scheme 1). Amorphous. $[\alpha]_D^{25}$ −104.4° (c = 0.16, $CHCl_3$). UV (EtOH) 212.0 nm (ϵ = 16000), 223.0 nm (ϵ = 16500). IR ($CHCl_3$) no OH, 3010, 1615, 720 (arom.), 1747, 1210 (COO) cm^{-1}. [^{1}H]NMR ($CDCl_3$) δ 1.69 (3H, d, J = 8 Hz, 10-H), 2.00, 2.30 (12H, 3H each, both s, OAc × 5), 2.40 (1H, dd, J = 14 Hz 10 Hz, 7-H_a), 2.73 (1H, dd, J = 14 Hz, 6 Hz, 7-H_b), 2.89 (2H, t, J = 6 Hz, 2′-H), 3.71 (3H, s, 12-H), 3.96 (1H, dd, J = 10 Hz, 6 Hz, 4-H), 4.05-4.37 (2H, m, 8′-H_{ab}), 5.66 (1H, s, 2-H), 5.96 (1H, q, J = 8 Hz, 9-H), 6.96 (2H, d, J = 10 Hz, 2′-, 6′-H), 7.16 (2H, d, J = 10 Hz, 3′-, 5′-H), 7.39 (1H, s, 6-H). [^{13}C]NMR (DMSO-d_6) δ 13.5 (q), 20.6 (q, C × 4), 21.1 (q), 30.2 (d), 34.4 (t), 39.9 (t), 51.4 (q), 61.7 (t), 64.9 (t), 68.2 (d),70.7 (d), 72.2 (d), 72.5 (d), 93.7 (d), 97.0 (d), 108.8 (s), 121.6 (d, C × 2), 124.8 (d), 128.0 (s), 129.8 (d, C × 2), 135.2 (s), 149.3 (s), 153.0 (d), 166.7 (s), 169.2 (s), 169.3 (s), 169.4 (s), 170.1 (s), 170.5 (s), 171.0 (s).

Dimethyl Ester (III). Amorphous. UV (EtOH) 233.0 nm (ϵ = 11000). IR (Nujol) 3520–3200 (br OH), 1730, 1700 (br COO), 1630 (br C = CH), 1010–1090 (br COC) cm^{-1}. [^{1}H]NMR (DMSO-d_6) δ 1.68 (3H, d, J = 7 Hz), 2.43 (1H, dd, J = 15 Hz, 10 Hz). 2.66 (1H, dd, J = 15 Hz, 4.5 Hz), 3.07 (1H, m), 3.04–3.13 (1H, m), 3.16–3.26 (2H, m), 3.46 (1H,m), 3.57 (3H, s), 3.65 (3H, s), 3.69 (1H, m), 3.86 (1H, dd, J = 10 Hz, 4.5 Hz), 4.47 (1H, t, J = 7 Hz), 4.65 (1H, d, J = 8 Hz), 4.98, 5.04 (both 1H, both br s), 5.17 (1H, d, J = 4 Hz), 5.85 (1H, s), 5.97 (1H, q, J = 7 Hz), 7.53 (1H, s), Assignments shown in Figure 3 are confirmed by decoupling experiments. [^{13}C]NMR (C_5D_5N) δ shown in Figure 3. EI-MS *m/z*: 256, 238, 196, 178, 165, 151.

Tetraacetyldimethyl Ester (IIIa). IIIa was obtained from III by the same way as for Ia. Physical and spectral data of IIIa agreed well with those of the authentic

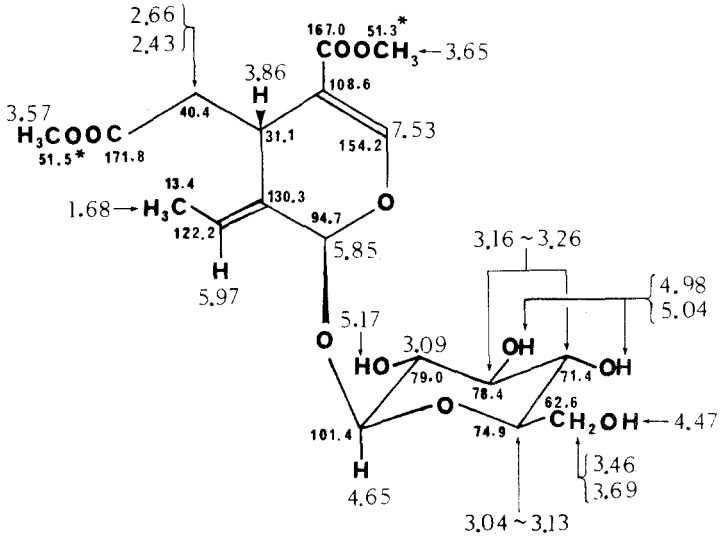


FIG. 3. ^{1}H NMR and ^{13}C NMR assignments for III. *Assignments may have to be interchanged. Decoupled experiments also confirmed the ^{1}H NMR assignments.

sample (Inoue et al., 1970). mp 112–114°C. $[\alpha]_D^{24} -138.5°$ (c = 0.2, $CHCl_3$). IR ($CHCl_3$) no OH, 1760, 1710 (COO), 1635 (C = C-H) cm^{-1}. UV (EtOH) 222 nm (ϵ = 15300). EI-Ms *m/z*: 587 (M + 1), 513, 332.

RLCC Separation of Fresh Methanol Extract. The RLCC separation was performed on a model RLCC-A (Tokyo Rikakikai Co., Tokyo, Japan). The solvent system of $CHCl_3$–CH_3OH–H_2O (13:7:4, v/v) in the ascending method was chosen. The methanol extract (1.0 g) was dissolved in the mobile phase and injected into the RLCC apparatus using a 3-ml sample chamber. The eluent was collected in 1.4-ml fractions which were monitored by TLC. The RLCC chromatogram is shown in Figure 4.

HPLC Analyses. Fresh leaves, fruits, and seeds (each amount weighed about 30 mg) were extracted with methanol for one week. After filtration and solvent removal, the methanol extract of each was passed through a SEP-PAC C_{18} cartridge (for rapid sample preparation, Water Associates, Inc.) with CH_3OH–H_2O (6:4, v/v) in order to remove some of the lipophilic portion of the extract. After evaporation of the solvent in vacuo, 10 μg of each residue was dissolved in methanol for injection into a HPLC apparatus (DuPont model 850 liquid chromatograph; DuPont Zorbax ODS, particle size 5–6 μm, 25 cm × 4.6 mm I.D). Compounds were detected by a DuPont variable-wavelength UV spectrophotometer and microflow cell at 254 nm using CH_3OH–H_2O (6:4, v/v) as a solvent at a flow rate of 1.5 ml/min. The results are listed in Table 1.

Antimicrobial Activity. Compounds I, II, and III were screened for antimicrobial activity by the paper disk bioassay.

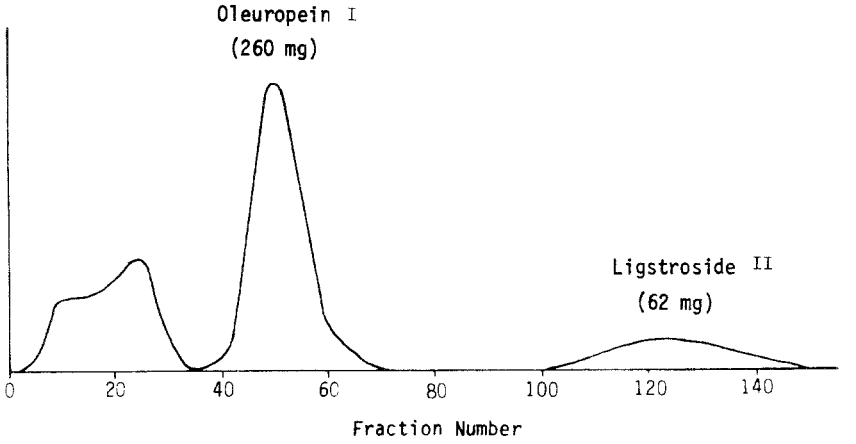


FIG. 4. RLCC of the methanol extract of *O. europaea* (1.0 g) with $CHCl_3$–CH_3OH–H_2O (13:7:4, v/v) by the ascending method; 1.4 ml/fraction; 3 days.

Cultures of *S. cerevisiae* X2180–1B used in this study was obtained from Professor J. Thorner, and both *B. subtilis* and *E. coli* W3100 were obtained from Professor H. Nikaido, U.C. Berkeley.

Medium for *S. cerevisiae* was a 0.67% aqueous solution (20 ml) of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Michigan) containing 1.0% agar (Difco Laboratories), pH controlled by 1 N NaOH and 1 N HCl, and was autoclaved at 120°C for 15 min. After the solution was cooled to 50°C, it was seeded with a cell suspension of *S. cerevisiae* (a 24–hr culture at 30°C at pH 7 on a 0.67% aqueous solution of yeast nitrogen base without amino acids at a concentration of 10^4 cells/ml). An aqueous solution (10 unit/ml) of β-glucosidase (Sigma Chemical Company, St. Louis, Missouri) was added to give a 0.5 unit/ml concentration in the medium. This mixture was then poured into a 90-mm-diameter Petri dish. The plating medium without added β-glucosidase was used as a control.

In the media for *B. subtilis* and *E. coli*, a 1% Pepton solution (Difco Lab-

TABLE 1.

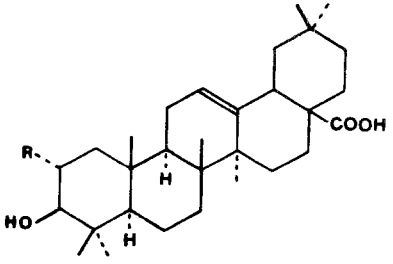
	Oleuropein I		Ligstroside II	
	In fresh olive (%)	In MeOH ext (%)	In fresh olive (%)	In MeOH ext (%)
Fruit	0.39	5.0	0.052	0.66
Seed	0.55	3.5	0.69	4.4
Leaf	0.42	3.5	not found	not found

oratories) was used instead of the yeast nitrogen base used for the medium of *S. cerevisiae*, and the cell suspensions of *B. subtilis* and *E. coli* were seeded at a concentration of 10^5 cells/ml for each medium. Otherwise the medium was the same as for *S. cerevisiae*.

To test for antimicrobial activity by paper disk bioassay, appropriate volumes of the solutions were pipetted onto 6.5-mm-diameter paper disks to give desired dry weight quantities. Solutions were allowed to evaporate before the disks were placed on the seeded agar surface. Zones of inhibition were observed after incubation at 30°C for 16 hr. No zone of inhibition was observed in any case without added β-glucosidase. The results under the influence of β-glucodase are listed in Table 2.

Oleanolic Acid (IV). Colorless needles (Scheme 2). mp 300–302°C. $[\alpha]_D^{20}$ + 86.3° (c = 0.13, $CHCl_3$). IR (Nujol) 3400 (OH), 1700 (COOH) cm^{-1}. [^{1}H]NMR (C_5D_5N + $CDCl_3$) δ 0.78, 0.87 (6H each, both S, OMe × 4), 0.94, 1.00, 1.16 (3H each, each s, OMe × 3), 2.79 (1H, dd, J = 14 Hz, 3 Hz, 18-H), 3.20 (1H, d, J = 8 Hz, 3-H), 5.28 (1H, t, J = 3 Hz, 12-H). [^{13}C]NMR (C_5D_5N + $CDCl_3$) δ 15.1 (q), 15.6 (q), 16.8 (q), 18.2 (t), 23.1 (t), 23.2 (t), 23.5 (q), 25.8 (q), 27.1 (t), 27.7 (t), 28.0 (q), 30.6 (s), 32.5 (t), 32.6 (t), 33.0 (q), 33.9 (t), 36.9 (s), 38.4 (t), 38.7 (s), 39.1 (s), 41.2 (d), 41.6 (s), 46.0 (t), 46.2 (s), 47.6 (d), 55.2 (d), 78.4 (d), 121.9 (d), 144.1 (s), 180.5 (s). EI-MS *m/z*: 456 (M^+), 423, 300, 203, 133, 119, 69.

Maslinic Acid (V). White powder (Scheme 2). mp 264–266°C. $[\alpha]_D^{20}$ + 60.0° (c = 0.01, $CHCl_3$). IR (Nujol) 3380 (OH), 1690 (COOH) cm^{-1}. [^{1}H]NMR (C_5D_5N + $CDCl_3$) δ 0.80, 0.84, 0.92 (3H each, each s, OMe × 3), 0.92 (6H, s, OMe × 2), 1.05, 1.15 (3H each, both s, OMe × 2), 2.95 (1H, dd, J = 14 Hz, 3 Hz, 18-H), 3.07 (1H, d, J = 10 Hz, 3-H), 3.73 (1H, dt, J = 10 Hz, 4 Hz, 2-H), 5.30 (1H, t, J = 3 Hz, 12-H). [^{13}C]NMR (C_5D_5N + $CDCl_3$) δ 16.4



IV : R=H, Oleanolic acid

V : R=OH, Maslinic acid

SCHEME 2.

TABLE 2. ANTIMICROBIAL SPECTRUM IN PRESENCE OF β-GLUCOSIDASE BY PAPER DISK BIOASSAY[a]

		Zone of inhibition (mm diameter)						
		Oleuropein I			Ligstroside II		Dimethyl ester III	
Microorganism	(mg/disk)	2.0 (mg/disk)	1.0 (mg/disk)	0.5 (mg/disk)	1.0 (mg/disk)	0.5 (mg/disk)	1.0 (mg/disk)	0.5 (mg/disk)
Bacillus subtilis	pH 8	trace	—[b]	—	8	7	—	—
	pH 7	14	11	8	16	12	10	trace
	pH 5	NG[c]	NG	NG	NG	NG	NG	NG
Saccharomyces cerevisiae	pH 8	25	20	14	—	—	—	—
	pH 7	16	13	—	—	—	—	—
	pH 5	—	—	—	—	—	—	—
Escherichia coli	pH 8	—	—	—	—	—	—	—
	pH 7	—	—	—	—	—	—	—
	pH 5	—	—	—	—	—	—	—

[a]No zone of inhibition was observed in any case without added β-glucosidase.
[b]— indicates no zone of inhibition.
[c]NG indicates that the bacteria did not grow in the medium.

(q), 16.7 (q, C × 2), 18.2 (t), 23.0 (t), 23.3 (t), 23.5 (q), 25.8 (q), 27.6 (t), 28.5 (q), 30.6 (s), 32.5 (t, C × 2), 33.0 (q), 33.9 (t), 38.1 (s), 39.0 (t), 39.2 (s, C × 2), 41.1 (d), 41.6 (s), 46.2 (t), 46.3 (s), 47.6 (d), 55.2 (d), 77.3 (d), 83.4 (d), 121.7 (d), 144.1 (s), 180.5 (s). EI-MS *m/z*: 472 (M^+), 426, 408, 300, 248, 203, 133, 119, 69.

RESULTS AND DISCUSSION

The preliminary antimicrobial screening of the methanol extract of the fruits of *O. europaea* showed activity against *B. subtilis* (the fruits had been kept in methanol more than six months). Testing after separating the crude extract into ether-, ethyl acetate-, and water-soluble fractions indicated the active components were in the ether fraction. Attempts to isolate the active components in the ether fraction failed. We then examined the biological activity of the main compounds found in the methanol extract.

The R_f values of the main compounds were determined to be 0.3–0.5 on a silica gel TLC plate using an organic layer of $CHCl_3$–CH_3OH–H_2O (13:7:4, v/v) mixture. Due to its polar nature, the methanol extract seemed ideally suited for further separation by countercurrent chromatography which has been previously applied to the resolution of many polar mixtures (Hostettmann, 1980; Kubo et al., 1983). The initial DCCC attempt using $CHCl_3$–CH_3OH–H_2O (13:7:4, v/v) by the ascending method was able to isolate compound I, but left II and III as a mixture (Figure 1). Reinjecting the mixture of II and III, and changing the solvent system to C_6H_6–$CHCl_3$–CH_3OH–H_2O (5:5:7:2, v/v) in the ascending method resulted in baseline separation of II and III (Figure 2).

Structural determination by $[\alpha]_D$, UV, IR, MS, [^{1}H]- and [^{13}C]NMR showed I and II to be the known bitter *seco*-iridoid glycosides oleuropein (Panizzi et al., 1960) and ligstroside (Asaka et al., 1972). This was confirmed by spectral studies ($[\alpha]_D$, UV, IR, MS, NMR) of their acetylated derivatives Ia and IIa. This is the first isolation of ligstroside from olive.

The structure of III was shown by [^{1}H]- and [^{13}C]NMR to have the same *seco*-iridoid skeleton found in I and II, but the 3,4-dihydroxyphenylethyl of I and the 4-hydroxyphenylethyl group of II were replaced by a methyl group (Figure 3). The acetylated compound of III (IIIa) was identified by a direct comparison with an authentic sample (Inoue et al., 1970) based on physical and spectral data (mp, $[\alpha]_D$, IR, UV, EI-MS).

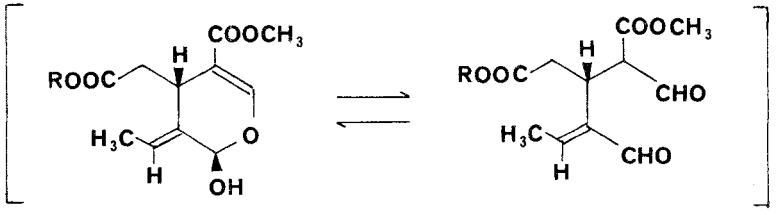
To see if compound III was an artifact arising from prolonged storage in methanol, fresh olive fruits were extracted, and the components were separated by rotation locular countercurrent chromatography (RLCC) (Kubo and Matsumoto, 1984. The RLCC separation took two days less than the DCCC separation and showed that compound III was an artifact that did not exist in the fresh fruit (Figure 4). HPLC was used to quantify the amounts of oleuropein (I) and ligs-

troside (II) in the fresh fruit body, seed, and leaf. The amounts of I and II from fresh and stored materials are listed in Table 1. No seasonal variation of these components was observed on a monthly basis over the period of a year. Oleuropein and ligstroside were found in the seed; I was the major compound in fruit and leaf (II was not detected in the leaf).

The presence of antimicrobial compounds in olive has been suspected for some time. Walter et al. (1973) and Fleming et al. (1973) reported the antimicrobial activity of oleuropein and its enzymatic hydrolysis poduct against bacteria and yeast. However, not enough data were available to identify the chemical structure of the enzymatic degradation product, nor could they examine the antimicrobial activity of the pure compound.

Cruess and Alsberg (1934) suggested that a much more active hydrolytic enzyme than emulsin was found in olive, and we examined the antimicrobial activity of I, II, and III with and without β-glucosidase (Table 2). Compounds I, II, and III showed no activity without β-glucosidase against *S. cerevisiae, B. subtilis,* and *E. coli* by the paper disk method. However, they inhibited the growth of *B. subtilis* at pH 7 in the presence of β-glucosidase. Oleuropein also inhibited the growth of *S. cerevisiae* at pH 7 and 8 with the enzyme (this conflicts with the results by Fleming et al., 1973). The data in Table 2 suggest, in the case of oleuropein, the active form is the aglycone hemiacetal (i) or the corresponding aglycone cleaved enal–aldehyde structure (ii) (Taniguchi et al., 1983), which is theoretically possible (Sticher, 1969) (Figure 5). Possibly the crude ether extract showed the antimicrobial activity previously mentioned because of the active aglycone, which would be easy to extract with ether but difficult to isolate because of its reactivity and instability.

Another defense mechanism against microbe attack of the olive leaf is a physical barrier on the leaf surface. The scanning electron micrographs show the leaf surface of *O. europaea* to be covered with crystals (Figure 6) (Kubo and Matsumoto, 1984). These crystals were carefully collected using a microscope and spatula without damaging the leaf's cuticle. TLC analysis ($CHCl_3$–CH_3OH, 20:1, v/v, silica gel) showed these contained a major and a minor component.



i ii

FIG. 5.

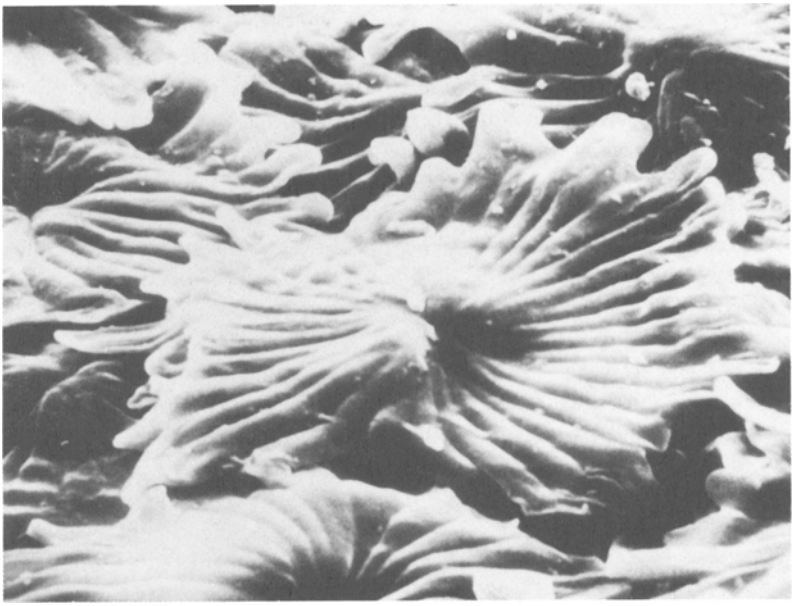


FIG. 6. The electron micrographs of the abaxial surface of the leaf of *O. europaea* (×550).

Purification by silica gel column chromatography gave two triterpenes. The major component was identified as oleanolic acid (IV, $C_{30}H_{48}O_3$, mp 300–302°C, $[\alpha]_D$ + 86.3°) from spectroscopic data (Roncero and Janer, 1969; Romualda et al., 1974). The very minor compound (V, $C_{30}H_{48}O_4$, mp 264–266°C, $[\alpha]_D$ + 60.0°) was identified by spectroscopic data as maslinic acid (Roncero and Janer, 1969; Romualda et al., 1974). The total yield of these triterpenes from the leaf surface (3.2% by weight) is comparable to the amount found for the whole leaf reported by Roncero and Janer (1969). Thus almost all oleanolic acid present is on the surface of the leaf. Oleanolic acid does not exhibit antimicrobial activity. However, this hydrophobic barrier on the leaf surface keeps water from collecting on the leaves and in this way inhibits the germination of microbes. This material also forms a physical barrier that might keep microbes from penetrating the leaf. This simple mechanism of secreting triterpenes onto the leaf surface must be considered as a part of a multichemical defense.

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REFERENCES

ASAKA, Y., KAMIKAWA, T., KUBOTA, T., and SAKAMOTO, H. 1972. Structures of seco-iridoids from *Ligstrum obtusifolium* Steb. et Zucc. *Chem. Lett.* 00:141–144.

CRUESS, W.V., and ALSBERG, C.L. 1934. The bitter glucoside of the olive. *J. Am. Chem. Soc.* 56:2115–2117.

FLEMING, H.P., WALTER, W.M., Jr., and ETCHELLS, J.L. 1973. Antimicrobial properties of oleuropein and products of its hydrolysis from green olives. *Appl. Microbiol.* 26:777–782.

HOSTETTMANN, K. 1980. Droplet countercurrent chromatography and its application to the preparative scale separation of natural products. *Planta Med.* 39:1–18.

INOUE, H., YOSHIDA, T., TOBITA, S., TANAKA, K., and NISHIOKA, T. 1970. Absolute Struktur des Oleuropeins und einiger verwandter Glucoside. *Tetrhaderon Lett.* 29:2459–2464.

KUBO, I., and MATSUMOTO, A. 1984. Secreted oleanolic acid on the cuticle of *Olea europaea* (Oleaceae); a chemical barrier to fungus attack. *Experientia.* 40: 937–938.

KUBO, I., KLOCKE, J.A., GANJIAN, I., ICHIKAWA, N., and MATSUMOTO, T. 1983. Efficient isolation of phytoecdysones from *Ajuga* plants by high-performance liquid chromatography and droplet countercurrent chromatography. *J. Chromatogr.* 257:157–161.

KUBO, I., MATSUMOTO, T., and KLOCKE, J.A. 1984. Multichemical resistances of the conifer *Podocarpus gracilior* (Podocarpaceae) to insect attack. *J. Chem. Ecol.* 10:547–559.

PANIZZI, L., SCARPATI, M.L., and ORIENTE, G. 1960. Constituzione della oleuropeina, glucoside amaro e ad azione iptensiva dell'olivo. *Gazz. Chim. Ital.* 90:1449–1485.

ROMUALDA, C., PIETRO, M., and LUCIO, P. 1974. Triterpenes in husks of *Olea europaea. Phytochemistry* 13:1551–1552.

RONCERO, A.V., and JANER, M.L. 1969. Acidos triterpenicos del olivo. *Grasas Aceites* 20:133–138.

STICHER, O. 1969. Iridoide. *Pharm. Acta Helv.* 44:453.

TANIGUCHI, M., ADACHI, T., HARAGUCHI, H., OI, S., and KUBO, I. 1983. Physiological activity of warburganal and its reactivity with sulfhydryl groups. *J. Biochem.* 94:149–154.

WALTER, W.M. Jr., FLEMING, H.P., and ETCHELIS, J.L. 1973. Preparation of antimicrobial compounds by hydrolysis of oleuropein from green olives. *Appl. Microbiol.* 26:773–776.