Preparation of Antimicrobial Compounds by Hydrolysis of Oleuropein from Green Olives[1]

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Oleuropein, an intensely bitter glucoside, was isolated from green olives. Hydrolysis products obtained from oleuropein in sufficient quantity for further tests were: (i) β-3,4-dihydroxyphenylethyl alcohol prepared by acid hydrolysis of oleuropein; (ii) elenolic acid obtained by methanolysis of oleuropein, isolation of the intermediate acetal, and subsequent acid hydrolysis; and (iii) oleuropein aglycone formed by the action of β-glucosidase on the parent glucoside. Mass spectral verification of the isolated compounds and ultraviolet absorption data are given. Oleuropein and its aglycone had similar threshold levels for detection of bitterness, whereas elenolic acid and β-3,4-dihydroxyphenylethyl alcohol were not judged to be bitter.

The bitter principle of olives, oleuropein, was named and studied by Bourquelot and Vintilesco (1). Later Panizzi et al. (8) showed that the oleuropein molecule contained glucose, β-3,4-dihydroxyphenylethyl alcohol, and an acid (Fig. 1). These workers suggested that this acid was identical to a hypotensive agent designated as elenolic acid (W. L. C. Veer, U.S. Patent 3,033,877, 1962; reference 10) which was prepared by hydrolysis of olive extracts with phosphoric acid.

Fleming et al. (5) isolated a compound from green olives that appeared to have the antimicrobial properties noted earlier during the fermentation of brined olives (3, 4). The compound, a bitter phenolic material, was considered to be an enzymatic degradation product of oleuropein (5). Others (8) proposed that oleuropein is hydrolyzed in vitro by β-glucosidase into glucose and a bitter tasting aglycone.

Because of the importance of a proper fermentation on the preservation of brined green olives, a complete understanding of oleuropein's role was needed. The present study was undertaken to develop a procedure to produce sufficient amounts of oleuropein and its hydrolysis products so that the chemical and antimicrobial properties could be determined. Effects of these compounds on selected species of bacteria and yeasts are reported in a separate paper (6).

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MATERIALS AND METHODS

Extraction and purification of oleuropein. Oleuropein was extracted from steam-heated (100 C, 20 min), green, Manzanillo variety olives (500 g) as described earlier (5) except that the residue from the methanol extraction was shaken with hexane to remove lipids before extraction with ethyl acetate. The lipid-free ethyl acetate solution was evaporated to 20 ml, and the components were separated by counter-current distribution (CCD) with the solvent system of ethyl acetate: 0.1 M potassium phosphate, pH 4.5. By using absorption at 280 nm, the major band was located and collected, and the solvent was removed by evaporation. The isolate was a crusty, light-yellow material (7.2 g), consisting mainly of oleuropein.

This dried extract was dissolved in methanol, and the oleuropein fraction was purified by preparative thin-layer chromatography (PLC) as described earlier (5) except that the solvent system was benzene-methanol-acetic acid (45:16:1). The oleuropein was purified further by CCD by using ethyl acetate as the mobile phase and distilled deionized water as the stationary phase. Oleuropein (2.01 g) purified in this manner was a light-yellow amorphous material. The final yield of purified oleuropein was about 0.4% of the weight of the pitted olives.

Acid hydrolysis of oleuropein and isolation of the products. Purified oleuropein (500 mg) was hydrolyzed in 100 ml of 1 N H_2SO_4 for 1 h at 100 C. The hydrolysate was cooled, adjusted to pH 2, and extracted with ethyl acetate. After drying, the solvent was removed in vacuo, yielding 304 mg of an oily product. The oil was dissolved in methanol and applied to thin-layer chromatography (TLC) plates coated with Silica Gel HF_{254}. After development in

benzene-methanol-acetic acid (45:8:1), the plate was observed under shortwave ultraviolet (UV) light, and three compounds were noted. Compound 1 (R_f 0.26) gave a positive reaction when sprayed with a phenol-sensitive reagent (5). The R_f was identical to that of authentic β-3,4-dihydroxyphenylethyl alcohol. Compound 2 (R_f 0.35) gave a faint blue color with the phenolic spray and was assumed to be the aglycone of oleuropein. Compound 3 (R_f 0.43) gave a negative phenol test and had an R_f value similar to that of elenolic acid.

With the compounds tentatively identified, components of the oleuropein hydrolysate were separated by PLC by using the solvent system described above for analytical TLC. Each of the three zones was collected and dissolved in the appropriate solvent for purification by CCD. The solvent system used for CCD purification of the aglycone and elenolic acid was ethyl ether-water (compounds located by absorption at 224 nm), whereas ethyl acetate-water was used for β-3,4-dihydroxyphenylethyl alcohol (located by absorption at 280 nm). Yields of the lyophilized isolated products were: β-3,4-dihydrolyphenylethyl alcohol, 65 mg; aglycone, 14 mg; and elenolic acid, 15 mg. High-resolution mass spectra were obtained on each of these compounds.

The glucose content in the hydrolysate, assayed by paper chromatography (5) followed by enzymatic glucose analysis (11), was 27.4% of the weight of the oleuropein sample. This value is similar to that previously reported (5) but less than the theoretical value of 33%.

Macropreparation of compounds. Because direct acid hydrolysis of oleuropein gave very small amounts of elenolic acid and oleuropein aglycone, alternate methods were used to prepare quantities sufficient for microbiological studies.

Elenolic acid was prepared by methylating a crude oleuropein extract with anhydrous methanolic hydrogen chloride. The resulting methyl-*o*-methyl elenolate (W. L. C. Veer, U.S. Patent 3,033,877, 1962) was isolated and converted into the free acid by hydrolysis in dilute mineral acid.

Crude oleuropein (3.4 g dry weight) from an ethyl acetate extract of olives was dissolved in 10 ml of anhydrous methanolic HCl (5%), sealed in an ampoule under nitrogen, and heated at 65 C for 3.5 h. The methyl-*o*-methyl elenolate (1.06 g) was obtained from the reaction mixture by extraction with ethyl acetate, followed by a CCD separation using hexane-methanol, 1:1, as the solvent system. The oily material (1.01 g) was dissolved in 5 ml of ether and added dropwise to 300 ml of 1 N H_2SO_4, and the mixture was stirred at 70 to 80 C for 1.5 h. The solution was cooled and extracted with ethyl ether. The extract was washed several times to remove H_2SO_4, dried, and the solvent was evaporated, leaving 0.65 g of a colorless, oily material which TLC analysis indicated was primarily elenolic acid. This material was purified further by PLC and finally by CCD to give 0.150 g of pure elenolic acid.

Oleuropein aglycone was prepared by enzymatic hydrolysis of oleuropein by using β-glucosidase. A filter-sterilized 1% solution of pure oleuropein (500 mg) in 0.1 M sodium acetate buffer, pH 4.1, was mixed with one-ninth volume of filter-sterilized 2% β-glucosidase solution (Sigma Chemical Co.) and incubated for 16 h at 32 C. The solution then was extracted with chloroform. After removal of the solvent, the resulting purple oil was purified by PLC followed by CCD. The purified aglycone was a light-brown oil (78 mg).

Analyses. Mass spectra were obtained at the facilities of the Research Triangle Institute on an AEI-MS-902 instrument. Elemental analyses and molecular weights were determined by Galbraith Laboratories, Knoxville, Tenn. UV spectra were recorded with a Cary model 15 instrument, and optical rotations were recorded with a Perkins Elmer model 141 polarimeter.

CCD. CCD was performed with a 50-tube Post-Craig apparatus (H.O. Post Co., Middle Village, N.Y.). Solvents were reagent grade and redistilled prior to use. Water was distilled and deionized.

TLC. Preparation of TLC and PLC plates using Silica Gel HF_{254} and Silica Gel PF_{254}, respectively, was described previously, as was the procedure for developing the plates and detection of phenolic compounds (5). Compounds also were located on TLC plates by spraying with 50% H_2SO_4 and heating at 170 C for 30 min. PLC plates, after developing, were dried under nitrogen, compounds were visualized under UV light at 254 nm, and zones were collected (5).

Reference compounds. A sample of elenolic acid as the calcium salt was provided by the Upjohn Company. A sample of β-3,4-dihydroxyphenylethyl alcohol was prepared by the Research Triangle Institute, N.C.

Bitterness test. The bitterness of oleuropein and its aglycone was evaluated by a taste panel consisting of eight individuals. Whatman no. 1 filter paper was washed in ethyl alcohol, dried, and cut into 1-cm squares. The compounds were applied to the paper as ethyl alcohol solutions, and then the alcohol was removed by evaporation. Panelists were instructed to hold the paper squares on their tongues until they could detect bitterness or decide that no bitterness was present. They were given a blank square of paper first and then a series of papers containing increasing levels of the compounds. They were asked to discontinue the test at the first level where bitterness was detected and to describe any unusual characteristics of the bitterness.

RESULTS AND DISCUSSION

Purified oleuropein was obtained from green olives, portions were hydrolyzed, and the major fragments were isolated therefrom. Physical properties of the compounds isolated are summarized in Table 1. Oleuropein isolated by our procedure was essentially identical to that previously described (8), except that we observed a specific optical rotation value of −178°, whereas they reported −158°.

The differences might be explained by the higher purity of our preparation. For example,

TABLE 1. *Physical properties of oleuropein and its hydrolysis products*

Compound	Elemental[a] composition	Molecular[a] weight	UV absorption[b] maxima (nm)	Mass spectral peaks[c]	
				M^+[d]	Highest-molecular-weight fragments
Oleuropein	$C_{25}H_{32}O_{13}$	540.5	232 (ϵ = 14,135) 282 (ϵ = 2,973)		360 (0.7), 225 (0.6), 178 (1.4), 165 (3.5)
β-3,4-Dihydroxyphenylethyl alcohol	$C_8H_{10}O_3$	154.0626	220 (ϵ = 4,994) 283 (ϵ = 2,426)	*154* (19.7)	123 (57.5), 113 (2.4), 105 (2.0)
Elenolic acid	$C_{11}H_{14}O_6$	242.0790	239 (ϵ = 10,729)	*242* (0.8)	224 (1.7), 211 (1.1), 196 (9.0)
Oleuropein aglycone	$C_{19}H_{22}O_8$	378.1321	225 (ϵ = 12,237) 285 (ϵ = 2,176)	*378* (5.5)	346 (7.5), 243 (3.5), 211 (5.5)

[a] Molecular weight of oleuropein was determined by vapor pressure osmometry in tetrahydrofuran, and the elemental composition was determined by combustion. Molecular weights and elemental analyses of the other compounds were based on high-resolution mass spectral data.

[b] Determined in ethanol.

[c] Numbers in parentheses refer to percent relative abundance.

[d] The molecular ion peak, M^+, was not obtained for oleuropein.

when oleuropein was first described in 1908 (1), the specific rotation was −127°. In 1934 Cruess and Alsberg obtained a value of −145 to −148°. Each investigator since Bourquelot and Vintilesco (1) has used improved purification methods and each has reported higher specific optical rotation values.

Our study confirms those of Panizzi et al. (8) and Cruess and Alsberg (2), who reported that oleuropein is hydrolyzed by β-glucosidase. Shasha and Leibowitz (9) reported that the olive bitter principle is not attacked by the enzyme. The ability of β-glucosidase to produce the aglycone from oleuropein is of considerable importance in olive fermentation due to the inhibitory nature of this moiety (6).

A molecular weight of 540 for oleuropein was obtained by vapor pressure osmometry. This value agrees with that reported by others (8). The mass spectrum did not exhibit any fragments above 360 mass units, probably because of the nonvolatility of the oleuropein molecule. Some of the higher-molecular-weight fragments are given in Table 1.

The mass spectrum for elenolic acid, whether from direct hydrolysis of oleuropein or from hydrolysis of methyl-*o*-methyl elenolate, was identical to the authentic reference compound. In addition, the mass spectrum of β-3,4-dihydroxyphenylethyl alcohol, from hydrolysis of oleuropein, was identical to that of the reference material. Some of the physical data are supplied in Table 1.

Panizzi et al. (8) reported the formation of oleuropein aglycone on the basis of paper chromatographic examination of the products resulting from treatment of oleuropein with β-glucosidase. No attempt was made by these workers to isolate or study the aglycone further. The structure given in Fig. 1, therefore, is tentative. Our preparation of the aglycone was subjected to several purification steps and is chromatographically pure (TLC). By the use of high-resolution mass spectrometry, we obtained an elemental composition of $C_{19}H_{22}O_8$ (molecular weight 378.132) for this compound, which corresponds to the product expected when glucose is hydrolytically cleaved from oleuropein. The highest-molecular-weight fragments and UV absorbance maxima are provided in Table 1.

The structures given in Fig. 1 are those of Panizzi et al. (8) and other workers (W. L. C. Veer, U.S. Patent 3,033,877, 1962; reference 10). A recent paper, however, proposed a slightly modified structure for elenolic acid (7). The tentative structures are provided as an aid in observing the origin of oleuropein hydrolysis products.

Oleuropein and its aglycone were bitter, the threshold levels for detection being about 50 μg for most of the panelists. Two individuals did not detect bitterness at a level of 200 μg per paper square, which was the highest level tested. Although the threshold levels for detection of bitterness of both compounds were similar, some panelists described the taste of the aglycone as having a stinging, biting, or sharp sensation associated with the bitterness. Neither elenolic acid nor β-3,4-dihydroxyphenylethyl alcohol was bitter at levels up to 200 μg.

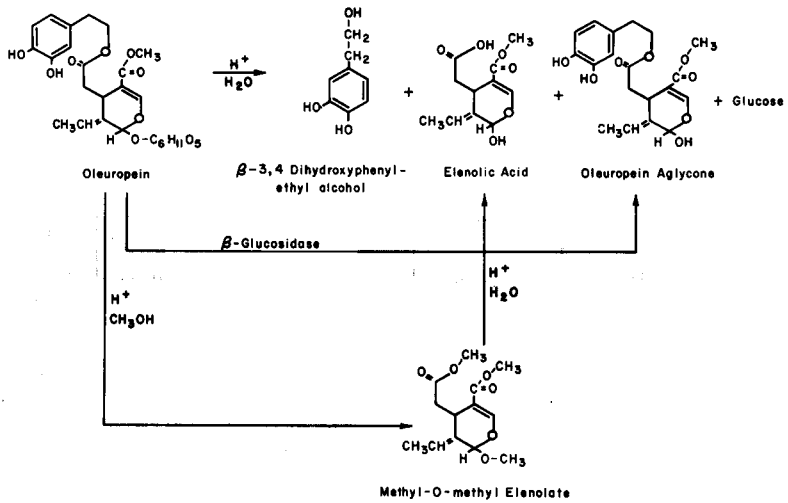

FIG. 1. *Structures of oleuropein and its hydrolysis products as proposed by Panizzi et al. (8). The structure for the aglycone was not reported by these authors but was assumed from the structure of oleuropein. The names, elenolic acid and methyl-o-methyl elenolate, were applied by Veer (W. L. C. Veer, U.S. Patent 3,033,877, 1962; reference 10).*

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