NMR Experiments of Oleuropein Biomimetic Hydrolysis

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The oleuropein hydrolytic conversion, under biomimetic conditions, induced by the endogenous enzymatic system of the olive fruit, has been monitored by the intermediate derivative formation through ^{1}H and ^{13}C NMR spectra; the assigned molecular structures reveal the identity of new bioactive biophenolic metabolites, the hemiacetal aglycon, and the two epimeric dialdehydes, which influence the pathogen antagonism of olive fruits and the hedonistic−sensorial characteristics of olive oil.

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

INTRODUCTION

The ortho-diphenolic functional groups characterize the biophenols (BP) (Bianco, 1997; Vekey, 1997), some of the minor molecular constituents found in the traditional Mediterranean foods (Romeo, 1996) of plant origin, e.g. table olives (Bianco, 1999a; Esti, 1998) and olive oil (Bianco, 1998a,b). They play an important role in the vegetable systems, acting in the defense mechanism against pathogen and insect attack on the original fruit (Kubo, 1995), i.e., the olives, and provide a synergistic contribution toward the antioxidant activity (Saija, 1998; Kohyama, 1997) and the hedonistic−sensorial quality of the agrifood products (Montedoro, 1993; Marsilio, 1996), which has favorable effect on human health (Saija, 1998).

The principal component among BPs contained in the olive fruit is the secoiridoid glycoside oleuropein **1** (Panizzi, 1960), the hydroxytyrosil ester of the 11-methyloleoside, which influences the table olive processing (Brenes, 1995) and the olive oil production, through its molecular transformation into hydrolytic metabolites (Bianco, 1998a,b).

The enzymatic (Marsilio, 1996; Panizzi, 1960; Bianco, 1993; Capasso, 1996; Limiroli, 1995) and chemical (Panizzi, 1960; Bianco, 1993; Walter, 1973; Gariboldi, 1986) reactivities of **1** have been previously investigated so as to understand molecular changes that might occur during the technological process of olive fruits, thus indicating the formation of some hydrolytic intermediates, which are then found in the agrifood products.

The detailed sequence of reactions, giving rise to intermediate derivatives from **1**, has now been followed by a biomimetic experiment, performed under NMR conditions, with the endogenous enzymatic system of olive fruit so as to discover the complete molecular transformations occurring in the abiotic system, which are similar to those taking place in the olive during fruit ripening or deterioration and during crushing and malaxation by the intervention of natural β-glucosidases.

To characterize thoroughly all the BP metabolites of **1** that might intervene during agrifood processing the enzymatic reactivity of **1**, with endogenous β-glucosidase, has been followed by monitoring all hydrolytic conversion steps of the original secoiridoid molecule. In this way, the structures of all BP intermediates formed have been investigated directly, in the same abiotic aqueous reaction medium. This affords, through the NMR probe, a straightforward response from the precursor molecules responsible for the metabolic derivatives found in Mediterranean agrifood, without any artifact due to the manipulation of sample.

Furthermore, since agrifood processing generates biphasic liquid system, i.e., the hydrophilic and lipophilic ones, the reaction mixture has been left to interact in polar aqueous medium with a less polar one, allowing the identification of all reaction products, partitioned in aqueous and lipophilic phases at different times.

^{1}H and ^{13}C NMR analysis gives rise to the detection and characterization of bioactive BP derivatives which populate the hydrolytic pathway (Scheme 1), thus providing the first report concerning the structural evidence of all chemically compatible and/or previously suggested, but unidentified, intermediates in the molecular transformation process of **1**.

EXPERIMENTAL PROCEDURE

Instrumentation. NMR measurements were performed on a Varian VXR-300 (Palo Alto, CA), equipped with a temperature control unit. Chemical shifts were referred to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) in water and to tetramethylsilane (TMS) in organic solvents.

Materials and Methods. *Hydrolysis of* ***1*** *in* D_2O *solution.* Thirty milligrams of **1** was dissolved in 0.7 mL of D_2O and 1−2 drops of olive juice were added to the solution. The ^{1}H NMR spectra were recorded at different times (2 min, 5min, 10min, 20min, 30 min, 1 h, 2 h, 5 h, 12 h, and 24 h).

Hydrolysis of ***1*** *in* $D_2O/CDCl_3$ *Mixture.* One hundred milligrams of **1** was dissolved in 20 mL of a $D_2O/CDCl_3$ 1:1 mixture, and 2 drops of olive juice were added. The two phases have been separated and independently analyzed at different times.

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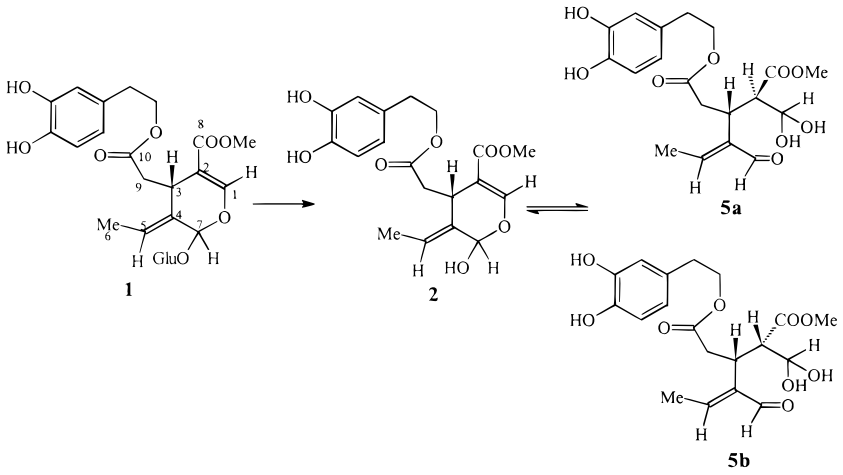
Scheme 1. Transformation Pathway of 1 in Water



*Detection of Compound **2**.* Compound **1** (1 g) in water (150 mL) was treated with endogenous β-glucosidase (2 drops of olive juice) from green olive fruit with stirring for 1 min at room temperature. Freeze-dried solvent sublimation and silica gel flash chromatography by overpurified chloroform/methanol (92:2) gave 20 mg of compound **2**. ^{1}H NMR (δ, $CDCl_3$): 7.55 (s, H-1), 6.80−6.72 (m, aromatic protons), 5.65 (q, H-5, J = 7.0 Hz), 4.25 (d, H-1, J = 5.0 and 0.7 Hz), 4.30 (m, H-1α and H-1′β), 3.60 (dd, H-3, J = 8.9 and 4.3 Hz), 3.58 (s, OCH_3), 2.82 (m, H-2″α and H-2″β), 2.75 (m, H-9α), 2.65 (m, H-9β), 1.65 (d, CH_3, J = 7.0 Hz). ^{13}C NMR: 169.20 (C-8), 168.5 (C-10), 155.70 (C-1), 142.80 (C-3′), 141.01(C-1′), 135.70 (C-4′), 132.05 (C-4), 121.25 (C-6′), 116 30 (C-5′), 116.30 (C-2′), 111.37 (C-2), 96.04 (C-5), 94.31 (C-7), 65.65 (C-11), 51.90 (C-13), 41.30 (C-9), 34.38 (C-12), 31.73 (C-3), 14.30 (C-6).

RESULTS AND DISCUSSION

The environmental conditions operating in olive fruits have been biomimed by performing the hydrolysis of **1** with the endogenous β-glucosidase direct action, as is present in olives, by adding olive juice to a D_2O solution of **1**.

The first experiment, achieved in the NMR tube by the addition of few olive juice drops, gives rise to a cloudy solution: the ^{1}H NMR spectrum, recorded 5 min after the dissolution, shows the presence of a complex signal pattern, due to the formation of more than one hydrolyte. This is clearly revealed by the appearance of several resonances in the range 8.8−9.0 ppm, attributable to aldehydic protons.

After the enzymatic action on the acetal functionality of **1**, the aglycon thus obtained appears to undergo a fast irreversible molecular transformation, which provides a mixture of products during the experimental NMR mode, with enhanced complexity in BP intermediate appraisal.

The NMR spectrum reveals the resonances of unaltered **1**, free β-glucose, resulting from the enzymatic hydrolysis on glucosidic acetal linkage; two epimeric aldehydes in hydrated form, previously identified in aqueous solutions as hydrated oleuropeindiale **5a** and **5b** (Limiroli, 1995); and a novel unidentified derivative oleuropeinhemiacetal, the aglycon **2** (Scheme 1).

Silica gel column flash chromatographic separation, with careful chosen experimental conditions, allows the isolation and characterization of this new BP intermediate of oleuropein hydrolysis, whose structure **2** has been assigned on the basis of NMR spectra analysis.

Thus, **2** contains a carbomethoxy group on an enol−ether double bond as in the case of **1**, while resonances in aldehydic range are absent. The hydroxytyrosil moiety, which is also found in **1**, has also been observed; coupling between the H-2 multiplet at 4.30 ppm and the two protons at 2.82 ppm along with aromatic proton pattern allows its identification.

The ^{1}H and ^{13}C NMR spectra reveal the presence of two olefinic protons. The singlet at 7.55 ppm is attributed to the vinyl hydrogen atom H-1, linked to a carbon bearing an oxygen atom, as in compound **2**; in fact, the chemical shift of C-1 (155.70) suggests its linkage with oxygen. The second olefinic proton appears to be a quartet at 5.65 ppm and consequently has to be attributed to a proton coupled to a methyl group; such a proton is in position 5, shifted upfield from 6.03 to 5.65 with respect to **1**.

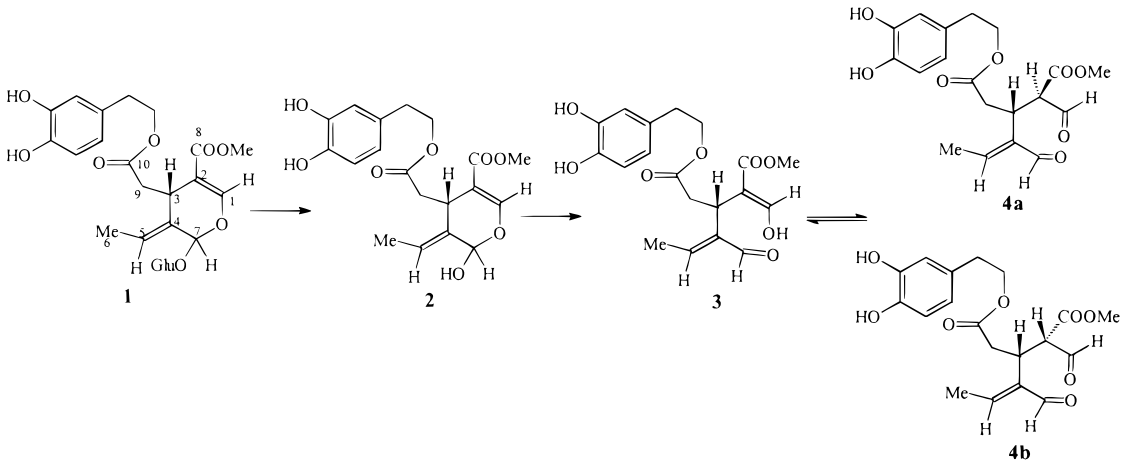
Irradiation of the methyl doublet at 1.65 ppm causes the collapse of the H-5 quartet, and, in contrast, when H-5 is irradiated, the doublet becomes nearly a singlet. Heteronuclear correlation experiments allows the C-5 resonance at 121.0 ppm to be assigned.

The acetalic proton H-7 resonates as a doublet at 4.25 ppm; a 0.7 Hz allylic coupling between H-7 and H-5 confirms the attribution. Furthermore, ^{1}H−^{1}H long-range couplings between H-5 and H-3 (dd, 3.6 ppm), H-1 and H-7, and H-1 and H-3 establishes the presence of a dihydropirane ring with a secoiridoid-type carbon atom as in **2**.

Detailed long-range coupling analysis observed in the ^{1}H−^{13}C HBMC confirms the attributions; thus, the long-range coupling between H-1 and H-9 (m, 2.55 and 2.75 ppm) and H-3 and the carboxylic C-10 (168.5) is in agreement with the proposed structure. Finally, a long-range coupling between the ester-like C-10 and H-1′, together with the COSY between H-1′ (3.60 ppm) and H-2′ (2.82 ppm), indicates the presence of a CH_2−CH_2 moiety, bonded to the ester function at C-10. The long-range coupling from H-2′ to the aromatic carbon atom at 141 ppm links this unit to the ortho-diphenolic ring, as required by structure **2**; the benzene substitution pattern is corroborated by similar ^{13}C chemical shifts of the corresponding unit in **1**.

In particular, the hemiacetalic isoprenoid derivative **2** can be considered to belong to the group of molecules known as natural chemical messenger, generally defined as semiochemicals (Pickett, 1997). The activity of this secondary metabolite can be synergized by other

Scheme 2. Transformation Pathway of 1 in $CDCl_3$



molecular component releases, vide infra, but together or alone, can give rise to a pathogen physiological response.

Compound **2** is stable for 2 days without solvents, undergoing a fast chemical conversion in water solution, leading to aldehydes **5a** and **5b**, while readily converting to a mixture of oleuropeinenol **3** and oleuropeindiales **4a** and **4b**, as explained below, in $CDCl_3$ and acid catalytic conditions.

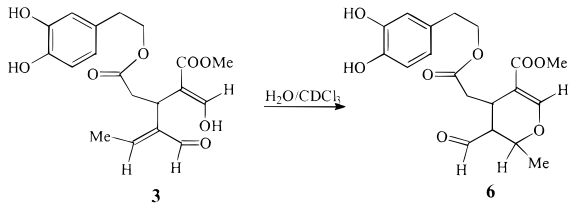
^{1}H NMR data of aldehydes **5a** and **5b** are consistent with the attributed structure. NOEDS experiments are in accord with structure **5**, as well. In particular, aldehydic resonance irradiation at 8.95 and 8.90 ppm, assigned to **5a** and **5b**, respectively, resulted in a NOE effect for the multiplet at 6.72 ppm and in a somewhat minor enhancement of two overlapping doublets around 1.9 ppm, thus confirming the presence of an α,β-unsaturated aldehydic system.

Compound **5a** is the most important aldehydic component which is formed as the kinetic one. Two compounds derive from the opening of the secoiridoid ring, through the corresponding enolic species **3**, previously detected in lipophilic phase (Bianco, 1999b) (Scheme 2). The presence of water gives rise to the formation of diastereoisomeric hydrated derivatives (Limiroli, 1995). The relative stereochemistry has already been assigned to the biomolecules under investigation (Limiroli, 1995): C-5 configuration, being *S* in **1**, remains unchanged, while C-4 acquires an *R* configuration in **5a**.

With time, the amount of **5a** diminishes, while **5b** increases. The biomimetic enzymatic reaction has been followed by monitoring the ^{1}H NMR spectra of the crude mixture at different times; after 5 h, the relative amounts of **5a** and **5b** are nearly the same and remain unaltered for prolonged times. Diastrereoisomers **5a** and **5b** appear to be stable at room temperature for several days; no trace of the ultimate conversion products, the elenolates **6**, has been observed after 3 days. At 60 °C, however, a rapid rearrangement into **6** has been verified (Scheme 3).

The biomimetic experiment thus accomplished supports the rapid, irreversible conversion of aglycon **2**, during the first enzymatic reaction step, as a molecular evolution consequence of the hemiacetal functionality of **2**, formed by glycosidic bond cleavage, without any secoiridoid ring opening and reclosure, since only C-7 appears as a point of chirality in the NMR practice. The BP derivatives of **1** first originated undergo further molecular modification with different lifetimes, according to the reaction system, before being transformed into final stable biomolecules, the elenolates **6**.

Scheme 3. Formation of Secoiridoid 6, the Ultimate Rearrangement Product



An analogous enzymatic hydrolysis experiment of **1** has been performed in a $CDCl_3/D_2O$ mixture; the two phases have been analyzed separately. Five minutes after endogenous β-glucosidase addition, the $CDCl_3$ solution shows the presence of enolic compound **3** and diastereomeric aldehydes **4.**

Aldehydes **4** were also obtained as an equilibrium mixture from acetal **2** by straightforward acid treatment. Their configurations have been assigned on the basis of the following considerations: the water phase shows the presence of **5a** only, 1 min after the β-glucosidase addition, while **4a** is unique in $CDCl_3$, showing a strictly similar stereochemistry at the C-4 chiral center.

In $CDCl_3$ solution, diastereoisomers **4a** and **4b** appear to be stable for several days: the atmospheric oxygen induces, however, a slow oxidation process at the aldehydic group level, as indicated by the resonance observed at 11.83 and 11.79 δ.

The above-discussed solution has been extracted with D_2O, followed by the t independent analysis of the two phases. The hydrophilic phase shows trace presence of aldehydes **4** in hydrated forms only, the *gem*-diols **5**, while $CDCl_3$ solution revealed, besides **4a** and **4b**, an increase of one component, identified as elenolates **6**, as previously reported.

The above-treated experimental findings clearly demonstrate that the lipidic/water interface promotes the rapid aldehyde **4** rearrangement into elenolates **6,** within 5 min; the hydratation process appears to be slow with respect to the elenolate formation in this environmental continuum.

The biomimetic experiment thus drawn allows the detailed description of the complete molecular transformation sequence, occurring during the enzymatic hydrolysis of **1**, as reported in Scheme 3.

The dialdehydic metabolites of **1** reveal molecular structures which are similar to those having related functional groups found in **4**, and deter plant consumption by modifying the taste of the plant (Luszniak, 1998). In this manner, bioderivatives **4**, being antifeedants produced by the olive fruit in response to damage caused by pathogen process, can act as signals to modify insect behavior.

The new metabolites evidenced in abiotic environment can be considered molecular derivative precursors, intervening as bioactive moiety in the defense mechanism of olive fruits against pathogens and in the olive oil organoleptic characteristics, e.g., the pungent and bitter flavor.

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