Oleuropein Site Selective Hydrolysis by Technomimetic Nuclear Magnetic Resonance Experiments

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Technomimetic NMR experiments were performed in accordance with the lye treatment adopted during table olive industrial procedures for the debittering process causing oleuropein degradation. The site selective hydrolysis of the two ester groups, characterizing the biophenolic secoiridoid molecule, was shown to be dependent on the different reactivities of these functionalities. The process is controlled by the experimental conditions exerted on the olive pulp and determined by the buffering capacity of the olive mesocarp and by the epicarp molecular components influencing the reactant penetration into the fruit pulp. The overall hydrolytic process of oleuropein, the bitter principle of olives, using the technomimetic experimental mode, gave rise to its catabolic derivatives, hydroxytyrosol, 11-methyloleoside, and the monoterpene glucoside, technomimetically produced, isolated, and structurally characterized by ^{1}H, ^{13}C, and COSY spectroscopy as the oleoside.

Keywords: *Basic oleuropein hydrolysis; technomimetic NMR experiments; biophenols and secoiridoids; table olive processing*

INTRODUCTION

Olives have long been processed as a traditional food (Connell, 1994) of the Mediterranean aliment culture (MAC) by several methods (Balatsouras, 1996; Borzillo et al., 2000). All of these employ a preliminary bio- or technotreatment to remove the bitter components. The most relevant is oleuropein (**1**) (Scheme 1), distinctive of the Oleacae fruits. **1**, because of its strong bitter taste (Bianco et al., 1998), with the molecular structure of biophenolic (BP) secoiridoid glucoside, must be removed, although it is not harmful to the health (Saija et al., 1998). Traditional recipes, the organic ones, and industrial procedures, the techno ones, treat suitably chosen olives with organic methods or with alkaline reagents, which can also affect further fermentation steps (Brenes et al., 1995).

An understanding of the relationship between the composition of olive raw materials and transformation and the final quality of MAC table olives is essential for exploiting scientific criteria to ensure consistency in manufacturing, for raw material specification, for the selection of crop varieties in olive plant breeding programs, and to identify suitable targets for genetic modification of olive cultural products.

The information acquired can be valuable to the final product of the table olive food chain (e.g., to the retail sector) and for understanding what determines table olive MAC quality by means of appropriate investigation at the biomolecular level of postharvest changes, particularly in relation to the composition of the raw material for table olives, with the focus on texture, color, and flavor attributes.

Appropriate experiments can disclose how molecular composition and modification of the raw material for olives, and postharvest treatment, affect the texture, color, and flavor of the MAC agrifood. They will explain the physical and molecular enzymatic principles underlying the development of these quality attributes in the olive plant foods and increase the current level of understanding of the technomolecular mechanisms involved. Molecular modification changes the composition and properties of the olive produce with respect to flavor generation processes.

The most highly developed industrial technology requires an alkaline reaction, with dilute soda solutions, as a debittering process of olive fruits by the BP degradation procedure (Brenes et al., 1992). Other methods, the more ancient and traditional ones, more readily adapt to bio-organic procedures, such as the Oinotria method (Borzillo et al., 2000) and the Ferrandina method (Brighigna, 1998).

Several relevant features of olive fruits can be related to their BP content (Bianco and Uccella, 2000; Romani et al., 1999), that is, bitterness (Tsimidou, 1998) and hedonistic−sensorial characteristics (Casuscelli et al., 1994), antioxidant activity (Visioli and Galli, 1998), inhibition of pathogen and insect attack (Lo Scalzo et al., 1994), and microbial activity (Bisignano et al., 1999).

Being natural hydroxy aromatic derivatives of low molecular weight, in the range of 500, olive BPs are characterized by multifunctional moieties, for example, secoiridoid monoterpene glucosides, although the term "polyphenols" can better be applied to biomolecular structures having 500−4000 MW, 12−16 phenolic groups, and 5−7 aromatic rings (Haslam, 1998).

As shown in Scheme 1, BP functionality, in the conjugated form **1** with the terpene unit and simple **2** form (Bianco and Uccella, 2000) can be responsible for free radical quenching and metal chelation (Saija et al., 1999) by the *o*-dihydroxy aromatic moiety (Russo et al., 1999). The other groups are also involved in several biological activities (Castelli et al., 1999). Thus, these

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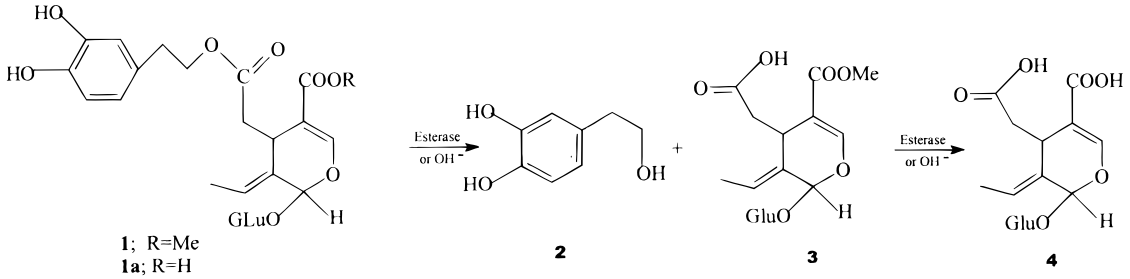
Scheme 1



natural secondary metabolites, found in olive fruits, can exhibit an important antimicrobial and antioxidant role (Bisignano et al., 1999) with the complexity of their molecular structure, comprised of the BP groups (Vekey et al., 1997; Bianco et al., 1997).

Complex BPs (Bianco et al., 1999b) can generate the sensory perception of the bitter and pungent throat-catching taste (Gutierrez et al., 1989), mainly because their secoiridoid original structure (Borzillo et al., 2000) undergoes further molecular modifications (Bianco et al., 1999a) after hydrolysis by endogenous β-glucosidase, occurring in the olive mesocarp (Bianco et al., 1999b).

The major BP component of olives has been found to be oleuropein (**1**) (Amiot et al., 1986). As described in Scheme 1, this is a bifunctional ester at the bident carboxylic moiety of oleoside (**4**) (Kuwajima et al., 1988), resulting from the conjugation with methanol and hydroxytyrosol (**2**) or esterified by **2** at the free position of 11-methyl oleoside (**3**) (Damtoft et al., 1992). Thus, the two pending carboxylic groups of the monoterpene unit **4**, having the proposed secoiridoid structure of oleoside (Inouye et al., 1974), should reveal different reactivities toward esterases and basic attack when conjugated as in the BP **1**.

BP **1** appears to be responsible for the bitter taste and for the fermentation control of the olive drupes (Marsilio et al., 1996). During fruit ripening, its amount decreases (Amiot et al., 1989) but is still relevant in table olive processing (Brenes et al., 1995), being degraded by enzymatic and chemical catalysis (Bianco et al., 1999a). Endogenous β-glucosidase attack releases the initial aglycon; this undergoes further complex molecular trasformation (Bianco et al., 1999b; Limiroli et al., 1995, 1996). Specific esterase enzymes could cleave the bident **1** at the ester groups, giving rise alternatively to **3** (Inouye et al., 1974) and to demethyloleuropein (Ragazzi et al., 1973), both found in ripe olives. The complete degradation of **1**, with subsequent sweetening of the fruit, leads to the hydrolytic derivatives **2** and **3** by alkaline treatment for several hours at room temperature, utilizing a large excess of the basic reagent (Garrido Fernandez et al., 1997a; Brenes et al., 1995; Brenes and de Castro, 1998).

Molecular transformation thus occurs from the BP precursor **1** to its already ascertained derivatives, **2** and **3**. The overall hydrolytic reaction and the byproduct **4** deserve great attention because of their antimicrobial, pathogenic, and free radical quenching action and due to their effects on the color, composition, and functional aspects (Goldberg, 1994) of the final processed table olives.

The table olive food constituents **1**−**4**, and other related ones, can reveal biological activities associated with anticarcinogenity, antimutagenity, and antiaging, recently attracting interest as the third molecular components of MAC foods after the nutrient and hedonistic ones (Wiseman et al., 1999).

A detailed investigation of the overall molecular transformation undergone by **1** during alkaline treatment can be performed by technomimetic experiments on **1** with the NMR mode (Bianco et al., 1999a), which provides valuable information significant for the processing technologies of table olives.

EXPERIMENTAL PROCEDURES

Instrumentation. NMR measurements for ^{1}H at 300.13 MHz and for ^{13}C at 72.42 MHz were performed on a Varian VXR-300 (Palo Alto, CA), equipped with a temperature control unit, and on a Bruker AC-300 spectrometer, controlled by Aspect 3000 computers. The sample (15 mg) was dissolved in deuterium oxide (0.6 mL). The COSY experiment was performed using standard Bruker programs, and most acquisition parameters were used as suggested by the programs. The ^{1}H−^{1}H COSY parameters were as follows: spectral width, 3597 Hz; acquisition time, 2.27 s; relaxation delay, 2 s. The FIDs were zero-filled to 512 data points prior to FT sine-bell window function for resolution enhancement. Chemical shifts were referred to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) in water and to tetramethylsilane (TMS) in organic solvents.

The technomimetic hydrolysis reactions were carried out directly in an NMR tube, as D_2O solution, at a probe temperature of 25 °C, by monitoring all of the hydrolytic conversion steps. In this way, the structures of products **2**−**4** formed were investigated in situ, in the same aqueous reaction medium, avoiding any further workup that might affect the nature of the process intermediates. The product evolution was followed in the same tube, without any molecular manipulation. The reaction course was monitored at different times.

Materials and Methods. *Technomimetic Hydrolysis of **1** in D_2O Solution.* The NMR samples were prepared, in a polypropylene micro sample tube (1.5 mL capacity), by dissolving **1** (22.5 mg) in D_2O (1 mL) and adding NaOD/D_2O solution (5 μL, 8.1 M), with the resulting measured pH* of 12.7. The apparent pH* of the final solution, not corrected for the isotope effect, was measured by means of a Crison GLP22 apparatus, equipped with a combined microelectrode Crison 52.08 (o.d. = 3 mm). The solution was then transferred in a 5 mm NMR tube for the spectroscopic analysis. The ^{1}H NMR spectra were recorded, every 5 min until 40 min, all **1** resonances being lost, and at 25 °C probe temperature.

The ^{1}H NMR spectrum, recorded starting 5 min after the addition of NaOD, showed signals of resonance disappearance at 1.55 (H-10, d, J = 7.2 Hz), 3.68 (s), 5.73 (H-1, s), and 7.48 (H-3, s) ppm and new resonance appearance at 1.68 (d, J = 6.9 Hz), 3.70 (s), 5.92 (s), 7.51 (s).

The hydrolysis of **1,** after 40 min, reveals the total absence of its original resonance, with the signals of free **2** and **3** sodium salt **5**. Derivative **3** was isolated, purified by acidification, flash chromatographed on a silica gel column with ethyl acetate/methanol (90:10), and structurally confirmed according to spectroscopic reference for methyl oleoside (Damtoft et al., 1992) by the ^{1}H NMR spectrum: δ (D_2O) 7.55 (s, H-3), 6.10

(q, H-9), 5.95 (s, H-1), 4.90 (d, H−G_1), 4.12 (dd, H-5), 3.91 (t, H-G_5), 3.71 (s, H-12), 3.65 (m, H-G_6), 3.40−3.50 (m, H-$G_{2,3,4}$), 2.71 (dd, H-6a), 2.39 (dd, H-6b), 1.67 (d, H-10).

After 24 h, NaOD/D_2O solution (5 μL) was added to the previously described D_2O solution, showing the occurrence of the hydrolytic derivative of **3** with the formation of the novel secoiridoid monoterpene salt **6** of oleoside **4**, revealing ^{1}H NMR signals at 7.20 (H-3, s), 5.89 (H-1, s), and 4.88 (anomeric proton, d, J = 5.1 Hz). Further resonances were at 3.85 (H-5, m), 6.05 (H-9, q, J = 6.3 Hz), 1.70 (CH_3-10, d, J = 6.3 Hz), 2.05 (H-6a, dd, J = 14.5 and 2.4 Hz), and 2.83 (H-6b, dd, J = 14.5 and 3.2 Hz); ^{13}C NMR δ (D_2O) 15.76, 34.19, 46.28, 63.42, 72.70, 75.49, 78.48, 79.26, 97.79, 102.46, 112.15, 126.41, 131.63, 156.75, 172.25, 182.65.

Oleuropein **1** (500 mg), dissolved in H_2O (20 mL), was then treated with NaOH (40 mg), 1:1 ratio, at pH 12.6, for 1 h at room temperature. The reaction mixture, neutralized, evaporated at reduced pressure, and flash-chromatographed as above-described, gave the methyl oleoside **3**. The same experiment, carried out with NaOH (80 mg), afforded the oleoside **4** after the same isolation and purification procedures.

Oleuropein **1** (250 mg) was dissolved in H_2O (10 mL) and treated with NaOH (40 mg), 1:2 ratio, pH 11.0, leading to **5**.

1 (100 mg) in H_2O (10 mL) with Na_2CO_3 (0.6 g) was left to react for 3 h at room temperature and pH 11.5. After workup, the secoiridoid derivative **3** was recovered only with 90% overall yield.

RESULTS AND DISCUSSION

The technomimetic reactivity of **1** was investigated by performing alkaline hydrolysis at different times, pH values, and molar ratios of the reactants, according to the technological procedure applied for table olive processing (Garrido Fernandez et al., 1997).

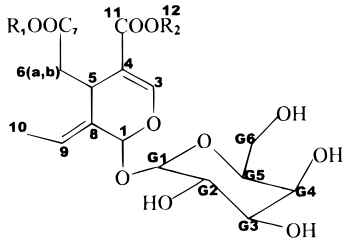
Therefore, the NaOD/D_2O solution was added to **1** and dissolved in D_2O to achieve a nearly equimolar ratio of reactants; the pH of the solution was 12.7.

The hydrolysis reaction was carried out directly in the NMR tube and D_2O solution, at a probe temperature of 25 °C; each step of the hydrolytic technoconversion was monitored. In this way, the structures of the products formed were able to be investigated in situ, in the same aqueous reaction medium, mimicking the technoprocedures and thus avoiding any further workup that might modify the molecular structure of the hydrolytes derived from the overall process. The obtained products **5** and **6** were thus analyzed without any further molecular manipulation before the corresponding free carboxylic acid **3** and **4** were isolated.

The reaction course, monitored at different times with the ^{1}H NMR spectrum and recorded 4 min after the addition of NaOD, showed signals indicating the occurrence of the hydrolytic process, easily observable by the disappearance of the resonance of **1** and the appearance of a new resonance set reported under Experimental Procedures.

After 40 min, the hydrolysis of **1** was nearly complete, as deduced from the disappearance of its resonances: the ^{1}H NMR spectrum revealed the signals of the free **2** and the salt **5** of methyl oleoside **3**, which was confirmed by spectroscopic methods and also by reference to the previously reported spectrum for **3** (Gariboldi et al., 1986), given under Experimental Procedures. The entire spectrum analysis indicated several difficulties in the region of 3.4−3.9 ppm, the signals of a glucose area. Under investigation, the precise assignment of the chemical shift to each proton in the molecule **3** (Chart 1 and Figure 1) could not readily be achieved. Two-dimensional (2D) methods were adopted to tackle the resolution problem of such complexity, offering advantages in terms of both efficiency and resolution over the 1D approach.

Chart 1



	R_1	R_2
3	H	CH_3
4	H	H
5	Na	CH_3
6	Na	Na

The 2D ^{1}H−^{1}H COSY homonuclear shift correlation, the most important measuring technique of the 2D experiment by NMR spectroscopy, refers to both frequency axes containing chemical shift and so-called cross-peaks, indicating which nuclei are spin−spin coupled. The detection and structural confirmation of the technomolecule, obtained by the hydrolytic process of **1**, were thus performed. Cross-peaks were observed between glucose protons. The axial H−G_1 and H−G_2 in the β-glucosidic linkage were doublets.

The connection of the H−G_5 and H−G_6 spin system can unequivocally be derived from its COSY spectrum. The protons of the free −CH_2OH group are equivalent, and the cross-peak correlation was easily achieved. The −CH_2OR group in **6** yields cross-peaks between the H-6 and diastereotopic protons H-6a and H-6b, the signal being two doublets.

Under the reaction conditions described above, the hydrolytic process did not proceed further: the ^{1}H NMR spectrum, recorded 24 h later, did not show any modification. The technomimetic experiment thus indicated that the most reactive ester group was actually the one at C-7 of the secoiridoid moiety. The addition of another aliquot of NaOD induced a fast hydrolysis of the less reactive estereal group, as shown by the disappearance of **5** signals at 7.51 (s), 5.92 (s), and 3.70 (s): the ^{1}H NMR spectrum gave the resonances of the free methanol at 3.35 ppm and the salt **6** of oleoside **4**. The chemical shift assigned to each proton of the molecular structure could provide the identification of **4**. This could be further improved by the performance of a 2D analysis of the whole spectrum (Figure 2), thus resolving the structural complexity reported in Chart 1. In fact, the same complications appeared in the 3.4−3.9 ppm region, as already found for **3**, because of the glucose signals. The COSY experiment prevented us from detecting and structurally identifying the novel secoiridoid hydrolytes of **1** and **3**, that is, the glucoside **4**, as the technoderivative obtained from the mimetic process in the chemically catalyzed debittering of olive raw material.

The reactions reported above, leading from **1** to **3** and **4**, according to the experimental conditions described, were also performed on a preparatory scale, thus allowing for the first time the isolation and identification of **4** among the minor components of technotreated table olives, which was only postulated previously (Inouye et al., 1974).

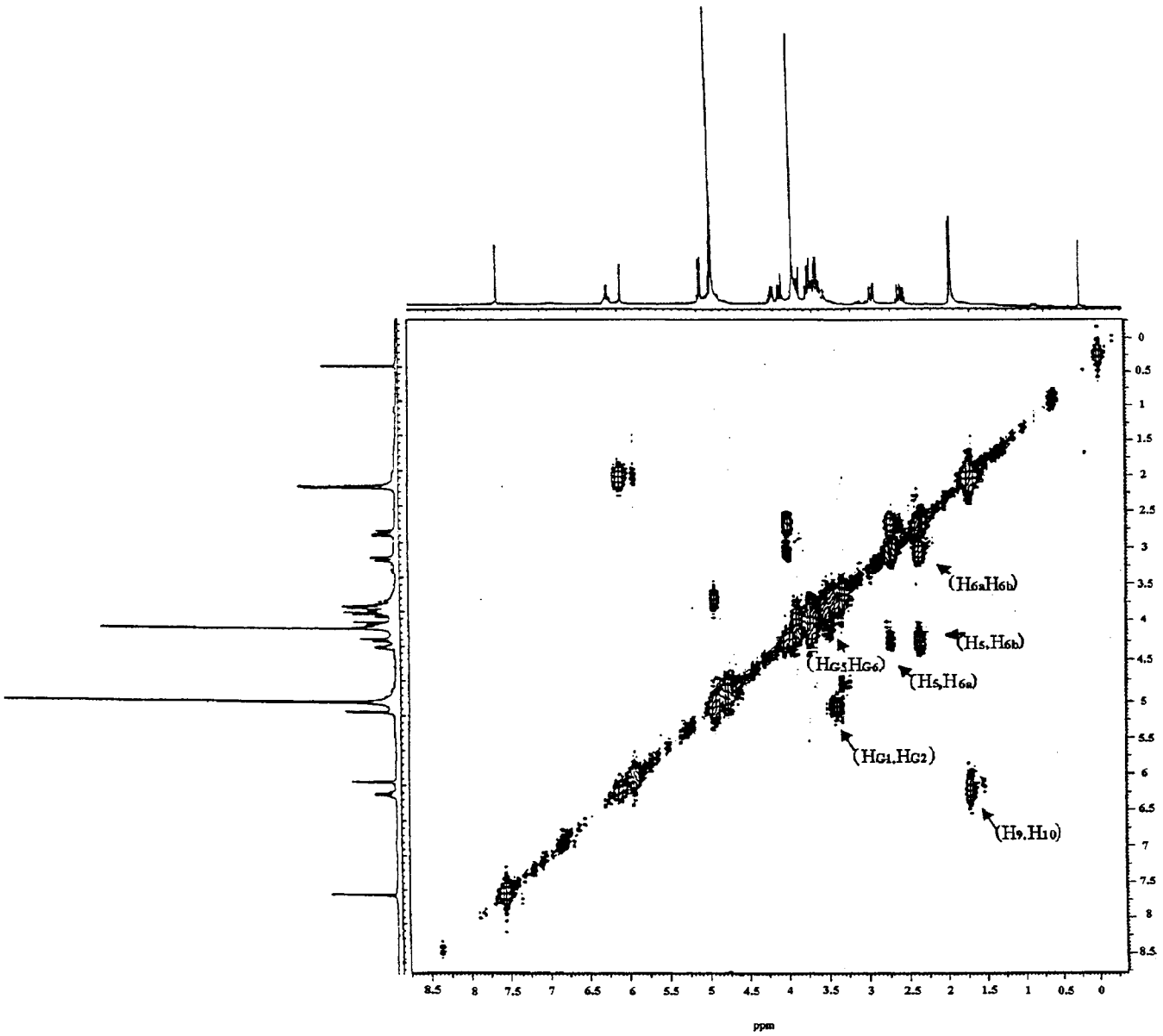


Figure 1. One-dimensional and COSY spectra of 11-methyloleoside **3**.

In a different set of experiments, under the conditions reported above, the hydrolysis reaction was not complete, leading to the formation of methyl oleoside sodium salt **5**.

To ascertain the effect of an excess of reactant on the overall hydrolytic degradation of **1**, as applied during the technology of table olive production, BP **1** was made to react as described in the experiment, thus showing that the overall hydrolytic process is controlled by the pH of the technomimetic procedure.

The technomimetic reactions performed on the olive component **1** indicate the exceptionally large site selectivity, occurring during base-catalyzed hydrolysis of BP **1**; this result appears to be similar to the physiological process, enzymatically catalyzed, which occurs as olive maturation proceeds, and to the technological treatment by alkaline solution. In all of the reaction media above considered, BP **1** offers a bident functional esteric site for the conversion to carboxylate, releasing **2** in one case and methanol in the other. The technomimetic model is under controlled experimental conditions; the pH has been varied in the range of 12.7 and 11.0 and the molar ratio in the range of 1:1 and 1:2. In effect, the hydrolytic conversion of the bident substrate **1** shows the expected dependence on hydroxide ion concentration, molar ratio, and substituent effect in the base-catalyzed process. The electron-releasing substituent, the vinyl ether one, on one acyl group, that is, that on C-4 of the heterocyclic ring, reduces hydrolytic reactivity, the transition state of this group being characterized by greater negative charge than the alternative reaction site on C-7. Basic hydrolysis of the two carboxylic ester moieties, respectively on C-4 and C-7, must follow a reaction mechanism similar to those already investigated (Isaacs, 1987). Entropies of activation for the technomimetic process thus described should be large, negative, and very sensitive to structure change of the reacting site, that is, C-4 and C-7 of BP **1**. Therefore, the rates of the hydrolytic reaction are strongly accelerated by an electron-withdrawing group, whereas the vinyl ether one, being an electron-releasing group, reduces the alkaline fission of **1** into its hydrolytes, methoxyde and demethyloleuropein anions. This is in agreement with the well-known behavior of substituent effects on nu-

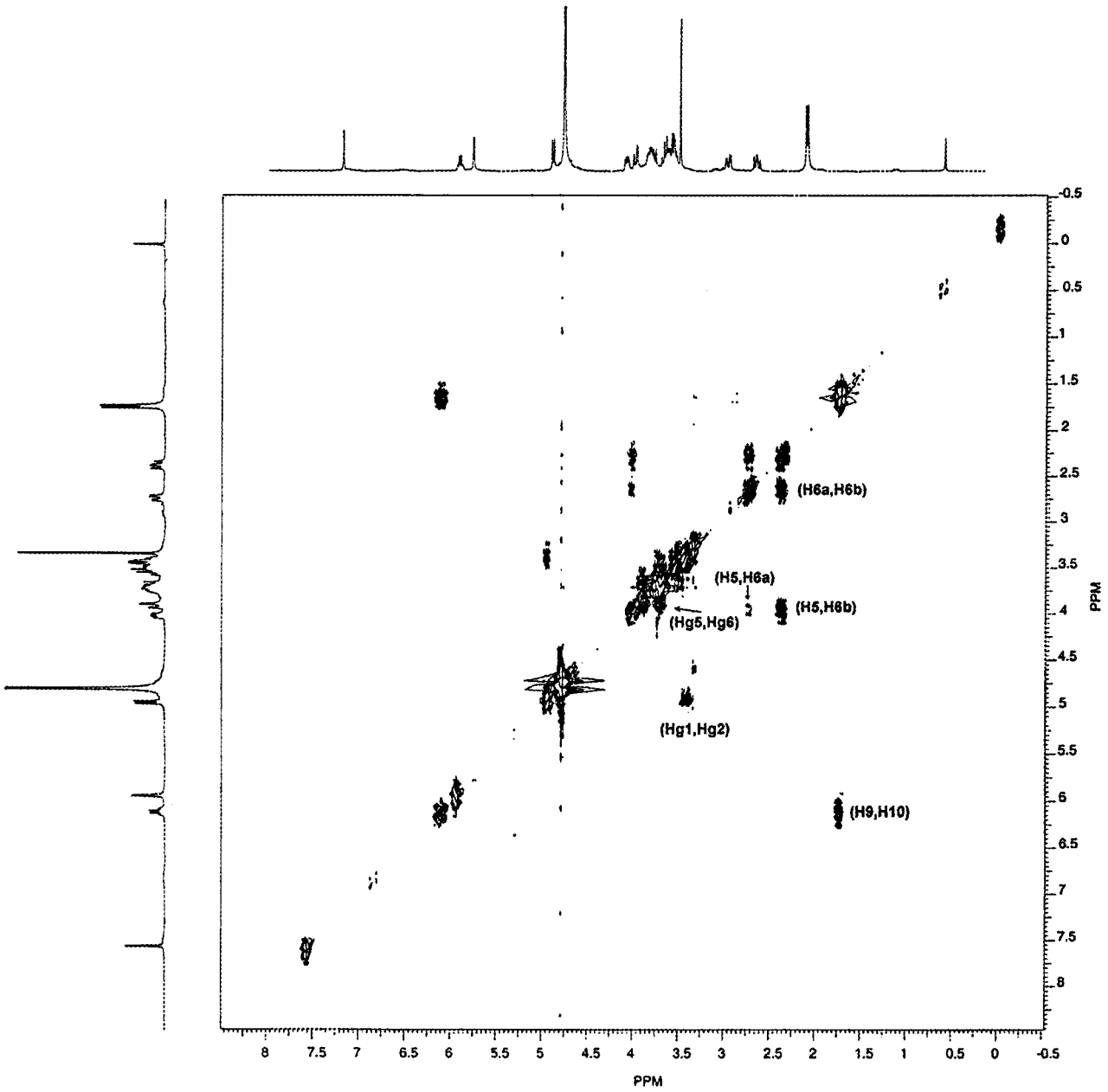


Figure 2. One-dimensional and COSY spectra of oleoside **4**.

cleophilic substitutions at the carbonyl carbons C-4 and C-7 of BP **1** (Isaacs, 1987).

Even though the BP **1** content changes during maturation of the olive fruits and diminishes during growth, there is still a considerable amount of it at harvest time, especially in the green-picked crop. In olive trees, a decrease of **1** coincides with the conversion to secoiridoidic glucosides **3** and **4** due to the biohydrolytic loss of one reactive site, that is, that esterified with the **2** moiety, whereas the enzymatic esterase cleavage can give rise to demethyloleuropein in the less reactive functional group of some olive cultivars. The biochemical relationship between the two hydrolytic derivatives must be linked to a different esterase reactivity responsible for the methyl ester scission. The latter cultivar characteristics can greatly influence the sensory attributes of the olive fruit produce, that is, olive oil and table olives. In effect, the olive produce could be affected by the occurrence of two different dialdehydic derivatives, one from the endogenous β-glucosidase attack on **1**, the other from the same enzyme on demethyl-**1**, giving rise to byproducts with exceptionally distinctive reactivities due to their molecular structures.

The table olive industrial procedure was performed, under NMR technomimetic experimental conditions, according to the debittering process caused by the degradation of **1**. The site selective hydrolysis of the BP secoiridoid derivative is shown to be dependent on the different reactive ester groups, controlled by the experimental conditions exerted on the olive pulp and determined by the buffering capacity of the olive mesocarp and by the epicarp molecular components, limiting reactant penetration into the fruit pulp.

The overall hydrolytic process of **1**, the bitter principle of olives, leads to its derivatives, **2**, **3**, and the secoiridoid monoterpene glucoside, the oleoside **4**, which were isolated and structurally characterized. During lye treatment for olive production, the epicarp molecular components can initially act as a water barrier and also as an important natural barrier against pest and microbial infection. They must be partially removed to increase skin permeability during processing. These

minor protective constituents of olive fruits also control penetration of the alkaline solution, thus affecting the hydrolysis of **1**, which determines most table olive treatments and produces the selective formation of **3** versus **4**.

The experimentally observed selection process from **1** to **3** and **4** can be interpreted on the basis of the buffer capacity of the olive flesh. In effect, because the total organic acid content of the olive mesocarp tends to increase during growth, the pH also increases slightly, due to the combined acidity changes of up to 157 mM (Garrido Fernandez et al., 1997b): these variations modify the buffering capacity of the table olive pulp, thus generating the appropriate condition for the selective reactivity of **1**.

During the debittering process, however, the cell wall molecular structure and composition of fresh fruits are influenced by enzymatic molecular degradation and solubilization, for example, by the alkaline treatment technomimetically performed. Therefore, an important role in table olive texture and changes during the fruit processing is revealed by the initial relatively powerful alkaline procedure, which brings about changes in the consistency and palatability of the final processed produce.

Skin permeability, thus related to the epicarp molecular constituents of the selected olive cultivar, could appear to be the major limit in the debittering process because the alkaline hydrolysis of BP **1** requires milder basic conditions than those industrially applied. Therefore, the concentration of the lye treatment mainly serves to penetrate the skin through the cutin, affecting the molecular structure of the wax esters, the morphological structure of the olive skin, and the overall texture of the final table olive products.

Thus, an initial treatment with a concentrated lye would reasonably be followed by a more dilute one or by a suitable basic solution. The critical biomolecular structure of the olive skin components deserves attention in the improvement of the debittering process, which would allow also for a better texture of the final product because the high NaOH concentration could give rise to β-elimination of pectic and cellulase moieties (Jimenez et al., 1997).

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