

# Isolation of Megaritolactones and Other Bioactive Metabolites from ‘Megaritiki’ Table Olives and Debitting Water

Evgenia Mousouri, Eleni Melliou, and Prokopios Magiatis\*

Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimiopolis Zografou, GR-15771 Athens, Greece

**S** Supporting Information

**ABSTRACT:** ‘Megaritiki’ is an olive cultivar widely used in Greece for the production of low polyphenol olive oil and table olives. To investigate possible metabolic differentiation in comparison with other varieties, the composition of ‘Megaritiki’ olive fruits and wastewaters from the debittering procedure was studied. Moreover, the recovery of bioactive metabolites from wastewater using adsorption resin was studied to exploit this byproduct. Metabolites in fruits and wastewaters were monitored using NMR spectroscopy. The major constituents of wastewater were hydroxytyrosol-4-*O*-glucoside, 11-methyl-oleoside, hydroxytyrosol, and tyrosol but not oleuropein. Furthermore, wastewater afforded rengyoxide and rengyoside B, which are for the first time isolated from olives. The final edible olives, besides hydroxytyrosol and tyrosol, contained rengyoxide and cleroidicin C, which are the first isolated from the species, haleridone for the first time isolated from edible olives, and four metabolites, which are the first reported as natural products, megaritodilactone, megaritolactonic acid, methyl ester of megaritolactonic acid B, and megaritolactonol.

**KEYWORDS:** *Olea europaea*, ‘Megaritiki’, table olives, iridoid, qNMR

## ■ INTRODUCTION

Table olives are used as a typical part of the Mediterranean diet since antiquity. They contain a large number of minor constituents<sup>1</sup> with interesting bioactivities, which are mainly dependent on the olive variety and the followed debittering process.<sup>2,3</sup> In general, olive fruits undergo a debittering procedure that removes totally or partially the natural bitterness that is mainly due to oleuropein, **1**. A few years ago, we performed a screening of the major table olive varieties found in the Greek market, and we identified varieties with high oleuropein, **1**, and/or hydroxytyrosol, **2**, content in the final edible product.<sup>3</sup> Both compounds have important antioxidant activities, and they have been correlated with protection from LDL oxidation.<sup>4</sup> The role of hydroxytyrosol and oleuropein derivatives has been recently recognized by the European Union for olive oil but not yet for edible olives. A significant observation<sup>3</sup> concerning the olive debittering method was that the use of dry salt and not brine can lead to table olives with high oleuropein content and potentially increased health protecting properties. However, one of the varieties that had been included in the previous study and that had been treated with dry salt without giving high oleuropein content was cv. ‘Megaritiki’. This fact had been initially attributed to a possible metabolic differentiation of that specific variety without further study.

Meanwhile, we studied extensively the olive oil produced by the major olive oil producing varieties in Greece, and again we found that the olive oil from cv. ‘Megaritiki’ presented very low concentration of secoiridoid derivatives (oleocanthal, oleacein, oleuropein aglycone, and ligstroside aglycone).<sup>5</sup> This specific variety is widely cultivated in regions like Attica for the production of both table olives and olive oil (dual use variety). The olive oil of that cultivar is known in the market as a low

bitterness oil, which is in accordance with the low recorded content of the usual secoiridoid derivatives responsible for pungency and bitterness. Moreover, the table olives from that variety belong to a group of cultivars that require traditionally little processing to debitter, indicating that oleuropein levels in the untreated fruit of this variety are lower comparatively to others. A simple method, known since antiquity for the debittering of this variety, is crushing and placement for a few days in water. The olives produced with this method are traditionally known as “klastades”.

All of the above observations led us to investigate that specific variety for possible metabolic differentiation. For this purpose, we studied the initial metabolic profile of the untreated olive fruits for the presence of oleuropein, and in a next step we studied the major compounds recovered from the debittering wastewater, and finally we studied the chemical constituents of the final edible product (Figure 1).

## ■ MATERIALS AND METHODS

**General Experimental Procedures.** NMR spectra were recorded on Avance 600 (with cryoprobe) and DRX400 spectrometers (Bruker, Rheinstetten, Germany); chemical shifts are expressed in ppm downfield from TMS. Column chromatography was performed on columns containing Si gel 60 (40–63 μm) (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on plates coated with Si gel 60 F<sub>254</sub> Merck, 0.25 mm. For HPLC–MS analysis, the column used was a 150 mm × 2.0 mm i.d., 5 μm, PolymerX RP-1 (Phenomenex, Torrance, CA). Samples (5 μL) were dissolved in methanol and injected onto HPLC–MS. A standard

**Received:** October 17, 2013

**Revised:** December 30, 2013

**Accepted:** January 2, 2014

**Published:** January 2, 2014

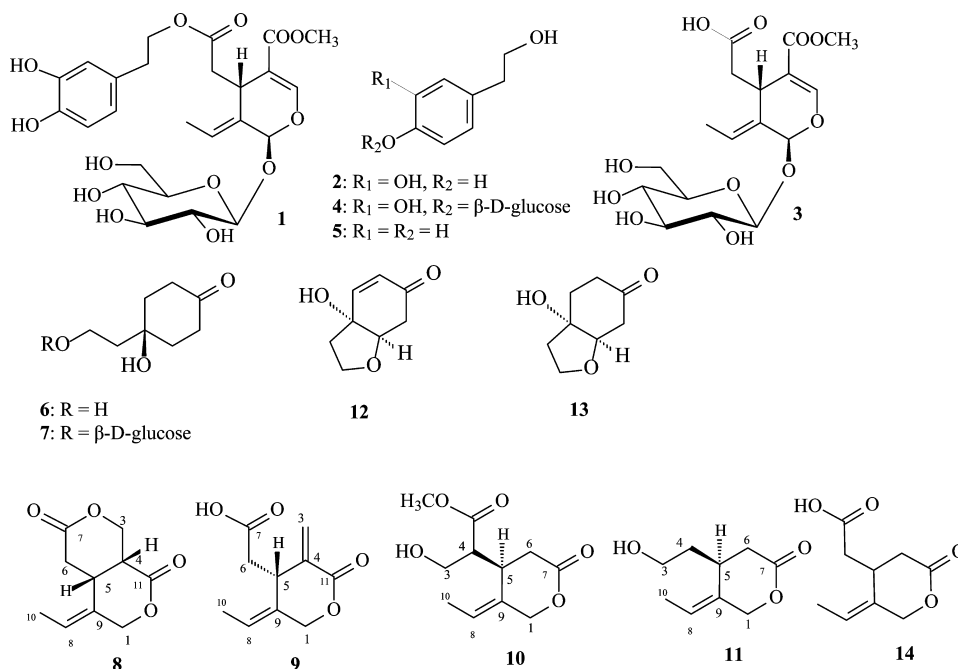


Figure 1. Structures of compounds 1–14.

reverse phase linear gradient with acidified water (0.1% formic acid) and acetonitrile was run over 30 min at a flow rate of 250  $\mu\text{L}/\text{min}$ , and the eluent was monitored for negative anions by a LTQ Orbitrap (Thermo Fisher Scientific, Waltham, MA) operated in the centroid mode. Source parameters were 5.5 kV spray voltage, capillary temperature of 275  $^{\circ}\text{C}$ , and nitrogen sheath gas setting of 20 mL/min. Data were acquired at a resolution setting of 60 000 fwhm with the lockmass feature, which typically results in a mass accuracy <2 ppm.

**Preparation of Table Olives.** Olives of the 'Megaritiki' cultivar used in this study were collected in August, October, and November 2011 from Salamina island. The olives collected in November were water-cured according to a traditional method of debittering as follows: olive fruits (3 kg) were soaked in water (3 L) for 1 day, and the second day each olive was removed, incised, and subsequently added to water (3 L). The water was replaced daily for 5 days. At the end of the sixth day, water-cured olives were placed in brine (salt solution of 10% NaCl) and stored until further analysis. Debittering wastewaters were stored separately for each day at  $-20^{\circ}\text{C}$  until analysis.

**Sample Preparation for NMR Analysis.** The fruits were collected and analyzed in the same day. The fruits were first washed with water, and then the stone and the flesh were separated and mashed using a laboratory blender. The olive flesh (10 g) was extracted with 25 mL of a MeOH/ $\text{H}_2\text{O}$  mixture (4:1) in a supersonic bath for 45 min. The supernatant was separated from the flesh by centrifugation at 3400g for 10 min. Next, 25 mL of hexane was added for oil removal, agitated for 30 s, and then the methanol extract was separated by subsequent centrifugation at 3400g for 3 min. The volume of the methanol phase was measured, and 1/20 of the extract was used for NMR analysis. The appropriate volume of methanol extract was mixed with 0.5 mL of the internal standard solution (0.5 mg of syringaldehyde/mL in acetonitrile) and evaporated under reduced pressure at 40  $^{\circ}\text{C}$ .

**Quantitative NMR Analysis of Oleuropein Content.** The dry extract was dissolved in 600  $\mu\text{L}$  of  $\text{CD}_3\text{OD}$ .  $^1\text{H}$  NMR spectra were recorded at 400 MHz. 32 scans were collected, and the spectra were phased corrected and integrated automatically using TopSpin software (Bruker). The quantitation was based on the integration ratio between the aldehydic proton signal of syringaldehyde at 9.75 ppm and the proton of oleuropein appearing at 5.91 ppm. Calibration curve of oleuropein was prepared at seven different concentrations ranging

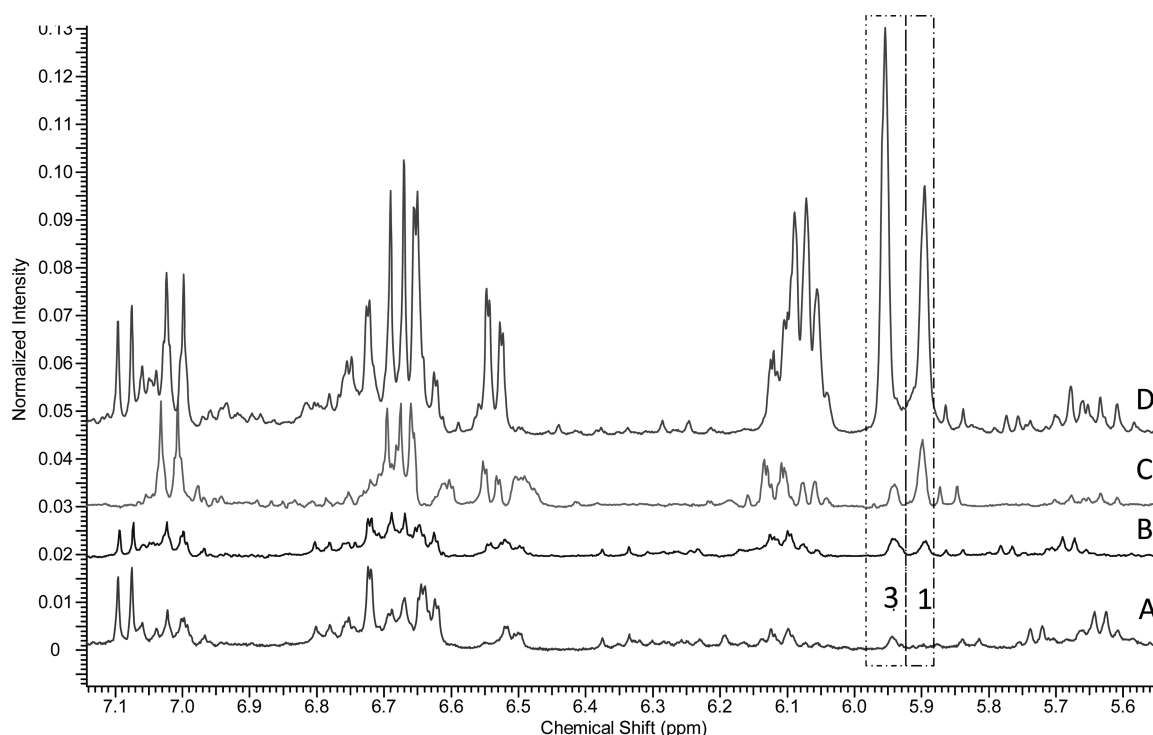
between 70 and 4500  $\mu\text{g}$  in tube. The solutions for the construction of the calibration curve were prepared by mixing appropriate volumes of a stock solution of pure oleuropein (Extrasynthese, Genay, France) (1 mg/mL in MeOH) with 0.5 mL of the internal standard solution (0.5 mg of syringaldehyde/mL in acetonitrile) and evaporation under reduced pressure at 40  $^{\circ}\text{C}$ . The equation used for the quantitation of oleuropein in tube was  $y = 0.512x + 0.0904$ , with  $r^2 = 0.995$ .

**Study of the Debittering Wastewaters.** *Treatment with Resins.* After filtration through filter paper, wastewater from each day was separately passed through a column containing adsorption resin. A column of 3.5 cm diameter filled with 250 g of Amberlite XAD-4 resin (Rohm and Haas, Philadelphia, PA) (BV = 375 mL), was used. The feeding flow rate was 750 mL/h (2 BV/h). The resin total feeding duration was 2 h. The procedure above refers to 1.5 L of wastewater. After the adsorption, the subsequent regenerating procedure consisted of three steps. The first one included resin wash by water until the water effluent was colorless. Afterward, the resin was regenerated by eluting the metabolites with methanol (500 mL). The methanol extract was evaporated to dryness and was used for subsequent analysis. Finally, the resin was washed again by water (500 mL). After these three steps, the resin was ready to be used again.

*Monitoring.* The debittering procedure was monitored using thin layer chromatography (TLC) and  $^1\text{H}$  NMR spectroscopy. More specifically, 20 mg of the dry methanol extract from each day of the debittering procedure was dissolved in 600  $\mu\text{L}$  of  $\text{CD}_3\text{OD}$ , and  $^1\text{H}$  NMR spectra were recorded at 400 MHz. The appearance, increase, or decrease from day to day of characteristic peaks corresponding to 11-methyl oleoside, 3 (5.95 ppm), hydroxytyrosol-4- $O$ - $\beta$ -D-glucoside, 4 (7.09 ppm), hydroxytyrosol, 2 (6.53 ppm), and tyrosol, 5 (7.02 ppm), were monitored.

*Isolation.* The extracts were combined, and low pressure column chromatography was performed on them leading to the isolation of five compounds. Three of them were already known, and the other two were for the first time isolated from olives. The structures of the already known compounds were identified using NMR spectroscopy, and the structure elucidation of the other two compounds was achieved both with NMR spectroscopy and with MS spectrometry.

A part (2.30 g) of the total extract (7.78 g) was placed into a 5 cm diameter column filled with silica gel 60 Merck (40–63  $\mu\text{m}$ ) up to 20 cm height. The eluent flowed through the column under low pressure. The column was eluted collecting fractions of 20 mL according to the following gradient: 1% (25 fractions), 2% (55 fractions), 3% (57



**Figure 2.** Monitoring of oleuropein in unprocessed olives of cv. 'Megaritiki' by quantitative  $^1\text{H}$  NMR. (A) 'Megaritiki', November; (B) 'Megaritiki', October; (C) 'Megaritiki', August; (D) 'Throuba Thassos', November. 1, oleuropein; 3, 11-methyl oleoside. Spectra are normalized according to the contained internal standard peak (not shown).

fractions), 4% (19 fractions), 5% (21 fractions), 6% (29 fractions), 7% (26 fractions), 8% (63 fractions), 10% (45 fractions), 20% (20 fractions), and 50% (25 fractions) MeOH in  $\text{CH}_2\text{Cl}_2$ . The obtained fractions were pooled using TLC (migration solvent 90:10  $\text{CH}_2\text{Cl}_2$ :MeOH) to give 32 final fractions. Fractions 8 and 9–14 afforded rengoxyde, **6**<sup>6</sup> (29.9 mg), and hydroxytyrosol, **2**<sup>7</sup> (43.6 mg), respectively. Fraction 19 was rechromatographed on a preparative TLC plate Merck (migration system 80:20  $\text{CH}_2\text{Cl}_2$ /MeOH) to give rengoside B, **7**<sup>8</sup> (6.8 mg), hydroxytyrosol-4-*O*- $\beta$ -D-glucoside, **4**<sup>9</sup> (12.4 mg), and 11-methyl oleoside, **3** (14 mg).<sup>10</sup> Other fractions containing the above compounds were extensively rechromatographed leading to a total yield of hydroxytyrosol (30 mg/g of total extract), hydroxytyrosol-4-*O*- $\beta$ -D-glucoside (82 mg/g), and 11-methyl oleoside (25 mg/g).

**Study of the Olive Flesh. Extraction and Resin Treatment.** Six months after the brine addition, 1 kg of the debittered olives was extracted at room temperature with water (2  $\times$  0.5 L) in a supersonic bath for 1 h. The water extract (1 L) was treated in the same way as the wastewater above to lead to an enriched extract. It was passed through a column of 3.5 cm diameter filled with 250 g of XAD-4 resin (BV = 375 mL). The feeding flow rate was 750 mL/h (2 BV/h). The resin total feeding duration was 1.3 h. The procedure above refers to 1.0 L of wastewater. The resin regeneration procedure was exactly the same as the corresponding for the case of the wastewater treatment described above. The MeOH eluent was evaporated under reduced pressure at 40  $^\circ\text{C}$ .

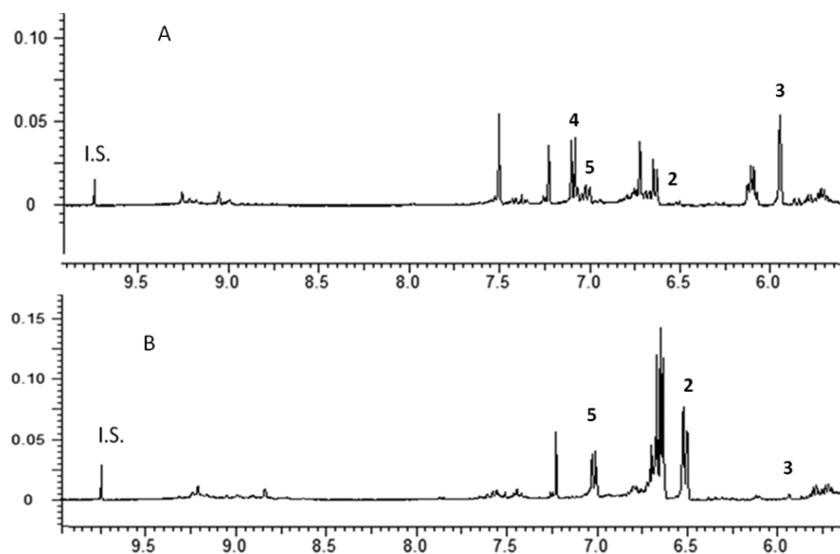
**Isolation.** The enriched extract of the olive flesh was fractionated with low pressure column chromatography, and this separation procedure led to the identification of nine compounds. Two of the isolated compounds were already known, three were for the first time isolated from table olives, and the other four are for the first time reported as natural compounds. The dry MeOH extract (1.98 g) was chromatographed under low pressure on a column similar to that above filled with silica gel 60 Merck (40–63  $\mu\text{m}$ ) up to 20 cm height. The volume of the collected fractions was 20 mL, and the solvent gradient was 1% (40 fractions), 2% (66 fractions), 3% (28 fractions), 4% (60 fractions), 5% (15 fractions), 6% (25 fractions), 7% (30

fractions), 8% (13 fractions), 10% (25 fractions), 20% (25 fractions), and 50% (25 fractions) MeOH in  $\text{CH}_2\text{Cl}_2$ . The obtained fractions were merged using TLC (migration solvent 90:10  $\text{CH}_2\text{Cl}_2$ /MeOH) to afford 27 final fractions. From fractions 4, 5, 6, and 8 were isolated the four new compounds, megaritodilactone, **8** (1.2 mg), megaritolactonic acid, **9** (2 mg), megaritolactonic acid B methyl ester, **10** (3 mg), and megaritolactonol, **11** (3 mg) respectively, after rechromatography on preparative TLC (migration systems 10:50:40  $\text{CH}_2\text{Cl}_2$ /hexane/EtOAc, 80:20 EtOAc/cyclohexane, 96.5:3.5  $\text{CH}_2\text{Cl}_2$ /MeOH, and 96:4  $\text{CH}_2\text{Cl}_2$ /MeOH, respectively). Fraction 12 was purified using preparative TLC to give tyrosol, **5**<sup>7</sup> (30 mg), and two compounds first isolated from olives: halleridone (rengyolone), **12**<sup>6</sup> (9.7 mg), and cleroidicin C, **13**<sup>11</sup> (16.1 mg). Finally, fraction 15 was rechromatographed on silica gel 60 Merck (40–63  $\mu\text{m}$ ) with  $\text{CH}_2\text{Cl}_2$ /MeOH (from 100:0 to 95:5 gradient) leading to the isolation of hydroxytyrosol, **2** (119 mg), and rengoxyde, **6** (78.4 mg).

**Megaritodilactone, 8.** White amorphous solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.74 (3H, d,  $J$  = 7.2 Hz,  $\text{CH}_3$ -10), 2.29 (1H, dd,  $J$  = 16.9, 12.1 Hz, H-6b), 2.84 (1H, dd,  $J$  = 16.9, 5.7 Hz, H-6a), 3.11 (1H, m, H-4), 3.51 (1H, dt,  $J$  = 11.8, 6.4 Hz, H-5), 4.46 (1H, dd,  $J$  = 11.8, 4.6 Hz, H-3b), 4.77 (1H, d,  $J$  = 13.4 Hz, H-1b), 4.80 (1H, d,  $J$  = 13.4 Hz, H-1a), 4.94 (1H, dd,  $J$  = 11.8, 3.1 Hz, H-3a), 5.71 (1H, q,  $J$  = 7.2 Hz, H-8).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 13.3 (C-10), 29.9 (C-5), 32.8 (C-6), 37.6 (C-4), 66.64 (C-3), 71.04 (C-1), 125.17 (C-8), 130.1 (C-9), 167.9 (C-7), 169.7 (C-11). HR-ESI-MS:  $m/z$  195.0716 [ $\text{M} - \text{H}$ ] $^-$ , [ $\text{C}_{10}\text{H}_{12}\text{O}_4 - \text{H}$ ] $^-$ , calcd 195.0657.

**Megaritolactonic Acid, 9.** White amorphous solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.75 (3H, dd,  $J$  = 7.0, 1.5 Hz,  $\text{CH}_3$ -10), 2.65 (1H, dd,  $J$  = 15.6, 7.3 Hz, H-6a), 2.70 (1H, dd,  $J$  = 15.6, 7.3 Hz, H-6b), 4.12 (1H, t,  $J$  = 7.6 Hz, H-5), 4.58 (1H, d,  $J$  = 12.9 Hz, H-1b), 4.97 (1H, dt,  $J$  = 12.9, 1.5 Hz, H-1a), 5.715 (1H, q,  $J$  = 6.8 Hz, H-8), 5.72 (1H, s, H-3b), 6.41 (1H, s, H-3a).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 12.8 ( $\text{CH}_3$ -10), 36.3 (C-5), 38.1 (C-6), 71.5 (C-1), 125.5 (C-8), 128.8 (C-3), 129.1 (C-9), 136.3 (C-4), 165.0 (C-11), 173.3 (C-7). HR-ESI-MS:  $m/z$  195.0702 [ $\text{M} - \text{H}$ ] $^-$ , [ $\text{C}_{10}\text{H}_{12}\text{O}_4 - \text{H}$ ] $^-$ , calcd 195.0657.

**Megaritolactonic Acid B Methyl Ester, 10.** White amorphous solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.75 (3H, dd,  $J$  = 6.9, 1.4 Hz,  $\text{CH}_3$ -10), 2.67



**Figure 3.** Monitoring of debittering evolution with  $^1\text{H}$  NMR. 2, hydroxytyrosol; 3, 11-methyl oleoside; 4, hydroxytyrosol-4-*O*-glucoside; 5, tyrosol. (A) First day. (B) Sixth day.

(1H, dd,  $J = 16.4, 7.0$  Hz, H-6a), 2.77 (1H, dd,  $J = 16.4, 6.7$  Hz, H-6b), 2.90 (1H, q,  $J = 6.6$  Hz, H-4), 3.32 (1H, q,  $J = 7.2$  Hz, H-5), 3.73 (1H, t,  $J = 7.8$  Hz, H-3a), 3.75 (3H, s,  $\text{CH}_3\text{O-12}$ ), 3.80 (1H, t,  $J = 5.3$  Hz, H-3b), 4.51 (1H, d,  $J = 12.5$  Hz, H-1a), 4.82 (1H, d,  $J = 12.5$  Hz, H-1b), 5.78 (1H, q,  $J = 6.7$  Hz, H-8).  $^1\text{H}$  NMR (acetone- $d_6$ )  $\delta$ : 1.73 (3H, d,  $J = 7.7$  Hz,  $\text{CH}_3-10$ ), 2.61 (1H, dd,  $J = 15.9/7.2$  Hz, H-6a), 2.82 (1H, dd,  $J = 15.9, 6.7$  Hz, H-6b), 2.99 (1H, q,  $J = 6.9$  Hz, H-4), 3.32 (1H, q,  $J = 6.9$  Hz, H-5), 3.67 (3H, s,  $\text{CH}_3\text{O-12}$ ), 3.72 (1H, dd,  $J = 10.5, 6.4$  Hz, H-3a), 3.79 (1H, dd,  $J = 10.5, 7.5$  Hz, H-3b), 4.44 (1H, d,  $J = 12.3$  Hz, H-1a), 4.82 (1H, d,  $J = 12.8$  Hz, H-1b), 5.78 (1H, q,  $J = 7.0$  Hz, H-8).  $^{13}\text{C}$  NMR (acetone- $d_6$ )  $\delta$ : 12.7 (C-10), 31.2 (C-5), 32.1 (C-6), 50.1 (C-4), 50.9 (C-12), 60.6 (C-3), 71.8 (C-1), 126.9 (C-8), 133.4 (C-9), 171.5 (C-7), 173.7 (C-11). HR-ESI-MS:  $m/z$  227.0915 [ $\text{M} - \text{H}$ ] $^-$ , [ $\text{C}_{11}\text{H}_{15}\text{O}_5 - \text{H}$ ] $^-$ , calcd 227.0919.

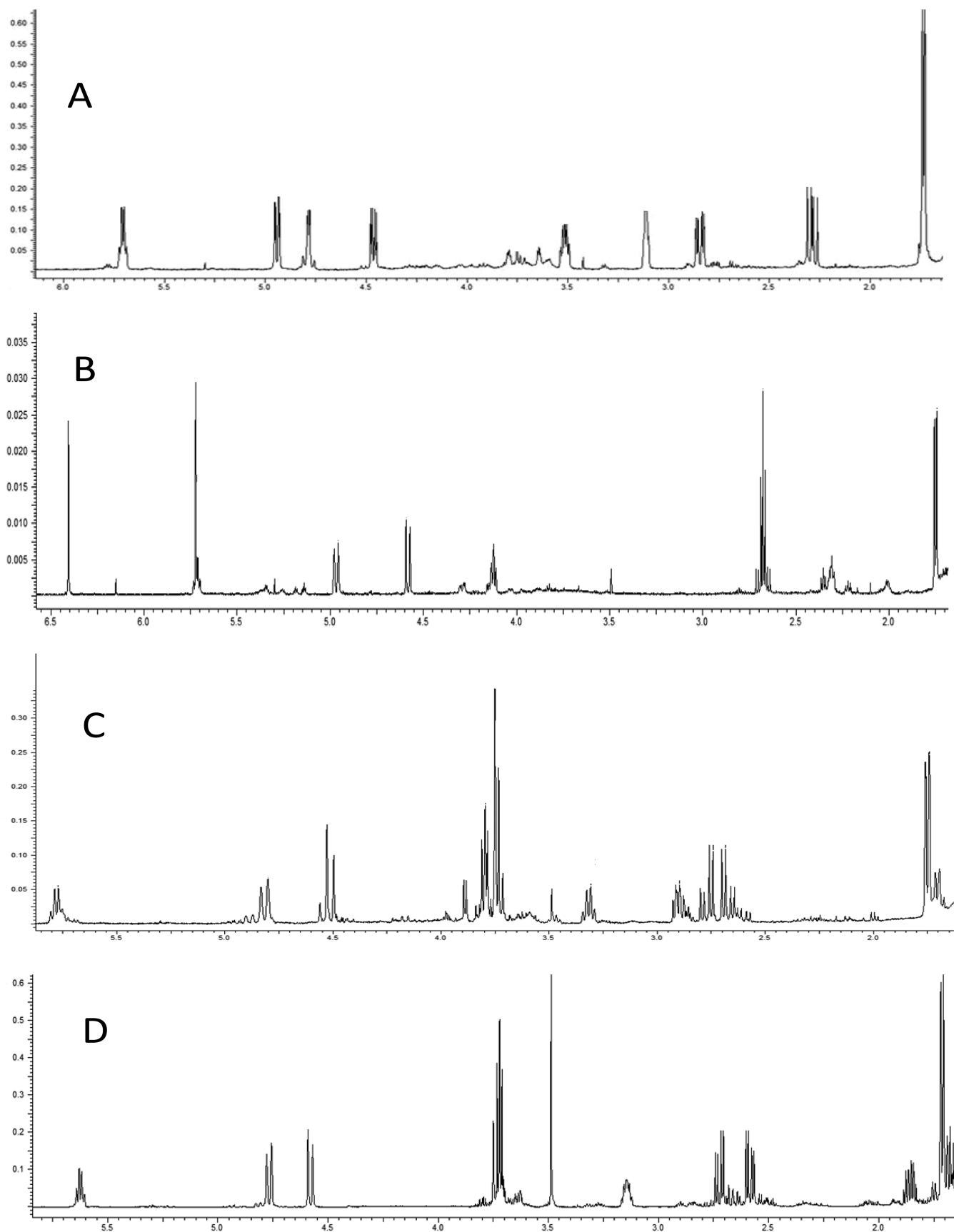
**Megaritolactonol, 11.** White amorphous solid,  $[\alpha]_D^{20} +8^\circ$  (c 0.15, MeOH).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.66 (1H, m, H-4a), 1.74 (3H, d,  $J = 6.8$  Hz, H-10), 1.86 (1H, m, H-4b), 2.58 (1H, dd,  $J = 15.9, 5.9$  Hz, H-6a), 2.72 (1H, dd,  $J = 15.9, 6.5$  Hz, H-6b), 3.14 (1H, m, H-5), 3.72 (2H, t,  $J = 6.3$  Hz, H-3), 4.58 (1H, d,  $J = 12.9$  Hz, H-1a), 4.77 (1H, d,  $J = 12.9$  Hz, H-1b), 5.62 (1H, q,  $J = 7.0$  Hz, H-8).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 13.6 (C-10), 29.6 (C-5), 35.5 (C-6), 37.1 (C-4), 60.1 (C-3), 72.2 (C-1), 124.2 (C-8), 133.4 (C-9), 172.5 (C-7). HR-ESI-MS:  $m/z$  169.0870 [ $\text{M} - \text{H}$ ] $^-$ , [ $\text{C}_9\text{H}_{14}\text{O}_3 - \text{H}$ ] $^-$ , calcd 169.0864.

## RESULTS AND DISCUSSION

**Oleuropein Monitoring in Untreated Olives.** The initial target was to monitor the presence of oleuropein, **1**, in the untreated olive fruit of cv. ‘Megaritikiki’. For this purpose, a simple method for the measurement of oleuropein was developed using quantitative  $^1\text{H}$  NMR. Using the integration ratio between the internal standard and the characteristic peak of oleuropein at 5.91 ppm, we constructed a calibration curve, which was used to measure quantitatively the levels of oleuropein in the olive fruit. The method was applied in the untreated fruits of cv. ‘Megaritikiki’ at three different stages of ripeness as well as in cv. ‘Throuba Thassos’ well-known<sup>3</sup> for producing oleuropein at harvest time. As it is presented in Figure 2, the peak of oleuropein in the case of cv. ‘Megaritikiki’ at usual harvest time (mid November, half green-half violet color) was almost absent corresponding to a concentration lower than 0.15 mg/g. In contrast, cv. ‘Throuba Thassos’ at the same harvest time and maturity stage presented significantly higher concentration of 9.1 mg/g, which is comparable with the

usually measured levels of oleuropein in untreated olives.<sup>12</sup> When the cv. ‘Megaritikiki’ olives were studied in August (fully unripe), oleuropein was present in higher levels (3.2 mg/g) than in November as expected, showing that the variety has the capability to biosynthesize oleuropein. In early October, the oleuropein level had been already reduced to 0.55 mg/g, showing a gradual but early oleuropein catabolism. In contrast with oleuropein, the peak of 11-methyl oleoside, **3**, was higher at harvest time than that of oleuropein showing that in the case of cv. ‘Megaritikiki’, the hydrolysis of oleuropein is activated much earlier than in other varieties, leading to a fruit that at harvest time has very low content of oleuropein. The peak of methyl oleoside at 5.95 ppm was clearly distinguished from that of oleuropein and was used for its monitoring. Several other characteristic compounds (such as hydroxytyrosol, **2**, hydroxytyrosol-4-*O*-glucoside, **4**, tyrosol, **5**, maslinic acid, etc.) could be simultaneously quantitated by combination of 1D and 2D qNMR, but this is a subject of a separate work. The characteristic peak for the measurement of oleuropein was not overlapping with signals from other major constituents as confirmed by extensive 2D NMR experiments. The observation of the very low content in untreated ‘Megaritikiki’ olives offers an explanation why the debittered olives with dry salt did not contain oleuropein in contrast with cv. ‘Throuba Thassos’, which after debittering with the same method contained high amounts of oleuropein.<sup>3</sup> Moreover, this finding shows that specific cultivars like ‘Megaritikiki’ present technological advantages concerning the removal of bitterness because oleuropein is already naturally hydrolyzed and the main bitter compound needing to be removed is 11-methyl oleoside. This compound is very soluble in water and can be easily dissolved out of the olive flesh simply by contact with water as explained below. A similar case of an olive cultivar in which the oleuropein level is very low at harvest time due to earlier activation of glucosidase and esterase has also been reported in cv. Dhokar from Tunisia,<sup>12</sup> but most probably there are also many other cultivars with similar behavior.

**Study of the Debittering Process with Water.** To study the evolution of the debittering process, table olives were prepared, according to a traditional water-curing method for 6 days, and the composition of the wastewater resulting from the



**Figure 4.**  $^1\text{H}$  NMR spectra of compounds 8–11. (A) 8, (B) 9, (C) 10, (D) 11.

debittering procedure as well as that of the final edible olive fruits was determined. To facilitate this study, we applied a

method for the recovery of metabolites from wastewater and then we monitored them using NMR spectroscopy. More

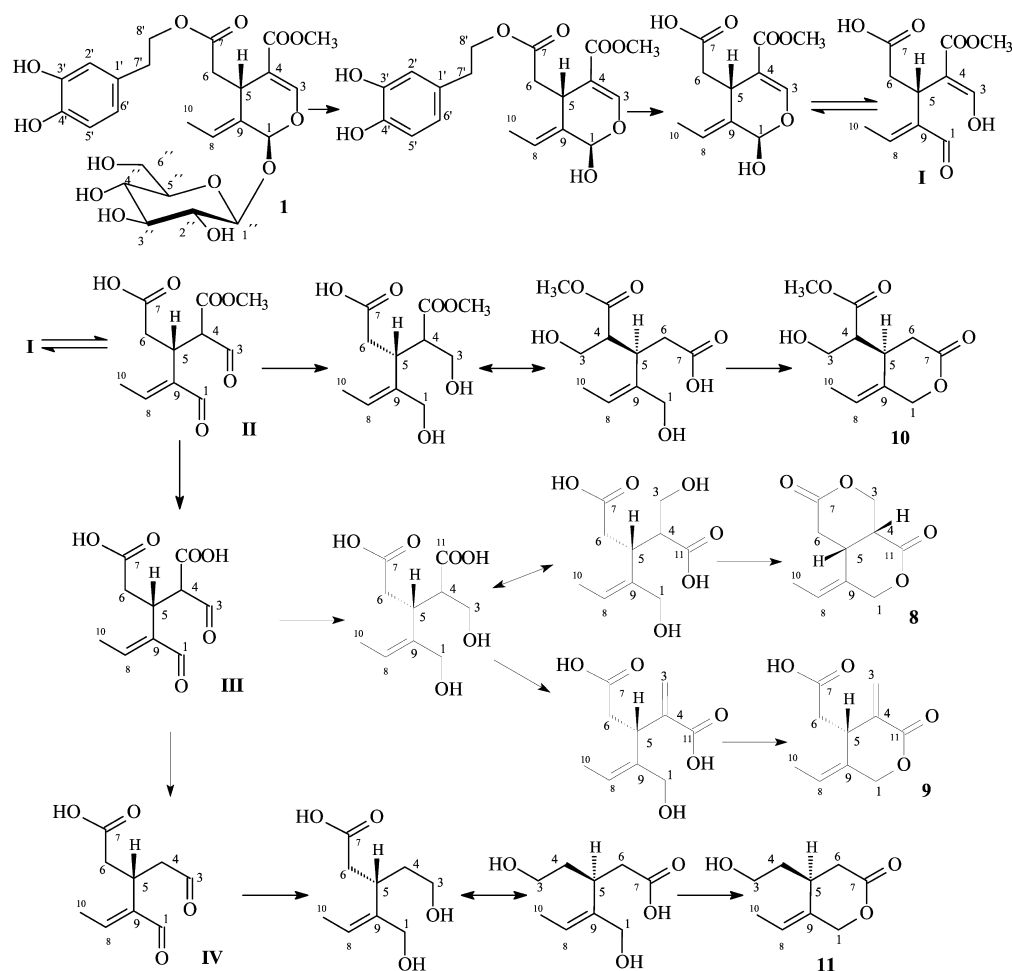


Figure 5. Proposed biosynthetic pathway of compounds 8–11.

specifically, we used a method similar to that developed a few years ago for the recovery of bioactive compounds from the olive mill wastewater.<sup>13</sup> In the current case, the debittering water was treated with adsorption resin XAD-4, and the adsorbed compounds were desorbed using a polar solvent such as methanol. The methanol extract was analyzed by TLC chromatography and NMR. This study showed that the major components of this specific wastewater are the bioactive metabolites: hydroxytyrosol, 2, 11-methyl oleoside, 3, hydroxytyrosol-4-*O*-glucoside, 4, and tyrosol, 5, but not oleuropein, 1. It is very interesting that the diffusion of each compound from the flesh to the debittering water was very different (Figure 3). Characteristically, 11-methyl oleoside was almost completely removed after the first two days, while hydroxytyrosol started to appear only after the third day. Additionally, the chemical analysis of the wastewater afforded two metabolites, rengyoxide, 6, and rengyoside B, 7, which are for the first time isolated from olives. Rengyoxide has been previously isolated from plants of the families Oleaceae (*Forsythia suspensa*),<sup>6</sup> Bignoniaceae,<sup>14</sup> Verbenaceae (*Clerodendrum indicum*),<sup>11</sup> Scrophulariaceae,<sup>15</sup> and Plantaginaceae.<sup>16</sup> Rengyoside B has been previously isolated from several Bignoniaceae (e.g., *Markhamia stipulata*)<sup>17</sup> and from *Forsythia suspensa* (Oleaceae).<sup>8</sup>

**Study of Metabolites Identified in the Final Treated Product.** The early hydrolysis of oleuropein in cv. 'Megaritiki' led us to assume that the olive flesh should be rich in compounds deriving from the breakdown of the oleuropein

secoiridoid skeleton. In particular, the less hydrophilic derivatives should stay in the flesh until the stage of the final edible product. Indeed, the water extract of the final olive flesh after enrichment with adsorption on XAD4 resin and several chromatographic purification steps led to the isolation of: two metabolites that are for the first time isolated from this species, rengyoxide, 6, and cleroindicin C, 13, one that is for the first time isolated from the edible fruit (rengyolone, 12) and four new natural products. The new natural products are all lactone derivatives coming from the skeleton of oleuropein after hydrolysis of the hydroxytyrosol moiety, cleavage of the glycosidic bond, removal or not of the carbomethoxy group, and subsequent reactions of reduction and/or dehydration and final lactonization. Their common characteristics prompted us to name them as megaritolactones. Currently, it is not known if they are common or not in other edible olive varieties.

**Structure Elucidation of New Compounds.** Megaritolactonol, 11, was isolated as an amorphous solid with molecular formula corresponding to C<sub>9</sub>H<sub>14</sub>O<sub>3</sub>. The <sup>1</sup>H NMR profile (Figure 4A) was very closely related to that of (5-ethylidene-2-oxo-tetrahydropyran-4-yl) acetic acid, 14, which had been described as a semisynthetic derivative<sup>18</sup> and isolated<sup>13</sup> in significant quantities from olive mill wastewater as a breakdown product of the secoiridoid skeleton of oleuropein after hydrolysis of the glycosidic bond and removal of the hydroxytyrosol moiety. The most characteristic signal revealing the similarity with lactone 14 is the methyl group observed as a

doublet at 1.74 ppm, which is attached to the exocyclic double bond observed as a quadruplet at 5.62 ppm. Moreover, two pairs of geminal protons H-1a,b and H-6a,b at 4.77/4.58 and 2.72/2.58, respectively, and one methine at 3.14 ppm (H-5) comprised the six-membered lactone ring, similar to lactone **14**. The presence of the lactone was confirmed by the carbonyl observed at 172.5 ppm. The main difference from lactone **14** is the side chain attached at position 5. While in the case of lactone **14** the side chain was ethanoic acid, in the case of compound **11** the side chain consisted of one oxygenated methylene at 3.72 ppm and one aliphatic methylene observed as two multiplets at 1.65 and 1.86 ppm. Sequential COSY correlations between H-6a,b/H-5, H-5/H-4a,b, H-4a,b/H-3 confirmed the positioning and the structure of the side chain. The structure was confirmed by the HMBC spectrum where the most important correlations were between H-1a,b and carbonyl C-7 and between H-5 with both the double bond carbon C-8 and the carbonyl C-7. The NOE correlation between H-8 and H-1 revealed the orientation of the methyl group of the double bond as depicted in Figure 1. On the basis of the above description, it was clear the compound **11** was a  $\delta$ -lactone with one ethylidene and one ethanol side chain for which we propose the name megaritolactonol. The absolute stereochemistry was deduced from the biosynthetic pathway (Figure 5).

Compound **10** was isolated as an amorphous solid with molecular formula  $C_{11}H_{16}O_5$ . The  $^1H$  NMR profile of **9** (Figure 4B) showed many similarities with **11**, making it obvious that it was also a  $\delta$ -lactone with one ethylidene group. The methyl group on the exocyclic double bond, two pairs of geminal protons, and one methine (3.32 ppm, H-5) bearing a side chain in combination with the carbonyl at 171.5 ppm made clear that **10** belonged to the same family of megaritolactones. The main difference from **11** was the size and the substitution pattern of the side chain. The side chain of **10** contained an oxygenated methylene H-3a,b at 3.73/3.80 ppm, one methine at 2.90 ppm (H-4), and a carbomethoxy group. In the COSY spectrum, H-3a,b was correlated with the methine at 2.90 ppm, and this methine (H-4) was correlated with the second methine at 3.32 ppm (H-5) belonging to the lactone ring. In the HMBC spectrum, both H-3, H-5 and the methoxy group were correlated with the carbonyl at 173.7 ppm, showing that the side chain of **10** consisted of a 2-yl-3-hydroxypropanoic acid methyl ester. The side chain was attached at the same position as in the case of **11**. The NOE spectrum showed also the same orientation for the methyl group of the ethylidene moiety. The absolute stereochemistry was also based on the biosynthetic pathway starting from the oleuropein secoiridoid skeleton as discussed below. Compound **10** was named as the methyl ester of megaritolactonic acid B.

Compound **9** was also obtained as a white amorphous solid with molecular formula  $C_{10}H_{12}O_4$ . Compound **9** showed also significant similarities with lactone **14** as well as **10** and **11** (Figure 4C). In comparison with **14**, the main difference was the presence of an exomethylene double bond observed as two singlets at 6.41 and 5.72 ppm and the absence of one pair of geminal protons. On the other hand, all of the other signals of the ethylidene group, the ethanoic acid side chain, and the oxygenated methylene of the lactone group were observed as expected. The third member of the megaritolactone family was also a product coming from the breakdown of oleuropein skeleton after hydrolysis, reduction, dehydration, and lactonization as explained in the biosynthetic pathway scheme (Figure

5). The exact placement of the exomethylene group was determined through the HMBC spectrum where the geminal protons of the double bond showed a  $^3J$  correlation with conjugated lactone carbonyl at 165 ppm and the methine carbon C-5 at 36.3 ppm. H-5 showed also critical correlations with both the lactone and the carboxylic carbonyls as well as the ethylidene double bond and the oxygenated methylene of the lactone ring. The lactone carbonyl was clearly discriminated from the carboxylic carbonyl through its correlation with the oxygenated methylene H-1a,b. The orientation of the methyl group was also the same as in **10** and **11**, highlighting their common biosynthetic origin. The orientation of the ethanoic acid side chain was opposite in comparison with **10** and **11** because the lactonization occurred between C-11 and C1 and not between C-1 and C-7 as in the case of **10** and **11**. In the case of **11**, C-11 is not present due to decarboxylation, while in the case of **10** C-11 is blocked as a methyl ester and cannot participate in the formation of the lactone. Compound **9** was named megaritolactonic acid.

The corresponding compound coming from the lactonization between C1 and C7 can be obtained in the case where C-11 is blocked as a methyl ester. It could be considered as a dehydration derivative of **10** leading to an exomethylene double bond on the side chain and not on the ring. This compound has been originally isolated from 'Throuba Thassos' table olives.<sup>19</sup> Although it has not yet been isolated from 'Megaritikiki' olives, its existence is reported herein to strengthen the discussion about the biosynthesis of the olive lactones and the possible occurrence of similar compounds in other table olives cultivars.

The last member of the megaritolactones family **9** had a molecular formula  $C_{10}H_{12}O_4$ . It was a compound closely related to megaritolactonic acid with the difference that the alcohol at position 3 was not dehydrated as in the case of **9** but lactonized with the carboxyl at C-7. This compound contained two  $\delta$ -lactone rings, and for this reason was named megaritodilactone. It presented all of the expected HMBC correlations, and more significantly the oxygenated methylene H-3 was correlated not only with C-11 and C-5 (as in **9**) but also with C-7 due to the formation of the second lactone ring. As in all of the previous compounds, the orientation of the methyl group was the same. Concerning the fusion between the two rings, it was found to be cis based on the small coupling constants of H-4 with H-5, H-3a, and H-3b, 6.4, 3.1, and 4.6 Hz, respectively. H-5 has clearly an axial orientation based on the large coupling constant with H-6b (12.1 Hz), and consequently H-4 has an equatorial orientation leading to a cis fusion between the two lactone rings (Figure 4D).

The proposed biosynthetic pathway (Figure 5) leading to megaritolactones starts from oleuropein (or ligstroside) first by hydrolysis of the glycosidic bond and then by hydrolysis of the ester at position 7. The obtained form I through keto-enol tautomerism leads to the key compound II. Reduction of aldehydes at positions 1 and 3 and subsequent lactonization between alcohol at position 1 and carboxyl at position 7 leads to **10**. Compound II after hydrolysis of the methyl ester at position 11 gives compound III from which starts the biosynthesis of **9** and **8**. Reduction of aldehydes at positions 1 and 3, dehydration of alcohol 3 to double bond, and lactonization between positions 1 and 11 leads to **9**. In the case of **8**, the biosynthesis occurs through double lactonization, between positions 1 and 11 and between 3 and 7. Compound III after decarboxylation leads to compound IV, which after reduction of the two aldehydes at positions 1 and 3 and

subsequent lactonization between positions 1 and 7 leads to **11**. In all cases, the absolute configuration at position 5 is already determined by the configuration of the corresponding carbon of oleuropein, which remains unchanged.

In conclusion, 'Megaritiki' table olives and debittering wastewater are useful sources of polyphenols and secoiridoids with unique structural characteristics. Quantitative NMR is a very useful tool for the rapid and easy measurement of key compounds in complex mixtures such as the olive extracts.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

NMR spectra of new compounds and compounds for the first time reported from table olives. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Tel.: +30-210-7274052. Fax: +30-210-7274594. E-mail: [magiatis@pharm.uoa.gr](mailto:magiatis@pharm.uoa.gr).

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We would like to thank Dr. Maria Nikolantonaki for recording NMR and MS spectra at University of California, Davis.

## ■ REFERENCES

- (1) Boskou, D. Phenolic compounds in olives and olive oil. In *Olive Oil: Minor Constituents and Health*, 1st ed.; Boskou, D., Ed.; CRC Press: New York, 2008; pp 12–36.
- (2) Charoenprasert, S.; Mitchell, A. Factors influencing phenolic compounds in table olives (*Olea europaea*). *J. Agric. Food Chem.* **2012**, *60*, 7081–7095.
- (3) Zoidou, E.; Melliou, E.; Gikas, E.; Tsaibopoulos, A.; Magiatis, P.; Skaltsounis, A.-L. Identification of Throuba Thassos, a traditional Greek table olive variety, as a nutritional rich source of oleuropein. *J. Agric. Food Chem.* **2010**, *58*, 46–50.
- (4) Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage. *EFSA J.* **2011**, *9*, 2033.
- (5) Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Direct measurement of oleocanthal and oleacein levels in olive oil by quantitative <sup>1</sup>H NMR. Establishment of a new index for the characterization of extra virgin olive oils. *J. Agric. Food Chem.* **2012**, *60*, 11696–11703.
- (6) Endo, K.; Hikino, H. Structures of renyol, renyoxide, and renyolone, new cyclohexylethane derivatives from *Forsythia suspensa* fruits. *Can. J. Chem.* **1984**, *62*, 2011–2014.
- (7) Park, C. H.; Kim, K. H.; Lee, I. K.; Lee, S. Y.; Choi, S. U.; Lee, J. H.; Lee, K. R. Phenolic constituents of *Acorus gramineus*. *Arch. Pharm. Res.* **2011**, *34*, 1289–1296.
- (8) Seya, K.; Endo, K.; Hikino, H. Structures of renyosides A, B and C, three glucosides of *Forsythia suspensa* fruits. *Phytochemistry* **1989**, *28*, 1495.
- (9) Bianco, A.; Mazzei, R. A.; Melchion, C.; Romeo, G.; Scarpati, M. L.; Soriero, A. Microcomponents of olive oil-III. Glucosides of 2 (3,4-dihydroxy-phenyl) ethanol. *Food Chem.* **1998**, *63*, 461–464.
- (10) Zhang, Y. J.; Liu, Y. Q.; Pu, X. Y.; Yang, C. R. Iridoidal glycosides from *Jasminum sambac*. *Phytochemistry* **1995**, *38*, 899–903.
- (11) Tian, J.; Zhao, Q. S.; Zhang, H. J.; Lin, Z. W.; Sun, H. D. New cleroidicins from *Clerodendrum indicum*. *J. Nat. Prod.* **1997**, *60*, 766–768.
- (12) Jemai, H.; Bouaziz, M.; Sayadi, S. Phenolic composition, sugar contents and antioxidant activity of tunisian sweet olive cultivar with regard to fruit ripening. *J. Agric. Food Chem.* **2009**, *57*, 2961–2968.

(13) Agalias, A.; Magiatis, P.; Skaltsounis, A. L.; Mikros, E.; Tsaibopoulos, A.; Gikas, E.; Spanos, I.; Manios, T. A new process for the integrated management of olive oil mill waste water, recovering natural antioxidants. *J. Agric. Food Chem.* **2007**, *55*, 2671–2676.

(14) Su, Y. Q.; Shen, Y. H.; Tang, J.; Zhang, W. D. Chemical constituents of *Incarvillea mairei* var. *Grandiflora*. *Chem. Nat. Compd.* **2010**, *46*, 109–111.

(15) Abdullahi, H.; Nyandat, E.; Galeffi, C.; Messana, I.; Nicoletti, M.; Bettolo, G. B. Cyclohexanols of *Halleria lucida*. *Phytochemistry* **1986**, *25*, 2821–2824.

(16) Breton, J. L.; Llera, L. D.; Navarro, E.; Trujillo, J. Photochemical synthesis of halleridone, hallerone, renyol and derivatives. *Tetrahedron* **1987**, *43*, 4447–4451.

(17) Kanchanapoom, T.; Kasai, R.; Yamasaki, K. Phenolic glycosides from *Markhamia stipulata*. *Phytochemistry* **2002**, *59*, 557–563.

(18) Gil, M.; Haidour, A.; Ramos, J. L. Two glutaric acid derivatives from olives. *Phytochemistry* **1998**, *49*, 1311–1315.

(19) Zoidou, E.; Agalias, A.; Magiatis, P.; Skaltsounis, A. L. New iridoid derivatives from table olives cv. 'Throuba Thassos' and olive mill waste waters. *Planta Med.* **2008**, *74*, 1081–1082.