Isolation and Characterization of the Antioxidant Component 3,4-Dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from Olive (*Olea europaea*) Leaves

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Storage of olive (*Olea europaea*) leaves for 22 h at 37 °C in closed plastic bags caused the content of a nonglycosidic secoiridoid, 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate (3,4-DHPEA-EDA) to rise from 15% to 50% of the phenolic extract with corresponding falls in the content of oleuropein and two oleuropeindials, which were identified as precursors of 3,4-DHPEA-EDA. Pure product was isolated from one set of stored olive leaves in a 0.16% yield. Storage of olive leaves under various conditions showed that the moisture present in closed plastic bags was important for the formation of 3,4-DHPEA-EDA. The time taken to reach the maximum concentration of the product varied widely for different samples of olive leaves, with a shorter time for the sample with lower initial oleuropein content. The oleuropeindial precursors of the product were readily hydrolyzed to carboxylic acid derivatives, which have been identified by NMR. The antiradical activity of 3,4-DHPEA-EDA, evaluated by scavenging of 2,2-diphenyl-1-picrylhydrazyl radicals, was comparable to that of α-tocopherol.

Keywords: *3,4-Dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate; 2,2-diphenyl-1-picrylhydrazyl radical; Olea europaea; Oleaceae; olive leaves; olive oil; oleuropein; oleuropein aglycon; secoiridoids*

INTRODUCTION

3,4-Dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate (**5** in Figure 1), also referred to as 3,4-DHPEA-EDA (*1*−*7*), is a compound that is of major interest because of its presence as one of the major secoiridoid antioxidant compounds in virgin olive oil (*2*, *8*). This oil is the major lipid component of the Mediterranean diet, and it can be distinguished from other seed oils by the composition of its triglyceride fraction and by its minor components, including polyphenols, which give the oil a characteristic taste, aroma, high resistance against oxidation (*9*), and health benefits (*10*−*12*). The major polyphenolic constituent in olives (*Olea europaea* L.) is oleuropein glycoside, but this compound is almost completely absent from olive oil because of its high water solubility. Phenyl acids and phenyl alcohols, including 3,4-(dihydroxyphenyl)ethanol (hydroxytyrosol) or *p*-hydroxyphenylethanol (tyrosol), have been found in virgin olive oil, but the prevalent phenolic compounds are secoiridoid derivatives of oleuropein such as the dialdehydic form of elenolic acid linked either to 3,4-(dihydroxyphenyl)ethanol (3,4-DHPEA-EDA, **5** in Figure 1) or to *p*-hydroxyphenylethanol, and an isomer of oleuropein aglycon (3,4-(dihydroxyphenyl)ethanol elenolic acid ester (3,4-DHPEA-EA, **4** in Figure 1) (*1*, *2*, *8*). These compounds are the most concentrated of those with a phenolic structure in virgin olive oil, and the 3,4-(dihydroxyphenyl)ethanol derivatives are of particular significance because of their strong antioxidant activity in several lipid systems including oil (*3*−*5*), emulsions (*5*), and low-density lipoprotein suspensions (*6*). However, studies of antioxidant and beneficial physiological effects of these individual compounds have been hindered by the difficulty of isolating them as pure compounds in sufficient amounts for further studies. In addition, accurate quantitative analysis of phenolic compounds in olive fruit and olive oil is hindered by the lack of sufficient pure standards to identify components and calibrate the response of chemical tests or instrumental methods. Olive polyphenols have rarely been obtained as pure samples, but very small quantities of pure polyphenols have been isolated occasionally − usually by preparative HPLC (*1*, *2*, *7*, *8*). Concentrations of polyphenols in oil and fruits have therefore been expressed as equivalents of gallic acid (*13*), caffeic acid (*14*), tyrosol (*15*), or oleuropein (*16*), but of these only oleuropein is a secoiridoid polyphenol similar to the major polyphenols of olive oil. A comparison of such data cannot be valid.

This paper reports the isolation of **5**, which is one of the major secoiridoids found in olive oil, and confirms that oleuropein and oleuropeindials are the natural precursors of this compound. The radical scavenging activity of this compound was also evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical method.

MATERIALS AND METHODS

NMR. ^{1}H, ^{13}C, ^{13}C DEPT-135, COSY, COSYLR, and HETCOR NMR spectra were recorded using a Brucker AC-200 spectrometer for samples dissolved in chloroform-*d* with TMS (Merck, Lisbon, Portugal) as internal standard.

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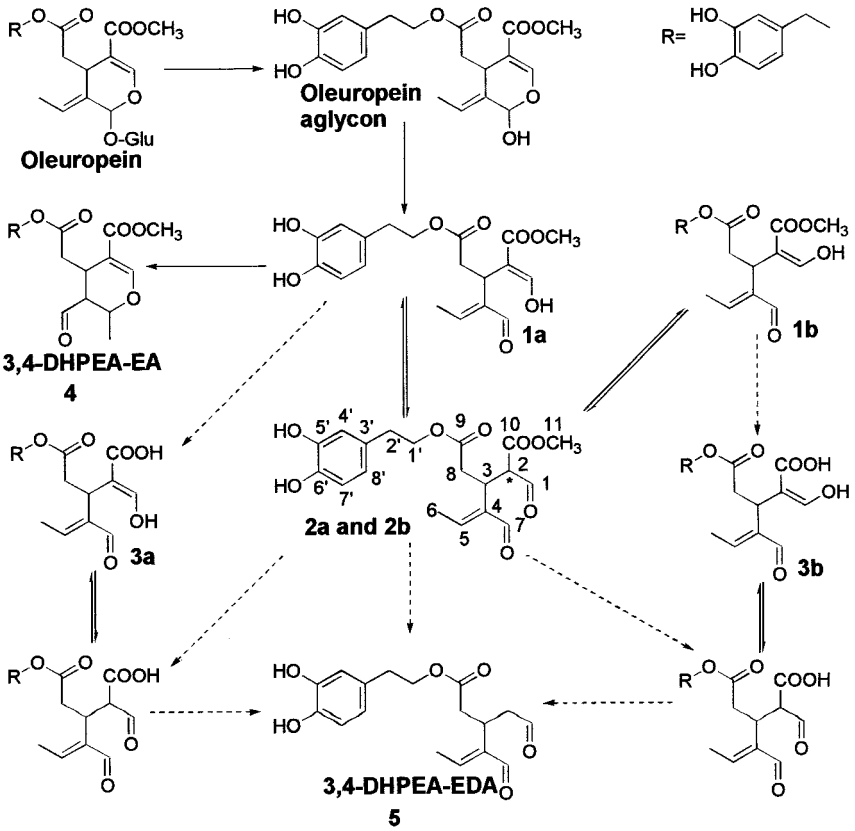

Figure 1. Possible transformation pathways of oleuropein in olive leaves and possible transformation pathway of compounds **2a** and **2b** in chloroform. (* = epimeric center in **2a** and **2b**).

HPLC. The HPLC system comprised a Merck-Hitachi chromatograph with a 250 mm × 4.6 mm Waters Spherisorb ODS2 5-μm column (Supelco Inc.), coupled to a Merck Hitachi L-4200 UV−Vis detector and with a Merck Hitachi L-6200 Intelligent Pump. Components were detected at 280 nm with elution at room temperature. The flow rate was 1 mL·min^{-1}; the mobile phase used was a mixture of 2% acetic acid (pH 3.1) in water (A) and methanol (B) with a total analysis time of 70 min; and the gradient changed as follows: 95% A/5% B for 15 min, 80% A/20% B in 15 min, 70% A/30% B in 10 min, 70% A/30% B for 5 min, 50% A/50% B in 5 min, 40% A/60% B in 5 min, 30% A/70% B in 5 min, 30% A/70% B for 5 min, 100% B in 1 min, and then held until the end of the analysis. Samples were analyzed in duplicate using 20 μL of each solution dissolved in methanol. Solvents were HPLC grade.

TLC. Silica gel GF_{254} (Merck); spots visualized by iodine; eluents: diethyl ether/methanol (1:1) and dichloromethane/methanol (5:1).

Preparation of 4 and Oleuropein. Compound **4** was obtained from oleuropein by enzymatic reaction using β-glycosidase (Aldrich, Spain) according to the procedure of Limirioli et al. (*17*). Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Gariboldi et al. (*18*).

Leaf Storage. Fresh olive leaves (50 g), harvested in October from the Trás-os-Montes region of Portugal, were stored in plastic bags (12.5 × 12.5 cm). Bags were pressed manually to reduce the air inside the bags before they were closed and kept at 37 °C in an oven protected from light. Two bags were frozen at time zero. Another two bags were removed from the oven at the time periods 15, 22, 40, and 48 h, and frozen.

Fresh olive leaves (50 g) harvested in the same region of Portugal in April were divided into three sets of storage conditions, and stored at 37 °C. One set of leaves was stored in bags without air; another set was stored in bags containing silica gel and flushed with nitrogen; and a third set of leaves was stored in an open vessel. Two samples of leaves (50 g) were frozen at time zero. Another two samples in each group were removed from the oven at time 10, 18, and 26 h, and frozen.

Isolation of Polyphenols from Leaves. Leaves from each sample were macerated in 250 mL of methanol for 5 days in the dark at room temperature. The extract was separated by filtration, and the solvent was evaporated under vacuum. The residue was taken up in 50 mL of acetone/water (1:1; v/v). The aqueous mixture was successively extracted with *n*-hexane and ethyl acetate. Each organic solution was washed with water, the solvent was evaporated, and the extracts were dissolved in methanol in volumetric flasks (250-mL) and analyzed by HPLC and TLC. The oleuropein concentration in each extract was calculated from a calibration curve.

Isolation of 3,4-Dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate (5) from Leaves. Isolation was carried out using leaves (400 g) harvested in October and stored in a closed bag (50 × 25 cm) at 37 °C for 22 h, followed by isolation of the polyphenol mixture as described above. The aqueous mixture was successively extracted with *n*-hexane followed by chloroform (Merck, Lisbon, Portugal), which was preferred to ethyl acetate because it reduced the content of oleuropein in the product and made it less sticky. After solvent evaporation, the extract (3.1 g) was purified by column chromatography using silica gel 60 (Merck, 230−400 mesh ASTM, 140 g) and eluted with diethyl ether/methanol (35:1) (Merck, Lisbon, Portugal). Combined fractions gave 0.64 g of a pure compound identified by NMR as **5**. This compound showed the same ^{1}H and ^{13}C spectral data as reported by Montedoro et al (*5*).

Isolation of Compounds 2a and 2b. The methanolic solutions of samples extracted from olive leaves before leaf storage were evaporated under vacuum and purified by column chromatography using dichloromethane/methanol (5:1) as eluent. Combined fractions gave 28 mg of a mixture of compounds including mainly compounds **2a** and **2b**. Compounds **3a**, **3b**, and **5** were also present, with the ratio of **2a**:**2b**:**3a**:**3b**:**5** being 1/1/0.21/0.25/0.15, respectively. Some signals

Table 1. ^{1}H and ^{13}C Nuclear Magnetic Resonance Data for Compounds 2a, 2b, 3a, and 3b in Chloroform-*d*

	^{1}H in ppm (*J* in Hz)		^{13}C in ppm (*J* in Hz)		^{1}H in ppm (*J* in Hz)		^{13}C in ppm (*J* in Hz)	
Atom no.[a]	2a	2b	2a	2b	3a	3b	3a	3b
1	9.65 d (J = 2.7)	9.48 d (J = 2.7)	196.10	195.32	7.39 d (J = 0.8)	7.33 d (J = 0.8)	163.86	163.86
2	4.06 d (J = 9.6)	4.03 d (J = 9.6)	59.20	60.18			103.41	103.41
3	3.85 m	3.75 m	29.88	29.88	4.21 m	4.17 m	31.70	31.35
4			141.97	141.97			140.77	140.18
5	6.59 m	6.56 m	154.94	154.94	6.72 m	6.72 m	157.11	156.60
6	2.02 d (J = 7.1)	2.00 d (J = 7.1)	15.32	15.21	2.05 d (J = 7.2)	2.05 d (J = 7.2)	15.32	15.20
7	9.16 d	9.15 d	195.82	195.52	9.16	9.15	195.82	195.52
8	2.80 m (8α) 2.60 m (8β)	2.80 m (8α) 2.60 m (8β)	35.15	34.10	2.75 m (8α) 2.90 m (8β)	2.75 m (8α) 2.90 m (8β)	35.62	34.42
9			172.06	171.91			172.06	171.91
10			172.62	171.41	11.82 s	11.76 s	171.62	171.41
11	3.77 s	3.64 s	52.79	52.50				
1′	4.22 m	4.16 m	65.18	65.18	4.22 m	4.16 m	65.18	65.18
2′	2.90 m (2′a) 2.75 m (2′b)	2.90 m (2′a) 2.75 m (2′b)	34.52	34.42	2.90 m (2′a) 2.75 m (2′b)	2.90 m (2′a) 2.75 m (2′b)	34.52	34.42
3′			130.05	130.05			130.05	130.05
4′	6.54 d	6.54 d	120.88	120.88	6.54 d	6.54 d	120.88	120.88
5′			142.77	142.77			142.77	142.77
6′			143.71	143.71			143.71	143.71
7′	6.78 d (J = 8)	6.78 d (J = 8)	115.19	115.19	6.78 d (J = 8)	6.78 d (J = 8)	115.19	115.19
8′	6.68 dd ($J_{7',8'}$ = 8) ($J_{4',8'}$ = 1.6)	6.68 dd ($J_{7',8'}$ = 8) ($J_{4',8'}$ = 1.6)	115.91	115.91	6.68 dd ($J_{7',8'}$ = 8) ($J_{4',8'}$ = 1.6)	6.68 dd ($J_{7',8'}$ = 8) ($J_{4',8'}$ = 1.6)	115.91	115.91

[a] Numbering of different carbons and protons is shown in Figure 1.

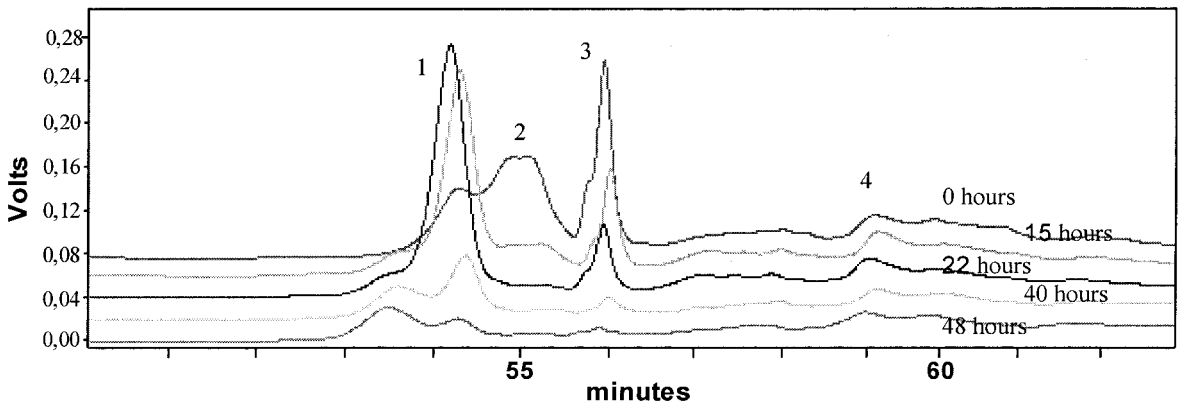

Figure 2. HPLC chromatograms of phenolic extracts isolated after 0, 15, 22, 40, and 48 h storage of olive leaves (October harvest) at 37 °C. Peak identification (t_r, min): 1 is **5** (54.3); 2 includes **2a** and **2b** (55.0); 3 is oleuropein (55.9); 4 is **4** (59.1).

from a small contamination by **4** could also be observed. Compounds were identified (Table 1) and quantified by NMR.

Determination of Radical Scavenging Activity. 1,1-Diphenyl-2-picrylhydrazyl radical was used as a stable radical (*19*). Several concentrations of **5** were tested. Phenolic compound solution (0.1 mL) was added to 3.5 mL of a 0.06 mM methanolic 1,1-diphenyl-2-picrylhydrazyl radical solution. The decrease in absorbance was determined at 515 nm at 0, 5, 15, 30, and every 30 min until 250 min. The exact initial 1,1-diphenyl-2-picrylhydrazyl radical concentration (C_{DPPH}) in the reaction was calculated from a calibration curve with the equation

$$Y = 0.1105x + 0.0001 \qquad r^2 = 0.9999$$

as determined by linear regression. The change in absorbance with time was plotted, and from this graph the percentage of 1,1-diphenyl-2-picrylhydrazyl radical remaining at several times was determined. The values were transferred onto another graph showing the percentage of residual 1,1-diphenyl-2-picrylhydrazyl radical as a function of the molar ratio of phenolic compound to 1,1-diphenyl-2-picrylhydrazyl radical. Antiradical activity was defined as the relative concentration of **5** required to lower the initial 1,1-diphenyl-2-picrylhydrazyl concentration by 50% [EC_{50} (mol/L **5** per unit 1,1-diphenyl-2-picrylhydrazyl concentration)]. The test was performed in quadruplicate. SPSS 10.0 software was used for statistical analysis by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$.

RESULTS AND DISCUSSION

Qualitative and quantitative changes in the secoiridoids present in *Olea europaea* leaves were determined during storage in closed bags without air at 37 °C. Changes in the ethyl acetate extract of the polyphenols isolated from leaves were analyzed by HPLC (Figure 2) and TLC. Three phenolic compounds in the secoiridoid region of the HPLC chromatogram were identified using standards as oleuropein, **5** and **4** (Figures 1 and 2). However, standards were not available to identify a mixture of compounds with retention time (t_r) around 55 min (compounds **2a** and **2b**) (Figures 1 and 2). During storage at 37 °C in closed plastic bags without air, the content of oleuropein decreased as the content

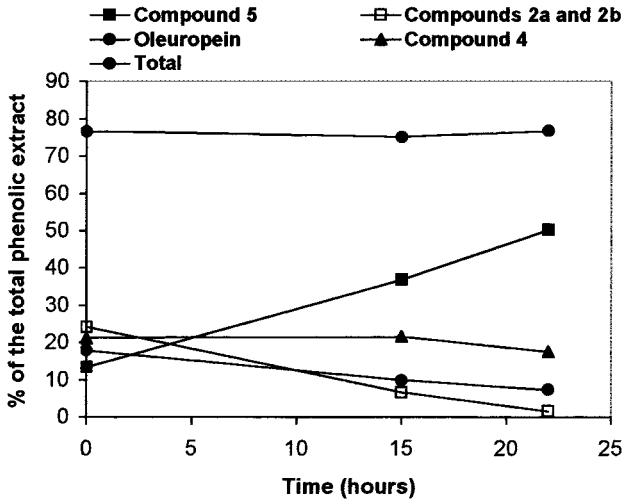

Figure 3. Qualitative and quantitative changes in the secoiridoid content of olive leaves (October harvest) during storage at 37 °C. Data are expressed as the mean of two samples, two determinations each (SD < 3%).

of **5** increased. The compounds with t_r between 54.5 and 55.5 min also decreased, falling to almost zero after 22 h of storage (Figure 3). The total secoiridoid polyphenol content of the leaves did not change significantly in the first 22 h, showing the interconversion of oleuropein and compounds **2a** and **2b** into **5** (Figures 1 and 3). Previous authors (*5*, *15*) have reported that virgin olive oil contained relatively high concentrations of **5** formed from oleuropein during the mechanical extraction of the oil. Compounds **5**, **2a**, and **2b** were extracted in low concentrations from olive leaves even before storage at 37 °C, but it is possible that these compounds were formed during maceration of the leaves in the extraction process. Because the leaves were frozen after storage at 37 °C and each batch was subjected to the same procedure, it can be concluded that the formation of **5** or its precursors in the leaves is enhanced by the storage in closed plastic bags.

Analysis of the ethyl acetate extracts by TLC with iodine detection confirmed the HPLC results. The TLC plate showed the presence of three main spots: one for oleuropein with the smallest R_f, another with an intermediate R_f for the mixture of **5**, **2a,** and **2b**, and a third one with the highest R_f for **4**. Compound **5** could not be well separated from **2a** and **2b** by TLC with any of the solvent mixtures tested. However, after 24 h of iodine detection, the color of the spot corresponding to **5** showed a yellow color, whereas spots richer in **2a** and **2b** showed a grayish brown color. A reduction in intensity of the brown color (due to **2a** and **2b**), located at the top of the spot, and an increase in intensity of the yellow color, due to **5**, was clearly evident as the time of storage at 37 °C of the corresponding leaves increased. Because the brown spot had a slightly higher R_f than that of **5** when dichloromethane/methanol (5:1) was used as eluent, a small amount of these compounds was separated by column chromatography and analyzed by NMR in order to identify the precursors of **5**. The ^{1}H, ^{13}C, ^{13}C−^{1}H J-modulated, ^{1}H−COSY, COSYLR, and HETCOR spectral data revealed the presence of two epimeric metabolites: the oleuropeindials **2a** and **2b** (Figure 1 and Table 1), which had been identified previously by Bianco et al (*20*, *21*) as intermediates formed following the enzymatic hydrolysis of the glucosidic linkage of oleuropein.

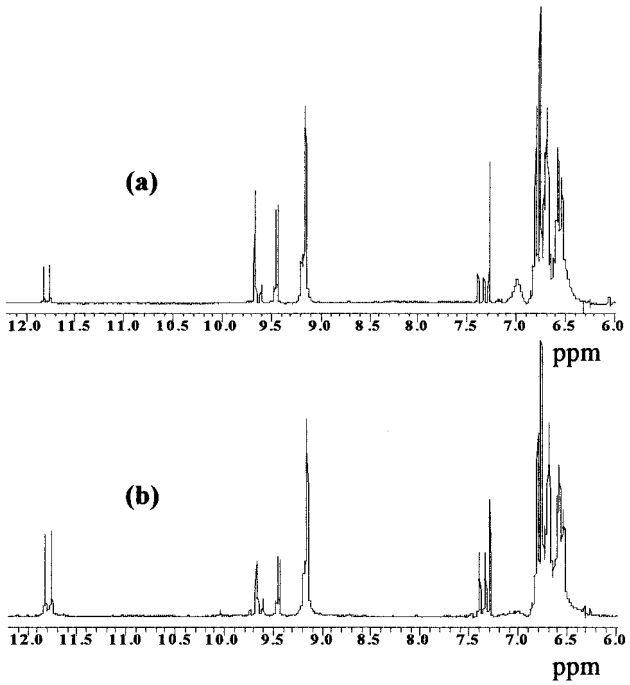

Figure 4. ^{1}H NMR spectra of compounds eluting with retention time around 55 min. (a) Region of the 200 MHz spectrum 24 h after chloroform-*d* solution preparation; (b) the same region of the spectrum as it appeared after 98 h.

Table 2. Oleuropein Concentration (mM)[a] in Leaf Extracts after 0, 10, 18, and 26 Hours of Storage at Different Conditions at 37 °C

	storage conditions	
time (hours)	in closed bags, full of N_2, with silica gel	in the open air
0	2.15 (± 0.04)	2.15 (± 0.04)
10	0.81 (± 0.00)	2.31 (± 0.09)
18	1.03 (± 0.03)	5.02 (± 0.20)
26	1.02 (± 0.05)	5.37 (± 0.16)

[a] Mean (± range) of two determinations.

Compounds **3a** and **3b** (Figure 1) would appear to be likely precursors of **5**, as these compounds could undergo rapid decarboxylation to give **5**. Decarboxylation may occur either in the leaves or during extraction. Even during storage in chloroform at room temperature, the NMR spectra showed that diastereoisomers **2a** and **2b** were hydrolyzed to **3a** and **3b**. As reported before by Bianco et al (*21*), a slow transformation process at the aldehydic groups was observed, as indicated by changes in the ^{1}H NMR spectrum at 11.82 and 11.76 ppm, which were ascribed in the literature to oxidation of the aldehyde groups (*21*). After 4 days in chloroform, compounds **2a** and **2b** decreased to half their initial concentration, calculated by the decrease of the aldehydic signals at 9.65 and 9.48 ppm, and the proportional increase of signals at 11.82 and 11.76 ppm (compounds **3a** and **3b**, Table 1). Moreover, a proportional increase in the signals at 7.39 and 7.33 ppm (Figure 4), attributed to the vinyl hydrogen atom H_1 linked to a carbon bearing an oxygen atom, could also be observed. In fact, the signals for C_1 at 163.86 ppm were coupled to H-1 of both isomers, suggesting its linkage to oxygen. However, the aldehydic signal at 9.16 ppm did not decrease, suggesting no change in this part of the molecule. The HETCOR spectral data revealed coupling between C-3 (31.70 and 31.35 ppm) and H-3 at 4.21 and

Table 3. 1,1-Diphenyl-2-picrylhydrazyl Radical-Scavenging Effects of 5, Oleuropein, and α-Tocopherol after Various Reaction Times (15, 60, and 250 min)[a]

	time 15 min[b]		time 60 min[b]		time 250 min[b]	
compound	EC_{50}[c]	no. of reduced radicals	EC_{50}[c]	no. of reduced radicals	EC_{50}[c]	no. of reduced radicals
5	0.28[a] (± 0.01)	1.8	0.25[a] (± 0.01)	2.0	0.19[a] (± 0.01)	2.6
oleuropein	0.22[b] (± 0.01)	2.2	0.18[b] (± 0.01)	2.6	0.12[b] (± 0.01)	4.0
α-tocopherol	0.25[c] (± 0.01)	2.0	0.25[a] (± 0.01)	2.0	0.24[c] (± 0.01)	2.1

[a] Superscripts within a column indicate samples that were significantly different ($p < 0.05$). [b] Mean (standard deviation in parentheses) of four determinations. [c] EC_{50} expressed as mol of antioxidant/mol of 1,1-diphenyl-2-picrylhydrazyl radical.

4.16 ppm, suggesting a molecular structure similar to that of **1b** (*21*). The two-dimensional ^1H (COSY) NMR spectra showed coupling between H-1 and the acidic protons and between H-1 and H-3 (4.21 and 4.17 ppm) suggesting the sequence CH−CCOOH═CHOH. Furthermore, the coupling between H-7 (9.16 ppm) and H-3 (4.21 and 4.17 ppm) proved the relationship between this sequence and the aldehydic group linked at C-4. This coupling (H-7, H-3) was also observed in the COSY spectrum of pure 3,4-DHPEA-EDA (**5**). Long-range couplings between H-7 (9.16 ppm) and H-6 (2.02 ppm) and between H-7 and H-5 (6.7 and 6.5 ppm) were also observed, confirming the sequence HCO−C═$CHCH_3$. These data indicate that diastereoisomers **2a** and **2b** have been hydrolyzed to the previously undescribed diastereoisomers **3a** and **3b** during storage in chloroform solution. Hydrolysis during storage in chloroform is unexpected. Possibly a trace of acid in the chloroform may have been responsible for this change.

To clarify the storage conditions needed for optimal formation of **5**, samples of olive leaves were stored under different conditions: samples in closed bags with a minimal amount of air, samples in bags containing silica gel and filled with nitrogen, and samples open to the air. The initial content of secoiridoid polyphenols in the leaves used for this experiment, which had been harvested in April, was much lower than that in the leaves harvested in October which had been used for the isolation of **5**. The initial polyphenol content and activity of relevant enzymes are likely to depend on the weather conditions during the period of growth of the leaves. Periods of sun before harvesting may be necessary for the increased formation of polyphenols in leaves (data not shown). This experiment confirmed the formation of **5** in leaves stored in closed bags. However, the time needed for samples to reach the maximum content of **5** was less for the leaves with the lower initial oleuropein content. The leaves harvested in April contained about 40% of the initial oleuropein level present in the leaves used in the first experiment (2.15 ± 0.04 mM compared to 5.53 ± 0.21 mM in leaf extract), and they also needed about 40% of the time to achieve the maximum concentration of **5** (9 h compared to 22 h). There was no detectable formation of **5** in leaves stored in closed bags with silica gel under nitrogen or in open vessels. Formation of **5** in closed bags is likely to be due to softening of the leaf tissues in the moist atmosphere, which allowed enzymes and substrate to come into contact. Samples stored under the other conditions would be exposed to reduced moisture levels. Leaves stored in open vessels would correspond to the lowest level of moisture, and these conditions appeared to enhance the formation of oleuropein. The rise in oleuropein content with storage time (Table 2) suggests the glycosidase enzyme is inactive under these conditions. In fact, oleuropein was the only secoiridoid found in significant quantities in the leaves stored at 37 °C in the open air for 18 and 24 h, and these storage conditions are recommended if oleuropein is to be isolated from olive leaves.

Compound **5** was examined for its radical scavenging activity toward the stable 1,1-diphenyl-2-picrylhydrazyl radical. This compound had significant radical scavenging activity comparable to that of α-tocopherol (Table 3), showing EC_{50} at 15 min just slightly lower. However, **5** showed radical scavenging activity significantly lower than that of oleuropein (21% lower at 15 min and 37% lower at 250 min; Table 3) but the change in radical scavenging activity with time indicated similar kinetic behavior. Compound **5** reacted rapidly with the 1,1-diphenyl-2-picrylhydrazyl radical in the first 15 min of reaction. However, a steady state was not reached by **5** and oleuropein at this time as it was for α-tocopherol, and **5** and oleuropein showed a further reduction in EC_{50} between 15 and 250 min which amounted to 30% and 45%, respectively.

This is the first report of isolation of pure **5** from olive leaves. The biosynthesis of **5** from its natural precursor oleuropein via the oleuropeindials **2a**, **2b** and **3a**, **3b** inside leaves is enhanced by storage of the leaves in closed bags containing little air at 37 °C. By this method, **5** may be isolated from polyphenol extracts from olive leaves by column chromatography in significant quantities without using preparative HPLC.

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