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Bioactive Derivatives of Oleuropein from Olive Fruits

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New bioactive epimeric derivatives of oleuropein have been detected in olive fruits and structurally characterized by ^{1}H and ^{13}C NMR. These hydrolytic metabolites, obtained by enzymatic catalysis, can be molecular microcomponents, present in Mediterranean food, table olives, and olive oil, responsible for complex sensorial attributes and for pathogen natural defense.

Keywords: *Oleuropein metabolites; biophenols; table olives; olive oil*

INTRODUCTION

Biophenolic (BP) minor components (Bianco, 1997; Vekey, 1997; Angerosa, 1995), found in olive fruits, have shown their relevance in the production of table olives (Marsilio, 1996) and olive oil, (Bianco, 1998a,b) typical foods of the Mediterranean culture (Casuscelli, 1994; Bianco, 1996; Romeo, 1996), because of their bioactive contribution to sensory characteristics (Bianco, 1997; Montedoro, 1993), to stability toward autoxidation (Montedoro, 1993; Castelli, 1997), and to human health beneficial effects (Castelli, 1997; Petroni, 1995).

The secoiridoid oleuropein **1**, the bitter glucoside present in the fruits of *Olea europea* L (Panizzi, 1960), being the dominant BP component (Amiot, 1986, 1989), can greatly influence the final product, also through its hydrolytic derivatives, spontaneously obtained by enzymatic and/or chemical catalysis (Walter, 1973; Scarpati, 1993; Capasso, 1996; Limiroli, 1996), undergoing further molecular transformations, via ring opening and rearranged reclosure of the original 11-methyloleoside moiety (Gariboldi, 1986; Limiroli, 1995). These derivatives of **1** can be performing a multichemical defense bioactivity (Kubo, 1985) against microbe and insect attack onto the olive fruits (Lo Scalzo, 1994), not shown by the direct secoiridoid glycoside.

The evaluation of the metabolic process of **1** at the molecular level can provide rational information on the BP components responsible for the bioactivity in the overall food characteristics and in the natural barrier mechanism of olive fruits against insect attack.

The structure elucidation of new epimeric BP metabolites, the oleuropeindiales **2a** and **2b**, has been carried out on samples isolated from methanol/acetone extracts of Cassanese cv. (cultivar) green mature olive fruits. Oleuropeindiales **2a** and **2b** have been previously postulated (Limiroli, 1995), but not identified, as intermediates in enzymatic hydrolysis of glucosidic linkage in **1**; the hydrated forms (oleuropeindiale gem-diols) **4a** and **4b** have, in fact, been evidenced in the mixture of aqueous reaction medium (Limiroli, 1995).

The detection of **2a** and **2b**, together with the precursor oleuropein-enolic form **3**, is the first direct observation of these biomolecules among the BP minor derivatives of olive fruits, confirming that **2a** and **2b** are the natural precursors of **4a** and **4b**, whose formation from **2a** and **2b** is amenable to presence of the water phase (Scheme 1).

EXPERIMENTAL PROCEDURE

Instrumentation. NMR measurements for ^{1}H at 300.13 MHz and for ^{13}C at 75.42 MHz were recorded on a Varian VXR-300 spectrometer (Palo Alto, CA) using TMS in deuteriochloroform or DDS in D_2O as internal standards. Two-dimensional COSY and inverse mode heteronuclear multiple-bond correlation (HMBC) spectra were determined in absolute value mode. Infrared spectra were recorded on a Perkin-Elmer 377 instrument.

The extracts were analyzed by TLC on silica gel GF 254 (Merck, Germany), and the spots were detected under UV light (254 nm). Flash chromatography was carried out with Kieselgel 60 (Merck).

All chemicals were analytical grade and used without further purification.

Materials and Methods. *Isolation of **2a**, **2b**, and **3**.* Cassanese cv. green olives (500 g) were frozen under liquid nitrogen and freeze-dried (Bianco, 1999a). Then, fruits were depitted by blending and homogenized in 200 mL of methanol/acetone (1:1), saturated with sodium disulfite, at top speed in an Ultraturrax homogenizer (Janke & Kunkel, IKA-Labortechnik, Germany) at 0 °C for 3 min, and centrifugated at 5000*g* for 20 min at 4 °C.

The supernatant was separated, and the pellet was resuspended (four times) in 200 mL of methanol/acetone (1:1) and saturated with sodium disulfite, until a colorless solution was obtained. The combined supernatants were evaporated to dryness, under vacuum at 45 °C. The dry residue was solubilized in water at pH 2 and centrifuged to separate a cloudy precipitate. The clear supernatant was extracted five times with hexane at a hexane to water phase ratio of 1:1, to remove free fatty acids and other lipid contaminants. The BPs were then extracted six times with ether/ethyl acetate (1:1) at a 1:1 solvent to water phase ratio.

The ether/ethyl acetate extracts were dehydrated with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 30 °C. The residue (6.5 g) was subjected to flash chromatography on silica gel column with chloroform/methanol 95:5 as eluent. The most important fraction (3.5 g) is the mixture of **2a**, **2b**, and **3**.

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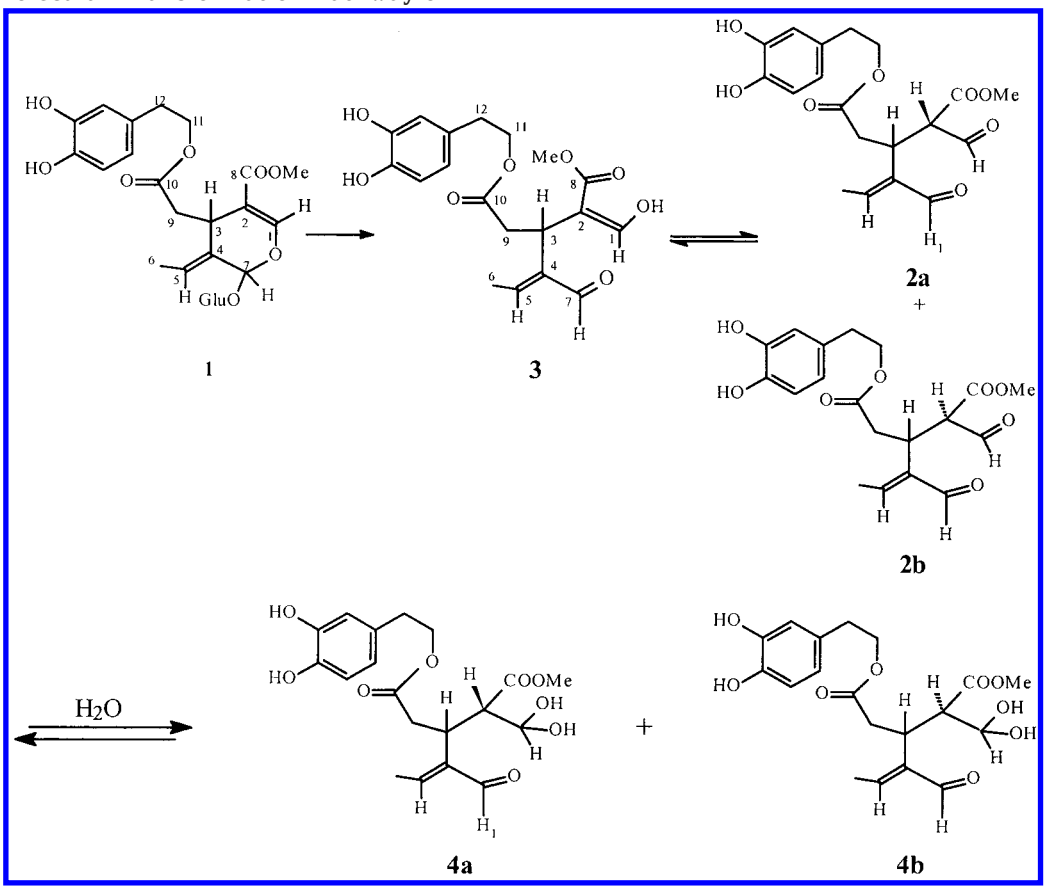
Scheme 1. Molecular Transformation Pathway of 1



Compound ***3.*** ^{1}H NMR (δ, $CDCl_3$): 2.03 (d, $J = 7.1$ Hz, CH_3), 2.70 (m, H-12α and H-12β), 2.75 (m, H-9α), 2.82 (m, H-9β), 3.79 (s, OCH_3), 4.10 (m, H-11α and H-11β), 4.19 (dd, $J = 7.5$, 7.0 Hz, H-3), 6.70 (q, $J = 7.1$, H-5), 6.6−6.85 (aromatic protons), 7.40 (bs, OH), 7.35 (d, $J = 6.2$ Hz, H-1), 9.20 (s, H-7). ^{13}C NMR: 195.31 (C-7), 171.81 (C-8), 171.10 (C-10), 156.93 (C-1), 155.08 (C-5), 143.70 (C-4′), 143.38 (C-4), 142.80 (C-3′), 140.02 (C-1′), 130.37 (C-2), 121.25 (C-6′), 116.30 (C-5′), 116.30 (C-2′), 65.65 (C-11), 51.90 (C-13), 37.15 (C-9), 34.38 (C-12), 31.03 (C-3), 15.30 (C-6).

Compound ***2a.*** ^{1}H NMR (δ, $CDCl_3$): 2.00 (d, $J = 7.0$ Hz, CH_3), 2.60 (m, H-9α), 2.78 (m, H-12α and H-12β), 2.80 (m, H-9β), 3.71 (s, OCH_3), 3.75 (m, H-3), 4.18 (m, H-11α and H-11β), 6.75 (q, $J = 7.0$, H-5), 6.6−6.85 (aromatic protons), 9.20 (s, H-7), 9.75 (d, $J = 2.72$ Hz H-1). ^{13}C NMR: 195.73 (C-1), 195.21 (C-7), 171.50 (C-8), 171.15 (C-10), 155.10 (C-5), 143.33 (C-4′), 142.95 (C-4), 142.74 (C-3′), 142.71 (C-1′), 119.25 (C-6′), 116 30 (C-5′), 115.94 (C-2′), 65.49 (C-2), 56.00 (C-11), 51.95 (C-13), 36.29 (C-9), 35.81 (C-12), 30.60 (C-3), 15.29 (C-6).

Compound ***2b.*** ^{1}H NMR (δ, $CDCl_3$): 1.98 (d, $J = 7.0$ Hz, CH_3), 2.60 (m, H-9α), 2.78 (m, H-12α and H-12β), 2.80 (m, H-9β), 3.78 (m, H-3), 3.85 (s, OCH_3), 4.18 (m, H-11α and H-11β), 6.75 (q, $J = 7.0$, H-5), 6.6−6.85 (aromatic protons), 9.20 (s, H-7), 9.48 (d, $J = 2.70$ Hz, H-1). ^{13}C NMR: 195.58 (C-1), 195.21 (C-7), 171.30 (C-8), 171.10 (C-10), 155.10 (C-5), 143.32 (C-4′), 142.80 (C-4), 142.74 (C-3′), 142.71 (C-1′), 119.29 (C-6′), 116 30 (C-5′), 116.00 (C-2′), 65.43 (C-2), 56.50 (C-11), 51.98 (C-13), 36.29 (C-9), 35.60 (C-12), 30.65 (C-3), 15.29 (C-6).

RESULTS AND DISCUSSION

The most important fraction (3.5 g), isolated as an oily product, reveals a mixture of three components **2a**, **2b**, and **3**, showing a nearly 1:1:1 relative ratio, as determined in solution from the ^{1}H NMR analysis. The BP hydrolytes of **1** could be formed during the isolation procedure, unless a careful treatment is adopted, as already described (Bianco, 1999a). The presence of an easily enolizable hydrogen atom in molecular structures **2a** and **2b** gives rise to a tautomeric equilibrium with the enolic forms **3**.

IR spectra (in $CHCl_3$) are consistent with the presence of aldehydic groups (2830, 1730 cm^{-1}), carbonyl groups and double bonds (1670−1720 cm^{-1}), and an aromatic ring (1630 cm^{-1}).

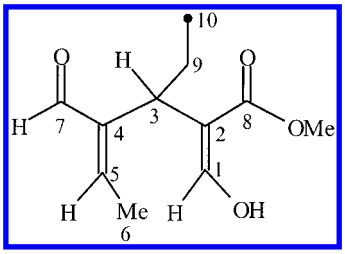
The ^{1}H NMR spectra confirm the occurrence of three components, as deduced from the presence of three carbomethoxy groups at 3.79, 3.71, and 3.85 ppm, with relative ratio 33:37:30, and three resonances in the range 9.2−9.8, attributable to aldehydic protons. In particular, the most relevant resonance appears at 9.20 ppm. All three biomolecules contain a hydroxytyrosil fragment, as in the case of **1**, and a methyl group, as a doublet, linked to a sp^2 carbon atom, as shown from the examination of ^{1}H and ^{13}C spectra and relative integrals.

Moreover, the ^{1}H NMR spectrum indicates the presence of a singlet at 7.35 ppm attributed to vinyl hydrogen atom H_1, linked to a carbon bearing an oxygen atom, as in BP **3**. In fact, the chemical shift of C-1 (156.93) suggests its linkage to oxygen. The ^{1}H NMR spectrum in D_2O enables observation of an exchangeable hydroxyl proton at 7.40 ppm, coupled to H-1.

One-dimensional ^{1}H and ^{13}C NMR spectral data and the phase-sensitive DQF−COSY reveal the two proton spin systems, from H-1 (br s, 7.35 ppm) to H-6 (d, 2.03 ppm) through H-3 (dd, 4.19 ppm) and H-5 (q, 6.70 ppm) and between H-3 and H-9 (m, 2.75 and 2.82 ppm).

Furthermore, ^{1}H−^{1}H and ^{1}H−^{13}C long-range couplings between H-5 and H-7 (s, 9.20 ppm), H-5 and C-7

Scheme 2. Substructure 5 of Enolic Form 3



(195.31), H-5 and C-1 (156.93), H-5 and C-3 (31.03), H-3 and C-2 (130.37), and H-9 and C-2 prove the relationship between these two sequences and the aldehydic group linked at C-4 (143.38).

Detailed analysis of long-range couplings, observed in the $^{1}H-^{13}C$ HMBC, reveals the substructure of **3**, including the carbomethoxy group. Thus, the long-range couplings from H-3 and H-9 to the carboxylic carbon atom C-10 (171.10 ppm) confirm the relationship between the two spin system sequences; furthermore, H-1 and H-3 are long-range coupled with the carboxyl group C-8 (171.81 ppm) and C-2 with the OMe group at C-8 (3.79 ppm), so indicating that the carbomethoxy group is linked to C-2, as shown in the substructure **5** (Scheme 2).

The other substructure of **3** is assembled as follows. The long-range coupling between the estereal C-10 and H-11, together with the COSY between H-11 (4.10 ppm) and H-12 (2.70 ppm), indicates the presence of a CH_2-CH_2 moiety, bonded to the ester function at H-9. Finally, a long-range coupling from H-11 to the aromatic quaternary carbon atom at 140.02 ppm links this unit to the *o*-diphenolic ring, as shown by the structure **3**; the benzene substitution pattern is corroborated by similar ^{13}C chemical shifts of corresponding unit in **1**.

Both ^{1}H and ^{13}C NMR spectra in $CDCl_3$ show the presence of a tautomeric equilibrium between the enolic structure **3** and the dialdehydic forms **2**, derived from the 1,3-prototropic shift of the enol hydrogen atom. The decrease of signals at 9.48 and 9.75 ppm and the corresponding increase of aldehydic resonance at 9.20 ppm and of singlet at 7.35 ppm, when acidified D_2O is added, provide a straightforward demonstration of biomolecular transformation among **2a**, **2b**, and **3**.

The observed experimental results can be rationalized by keto−enol tautomerism causing racemization at C-2, with formation of the two diastereoisomers **2a** and **2b**; the absolute configuration of C-3, being S in the original biomolecule **1** (Inouye, 1974), remains unchanged in the new hydrolytes.

The two aldehydes show two sets of NMR signals with similar spin pattern; 2D spectra are characterized by two sets of signals with identical correlations. The aldehydic protons of **2a** and **2b** are assigned resonances at 9.75 (H-1), 9.20 (H-7), and 9.48 ppm (H-1) and 9.20 ppm (H-7), respectively. The corresponding ^{13}C chemical shifts are 195.73 (C-1) and 195.21 (C-7) for **2a** and 195.58 (C-1) and 195.21 (C-7) for **2b**.

The $^{1}H-^{13}C$ HBMC spectrum of **2a** shows aldehydic correlations between C-1 (195.73) with H-2 (dd, 4.05) and H-3 (m, 3.75) and between C-7 (195.21), H-5 (q, 6.75), and H-3. For **2b**, analogously, correlations are evidenced between C-1 (195.58) with H-2 (dd, 4.07) and H-3 (m, 3.78) and between C-7 (195.21) with H-5 (q, 6.75) and H-3. C-6 methyl groups resonate at 2.00 and 15.29 for **2a** and 1.98 and 15.29 for **2b**.

The multiplicity of C-2, being a CH and not a CH_2, indicates a carbon atom still carrying CO_2Me group, as confirmed by long-range coupling between C-11 (56.0 ppm) and H-2.

Coupling between the signals at 3.75 ppm (H-3) and those at 2.80 and 2.60 (H-9) confirm the sequence C-3, C-9, C-10, reported in structures **2a** and **2b**. Finally, coupling between the two H multiplet at 4.18 and the two protons at 2.78 along with the aromatic pattern allows the identification of the 3,4-dihydroxyphenylethanol moiety, also present in **1**.

The neat metabolites **2a** and **2b**, thus described, are shown to be sufficiently stable under nitrogen for several days, (Bianco, 1999b), before further molecular transformations (Gariboldi, 1986). This indicates their possible bioactive contribution to the natural defense mechanism of olive drupes (Kubo, 1985). In fact, antimicrobial components, found in olive fruits, have been demonstrated to be active against bacteria and yeast after precursor BP **1** enzymatic hydrolysis (Walter, 1973). The chemical structure assignment to enzymatic hydrolytic product has not been previously arranged, lacking of reliable results. Thus, the above-discussed experiments on olive drupes now render available the molecular structure of bioactive derivatives as **2a**, **2b**, and **3**.

BP original molecule **1** is not effective against *S. cerevisiae*, *B. subtilis*, and *E. coli*, unless in the presence of β-glucosidase at pH 7 (Kubo, 1985), so inferring the hydrolytes **2a**, **2b**, and **3** being active in crude ether extracts.

The complex sensorial characteristics of food products from olive drupes, pungent and strongly bitter flavor (Gutierrez, 1992), can also be due to BP **1,** through its biomolecular derivatives, obtained during the olive fruit processing, i.e., milling, malaxation, centrifugation for olive oil production and for table olive debittering (Marsilio, 1996).

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