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Synthesis, Biological Evaluation, and Molecular Modeling of Oleuropein and Its Semisynthetic Derivatives as Cyclooxygenase Inhibitors

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Oleuropein, the main phenolic compound in virgin olive oil, and several of its derivatives such as oleuropein aglycone, hydroxytyrosol, and their respective acetylated lipophilic forms were obtained by simple and environmentally friendly semisynthetic protocols. The same molecules were then tested in vitro and in vivo, comparing their intriguing anti-COX-1 and anti-COX-2 properties to those of well-known anti-inflammatory drugs such as ibuprofen and celecoxib. Finally, molecular modeling experiments displaying the most probable binding modes within the classical binding clefts of the enzymes suggest the heme moiety as a potential alternative target.

KEYWORDS: Oleuropein; hydroxytyrosol; COX-1 and COX-2 inhibitor; lanthanoid triflates; Lewis acid catalyst; molecular modeling

INTRODUCTION

Epidemiological studies have shown a relationship between the Mediterranean diet and a lowered incidence of pathologies such as cardiovascular diseases, cancer, and diabetes (*1*). Several studies attribute these health benefits to high consumption of virgin olive oil—rich in phenols and flavonoids, as well as in other typical components of the Mediterranean diet. The main phenolic compounds in virgin olive oil are secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (HTyr) and 2-(4-hydroxyphenyl)-ethanol (Tyr) that occur as either simple phenols or esterified with elenolic acid to form, respectively, oleuropein (Ole) and ligstroside aglycones (**Figure 1**) (*2*). Many of the biological activities attributed to these or to other natural phenolic derivatives have anti-inflammatory components (*1*, *3*, *4*), so various health benefits seem to overlap with those attributed to non-steroidal anti-inflammatory drugs (*5*, *6*). Recently, Bauchamp et al. (*7*) reported that oleocanthal, the dialdehydic form of deacetoxy-ligstroside aglycone, a well-known phenolic compound contained in virgin olive oil, can inhibit cyclooxygenase enzymes COX-1 and COX-2. Conclusions made in that paper triggered a great interest in the scientific community. Indeed, some very long multistep syntheses of oleocanthal were proposed recently (*7*−*9*). The principles of green chemistry have well emphasized the need to address increasing environmental concerns by adopting more sustainable strategies in fine synthetic processes, such as reducing the amount of chemicals, performing neat reactions, and adopting environmentally benign solvents (e.g., water, nonclassic solvents, etc.) (*10*, *11*). The use of mild Lewis acid catalysis has quickly increased in recent years (*12*). For example, we proposed a series of triflate derivatives as reagents, allowing several typical organic chemical transformations to occur in very mild conditions (*13*−*15*).

Thus, looking for a tangible application of these green chemical methodologies, we decided to apply our protocols to the chemical manipulation of a known natural product such as oleuropein. In this paper we report biological evaluation and molecular modeling results carried out with some natural and semisynthetic oleuropein derivatives obtained by means of chemical manipulation of compound **3**.

MATERIALS AND METHODS

LC/MS/ESI analyses were performed on a Dionex NANO LC Ultimate working with a C-18 column (i.d. 3 μm, 75 μm × 15 cm) and a mobile phase consisting of the following solvents: A (5% acetonitrile, 95% water + 0.1% formic acid, and 0.02% TFA); B (95% acetonitrile, 5% water + 0.1% formic acid, and 0.02% TFA). The gradient was as follows: 0−90 min, 70−10% A, 30−90% B; flow rate = 300 μL/min. Mass spectra were recorded on an Applied Biosystem ibrid Q-STAR XL spectrometer working in a positive mode. An Applied Biosystem DE-STR/MALDI/TOF spectrometer was used for MALDI/MS spectra. Samples were dissolved in a mixture 1:1 of water/acetonitrile + 5 mmol of NaCl and suspended in a matrix solution of 2,5-DHB (40 mg/mL). ^{1}H spectra were recorded on a Bruker WM 300 instrument on samples dissolved in $CDCl_3$. Chemical shifts are given in parts per million (ppm) from tetramethylsilane as the internal standard

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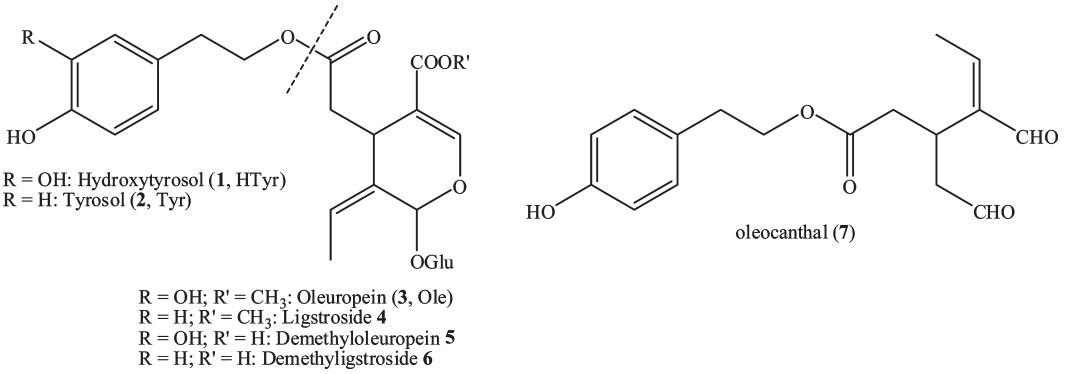

Figure 1. Chemical structures of compounds **1**−**7**.

(0.0 ppm). Coupling constants (*J*) are given in hertz. Reactions were monitored by a GC-MS Agilent workstation, formed by a GC-6890N (30 m Restek-5SIL capillary column, working in spitless mode, 1 mL/min He as carrier gas) and by an 5973N mass detector. MW-assisted reactions were performed in a domestic oven Daevoo KOR-63A5 with tunneling power from 200 to 800 W. TLC was performed using silica plates 60-F264 on alumina, commercially available from Merck. Liquid flash chromatography was performed on a Supelco versa flash HTFP station on silica cartridges commercially available from Supelco. A Jasco liquid chromatography instrument consisting of a ternary pump PU-1580, a Jasco MD-1510 diode array detector (DAD), and a manual injection valve (100 μL) was used for HPLC purity analysis. The mobile phase consisted of the following solvents: A ($H_2O + CF_3COOH$, pH 3.2) and B (CH_3CN). The gradient was as follows: 0−5 min, 100% A; 5−10 min, 100% B; 10−15 min, 100% B; 15−20 min, 100% A; flow rate = 1 mL/min. An Adsorbosphere 5U C-18 column (250 mm × 4.6 mm) was also used, and all chromatograms were recorded at 254 nm. All solvents were distilled before use according to standard methods. All chemicals were used as commercially available. [All reported compounds possess a purity of ≥95% as established by LC/MS analysis (see Supporting Information).]

Extraction MW-Assisted from Olive Leaves. *Samples*. The samples used for the extraction were olive leaves from Coratina cultivar of *Olea europaea*, collected, dried for 48 h at 50 °C, milled, and kept at room temperature until use.

Conventional Extraction Procedure. One hundred grams of dried and milled leaves and 1 L of water were refluxed at 100 °C for 8 h. Leaves were filtered, and the solution was dried under reduced pressure. The mixture was treated with acetone and filtered to eliminate the solid, and the solution was evaporated under reduced pressure to obtain the crude product. Oleuropein was separated by means of liquid chromatography on a Supelco Versa-Flash station equipped with a silica cartridge and eluted with a mixture of CH_2Cl_2/MeOH 8:2 v/v as the mobile phase.

MW-Assisted Extraction Procedure. One hundred grams of dried and milled leaves and 800 mL of water were placed in a Pyrex round-bottom flask equipped with a jacketed coiled condenser in a domestic microwave oven. After extraction [10 min of microwave irradiation at 800 W, shorter or longer extraction times did not improve the amounts of oleuropein obtained (**Table 1**)], leaves were filtered and the solution was dried under pressure. The mixture was filtered with acetone to eliminate the solid residue, and the solution was evaporated under pressure to obtain the crude product. Oleuropein was separated by liquid chromatography on a Supelco versa-flash station equipped with a silica cartridge and a mixture of CH_2Cl_2/MeOH 8:2 as mobile phase. The purity was determined by HPLC and NANO/LC/MS/ESI. LC/MS/ESI (+), *m*/*z* 558 (31) $[M + NH_4]^+$, 541 (16) $[M + H]^+$, 379 (76) $[M - Glu + NH_4]^+$, 361 (100) $[M - Glu + H]^+$

Synthesis of Hydroxytyrosol (***1***). The synthesis of hydroxytyrosol was performed in a classical manner starting from the treatment of 3,4-dihydroxyphenylacetic acid with EtOH in the presence of 10% v/v of H_2SO_4 and reduction of the ethyl ester with $NaBH_4$, as reported in the literature (*1*): ^{1}H NMR ($CDCl_3$) 2.53 ppm (t, 3H, H_2, $J_{H_2H_3}$ = 7.44 Hz), 3.17 ppm (s, 1, OH), 3.49 ppm (t, 2H, H_3, $J_{H_3H_2}$ = 7.44 Hz), 6.66−6.41 ppm (m, 1H, H_{Ar}), 6.60−6.58 ppm (m, 2H, H_{Ar}); MS/EI, *m*/*z* = 136 (100) $[M - H_2O]^+$, 43 (17) $[MeCO]^+$.

Table 1. Microwave Extraction Protocols of Oleuropein in Water

entry	time (min)	% w/w oleuropein
1	5	0.88
2	10	2.17
3	15	2.00
4	20	2.00
5	30	2.00
6	480[a]	0.5

[a] Performed in refluxing water by conventional heating.

Synthesis of Aglycons (***8a*−*e***). Oleuropein **3** (740 mg, 1,34 mmol) was dissolved in aqueous CH_3CN (12,7 mL) in the presence of 10 mol % of $Er(OTf)_3$ (123.5 mg, 0.20 mmol) and refluxed for 8 h at 80 °C. At the end of the conversion, the hydrolysate was cooled, 5 mL of water was added, and the mixture was extracted with CH_2Cl_2. After drying on Na_2SO_4, the organic solvent was removed in vacuo and the crude product was purified by flash chromatography (mobile phase CH_2Cl_2/MeOH 8:2 v/v), obtaining the aglycon as a mixture of three isomers (**8a**−**c**), a hydrated form (**8d**), and a methanolate form (**8e**), which were all characterized by HPLC, LC/MS/ESI, and ^{1}H NMR (total yield = 70%). ^{1}H NMR ($CDCl_3$) δ 1.40 [d, 3H, CH_3 (8c), $J_{H_{CH_3}H_1}$ = 6.63 Hz], 1.40 [d, 3H, H_{10} (8b), $J_{H_{10}H_8}$ = 6.75 Hz], 2.13−2.28 [m, 2H, H_6 (8c), H_{6a} (8c)], 2.50−2.63 [m, 2H, H_6 (8b), H_{6a} (8b)], 2.78−2.85 [t, 2H, H_7 (8b), $J_{H_7H_8}$ = 5.77 Hz], 2.87 (t, 2H, H_7, $J_{H_7H_8}$ = 5.85 Hz), 3.35 [m, 1H, H_4 (8b), $J_{H_4H_5}$ = 8.89 Hz], 3.74 [s, 3H, OCH_3 (8c)], 3.77 [s, 3H, OCH_3 (8b)], 4.10−4.50 [m, 6H, H_5 (8c), H_5 (8b), $2H_8$ (8c), $2H_8$ (8b)], 6.62[m, 2H, $H_{aromatic}$ (8b)], 6.75 [m, 2H, $H_{aromatic}$ (8c)], 7.26 [s, 2H, $H_{aromatic}$ (8b, 8c)], 7.58 [s, 1H, H_3(8c)], 9.52 [s, 1H, H_1(8b)], 9.60 [s, 1H, H_3 (8b)], 9.80 [s, 1H, H_{10} (8c)]. The cyclic isomer **8a** is present only in trace in the ^{1}H NMR spectrum. LC/MS/ESI (+), t_r = 22.70 min **8a**−**c**, *m*/*z* 379 (100) $[M + H]^+$, 347 (11) $[M - MeOH + H]^+$, 243 (4) $[M - H]^+$, 225 (12), 135 (26); t_r = 27.08 min **8d** (R = OH), *m*/*z* 361 (100) $[M - OH + H]^+$, 137 (40); t_r = 28.11 **8e** (R = OMe), *m*/*z* 393 (1) $[M - OH]^+$, 361 (100) $[M - MeOH - OH]^+$, 137 (11).

Synthesis of Peracetylated Hydroxytyrosol (***9***). Purified hydroxytyrosol (200 mg, 1,3 mmol) was reacted for 2 h at room temperature with an excess of dry acetic anhydride (5 mL) under N_2 atmosphere in the presence of 2 mol % of $Er(OTf)_3$ (0.026 mmol, 15.96 mg). At the end of the conversion, 12 mL of MeOH was added to the mixture and stirred for 1 h. Solvent was removed under vacuum, and the residue was solubilized in CH_2Cl_2. The mixture was extracted with a satured solution of $NaHCO_3$ until complete elimination of the acetic acid; the organic phases collected were dried on Na_2SO_4, and filtered, and the solvent was evaporated under pressure. The crude was purified by flash chromatography on silica gel (mobile phase CH_2Cl_2/MeOH 8:2) to isolate the pure product (80% yield): ^{1}H NMR ($CDCl_3$) 2.03 ppm (s, 3H, H_{Ac}), 2.28 ppm (s, 2 × 3H, $H_{Ac.ar.}$), 2.93 ppm (t, 2H, H_7, $J_{H_7H_8}$ = 6.90 Hz), 4.27 (t, 2H, H_8, $J_{H_8H_7}$ = 6.90 Hz), 7.05−7.11 ppm (m, 3H, H_{Ar}); MS/EI, *m*/*z* = 238 (1) $[M - CH_2CO]^+$, 220 (6) $[M - CH_3COOH]^+$, 178 (18) $[M - CH_3COOH - CH_2CO]^+$, 136 (100) $[M - CH_3COOH - 2 \times CH_2CO]^+$, 43 (24) $[MeCO]^+$.

Synthesis of Peracetylated Oleuropein (***10***). Purified oleuropein **3** (250 mg, 0,463 mmol) was allowed to react for 2 h at room temperature with an excess of dry acetic anhydride (5 mL) under N_2 atmosphere in the presence of 2 mol % of $Er(OTf)_3$ (0.009 mmol, 6.5 mg). At the end of the conversion, 12 mL of MeOH was added to the mixture and stirred for 1 h. The solvent was removed in vacuo, and the residue was solubilized in CH_2Cl_2. The mixture was extracted with a saturated solution of $NaHCO_3$ until complete elimination of the acetic acid; the organic phases collected were dried on Na_2SO_4 and filtered, and the solvent was evaporated under pressure. The crude was purified by flash chromatography on silica gel (mobile phase CH_3Cl/MeOH 98:2) to isolate the pure product (65% yield): ^{1}H NMR ($CDCl_3$) δ 1.69 (d, 3H, H_{10}, $J_{H_{10}H_8}$ = 7.13 Hz), 2.01−2.02−2.03 (s, 5 × 3H_{Ac}), 2.28 (s, 2 × 3H_{Ac}), 2.72 (dd, 1H, H_6, $J_{H_6H_{6a}}$ = 14.58 Hz, $J_{H_6H_5}$ = 4.41 Hz), 2.41 (dd, 1H, H_{6a}, $J_{H_{6a}H_6}$ = 14.58 Hz, $J_{H_{6a}H_5}$ = 8.81 Hz), 2.91 (t, 2H, H_7, $J_{H_7H_8}$ = 7.13 Hz), 3.72 (s, 3H, CH_3OCO), 3.96 (dd, 1H, H_5, $J_{H_5H_{6a}}$ = 8.81 Hz, $J_{H_5H_6}$ = 4.41 Hz), 4.06−4.28 (m, 4H, $H_{6'}$, $H_{4'}$, H_8), 5.03 (d, 1H, $H_{1'}$, $J_{H_{1'}H_{2'}}$ = 7.80 Hz), 5.12 (dd, 1H, $H_{2'}$, $J_{H_{2'}H_{3'}}$ = 9.49 Hz, $J_{H_{2'}H_{1'}}$ = 7.80 Hz), 5.27 (t, 1H, $H_{3'}$, $J_{H_{3'}H_{4'}}$ = $J_{H_{2'}H_{1'}}$ = 9.49 Hz), 5.69 (s, 1H, H_1), 5.94−6.03 (m, 1H, $H_{5'}$), 7.46 (s, 1H, H_3), 7.02−7.14 (m, 3H, $H_{aromatic}$); LC/MS/ESI, *m*/*z* 810 $[M + NH_4]^+$, 793 $[M + H]^+$.

Synthesis of Peracetylated Aglycons (***11a−e***). Aglycon (297 mg, 0,78 mmol) was reacted for 3 h at room temperature with an excess of dry acetic anhydride (5 mL) under N_2 atmosphere in the presence of 3 mol % of $Er(OTf)_3$ (0.023 mmol, 14.4 mg). At the end of the conversion, 12 mL of MeOH was added to the mixture and stirred for 1 h. Solvent was removed in vacuo, and the residue was solubilized in $CHCl_3$. The mixture was extracted with a saturated solution of $NaHCO_3$ until complete elimination of the acetic acid; the organic phases collected were dried on Na_2SO_4 and filtered, and the solvent was evaporated under pressure. The crude was purified by flash chromatography on silica gel (mobile phase CH_2Cl_2/MeOH 8:2) to isolate the pure product as a mixture of different peracylated forms (**11a−e**) (total yield = 79%) analyzed by LC/MS/ESI, MALDI/MS, and ^{1}H NMR.

11a: ^{1}H NMR ($CDCl_3$) 9.6 ppm (m, 1H, H_{al9}), 9.7 ppm (m, 1H, H_{al1}), 2.28 ppm (s, 3H, H_{Ac23}), 2.30 ppm (s, 3H, H_{Ac22}); MALDI/MS (2,5-DHB), *m*/*z* 484 (11) $[M + Na]^+$; LC/MS/ESI (+), t_r = 30.406 min, *m*/*z* = 485 (21) $[M + Na]^+$, 480 (100) $[M + NH_4]^+$, 463 (52) $[M + H]^+$, 445 (51) $[M - OH]^+$, 431 (43) $[M - MeOH]^+$.

11b: ^{1}H NMR ($CDCl_3$) 9.2 ppm (m, 1H, H_{al1}), 2.30 ppm (s, 3H, H_{Ac22}), 2.28 ppm (s, 3H, H_{Ac23}), 2.19 ppm (s, 3H, H_{Ac9}), 1.75−1.87 ppm (m, 1H, H_5 × 3); MALDI/MS (2,5-DHB), *m*/*z* 526 (12) $[M + Na]^+$; LC/MS/ESI (+), t_r = 29.372 min, *m*/*z* 527 (25) $[M + Na]^+$, 522 (100) $[M + NH_4]^+$, 505 (48) $[M + H]^+$, 445 (57) $[M - COOMe]^+$.

11c: ^{1}H NMR ($CDCl_3$) 660 ppm (m, 1H, OH_2), 2.30 ppm (s, 3H, H_{Ac23}), 2.28 ppm (s, 3H, H_{Ac24}), 2.20 ppm (s, 3H, H_{Ac10}), 2.07 ppm (s, 3H, H_{Ac1}), 1.66−1.73 ppm (m, 3 H, $(CH_3)_6$); MALDI/MS (2,5-DHB), *m*/*z* 586 $[M + Na]^+$; LC/MS/ESI (+) t_r = 27.587 min, *m*/*z* 587 (31) $[M + Na]^+$, 582 (98) $[M + NH_4]^+$, 445 (100) $[M - COOMe - HOAc]^+$.

11d: ^{1}H NMR ($CDCl_3$) 3.70 ppm (s, 3 H, $(OCH_3)_3$), 2.30 ppm (s, 3H, H_{Ac23}), 2.28 ppm (s, 3H, H_{Ac24}), 2.22 ppm (s, 3H, H_{Ac10}), 1.97 ppm(s, 3H, H_{Ac1}); MALDI/MS (2,5-DHB), *m*/*z* 600 (30) $[M + Na]^+$; LC/MS/ESI (+) t_r = 26.8, 28.742 min, *m*/*z* 601 (15) $[M + Na]^+$, 596 (100) $[M + NH_4]^+$, 579 (91) $[M + H]^+$, 445 (73) $[M - COOMe - MeOAc]^+$.

11e: ^{1}H NMR ($CDCl_3$) 2.30 ppm (s, 3H, H_{Ac23}), 2.28 ppm (s, 3H, H_{Ac24}), 2.19 ppm (s, 3H, H_{Ac10}), 2.02 ppm (s, 6H, H_{Ac1} + H_{Ac3}), 1.73−1.80 ppm (m, 3 H, $(CH_3)_6$); MALDI/MS (2,5-DHB), *m*/*z* 628 (29) $[M + Na]^+$; LC/MS/ESI (+) t_r = 33.702, 34.551, 35.083, 38.512 min, *m*/*z* 629 (15) $[M + Na]^+$, 624 (86) $[M + NH_4]^+$, 582 (13) $[M - Ac + NH_4]^+$, 547 (86) $[M - COOMe]^+$, 505 (100) $[M - COOMe - Ac]^+$, 445 (38) $[M - COOMe - Ac - HOAc]^+$.

RESULTS AND DISCUSSION

First, we decided to improve the oleuropein (**3**) extraction protocol from olive leaves by using microwave heating and environmentally friendly solvents. By virtue of this approach, maximum yield was obtained in a very short extraction time, 10 min, and using plain water as the solvent. It should be noted that the same results can be obtained with a conventional heating system, but only by means of refluxing water/methanol or an ethanol mixture and prolonged extraction times (entry 6 in **Table 1**).

Examining the molecular structure of this secoiridoid, it is quite evident that its aglycone form can be easily obtained by simple acetal hydrolysis, mimicking the natural glucosidase enzyme action (**Figure 2**). We successfully tested, and then patented (*16*), a very gentle method to realize the selective Lewis acid catalyzed oleuropein hydrolysis in water, applying one of the protocols we recently published for simple acetals (*13*). Selective hydrolysis of oleuropein is a challenging aim to achieve, due to the multifunctional chemical nature of the molecule. In the practical approach reported in **Figure 2**, an aqueous acetonitrile oleuropein solution was treated with 10 mol % of a lanthanide(III) salt under reflux until complete disappearance of the starting material, monitored by thin layer chromatography (tlc) analysis. The complex product characterization was performed by means of LC-MS and ^{1}H NMR analysis. As shown in **Figure 2**, the hemiacetal aglycone product was a mix of four tautomeric forms (aglycones **8a−d**): the enolic and the dialdehydic forms, **8a** and **8b**, respectively, as well as the ring-closed species **8c** and the hydrated aldehydic derivative **8d**. Moreover, LC-MS analysis detected the methanolic derivative **8e**, probably arising from a reaction with the mobile phase (see the Supporting Information). Several studies carried out with human and animal models have shown good bioavailability of olive oil phenols (*17*). Nevertheless, these active substances are effective at concentrations that are improbable to be achieved in vivo. On the other hand, no data are available about the bioavailability of the various aglycons, including oleocanthal, and contrasting evidence has been reported regarding their in vivo stability (*18*, *19*).

On the basis of these considerations, it would be useful to conveniently produce these species as chemically more stable derivatives that are able to be biochemically converted in vivo into their original active forms. It was already reported that some acetyl derivatives of phenolic compounds maintain biological antioxidant activity compared to that of the parent compound, probably because of extensive deacetylation of hydroxytyrosyl acetate by carboxylesterases. Such deacetylation can take place either within the cell, upon absorption of the acetylated molecule, or in the extracellular space by secreted esterases (*17*), generating free hydroxytyrosol, which is the effective antioxidant compound. Considering that the acetyl group is a ubiquitous substrate in the biochemical processes and that the acetylating agents are very common and manageable, we planned to prepare the acetyl derivatives of **1**, **3**, and **8a−e**. To obtain chemically stable forms of oleuropein and its derivatives, we successfully tested and then patented (*20*) the simple acetylation protocol, reported in **Figure 3**, derived by applying methods we previously developed (*14*, *15*). The acetylated analogues of **1**, **3**, and **8a−e** were synthesized in acetic anhydride in the presence of lanthanide(III) catalysts and then characterized spectroscopically (**Figures 3** and **4**). All synthesized compounds showed a high purity grade, as shown in the corresponding HPLC chromatograms (see the Supporting Information). To demonstrate the improved lipophilic character of the acetylated species obtained from **1**, **3**, and **8a−e**, that is, triacetylated hydroxytyrosol **9** and peracetylated oleuropein **10**, their octanol−water partition coefficients were determined using a modification of the experimental method described by Namjesnik-Dejanovic et al. (*21*). All acetylated species were much more lipophilic than their corresponding nonacetylated original molecules. A good correlation was observed between experimental and theoretical values of log $K_{o/w}$ (see the Supporting Information) (*21−23*).

In the next step, the same molecules (**1**, **3**, **8a−d**, **9**, **10**, and **11a−d**) were in vitro tested as COX-1 and COX-2 inhibitors by

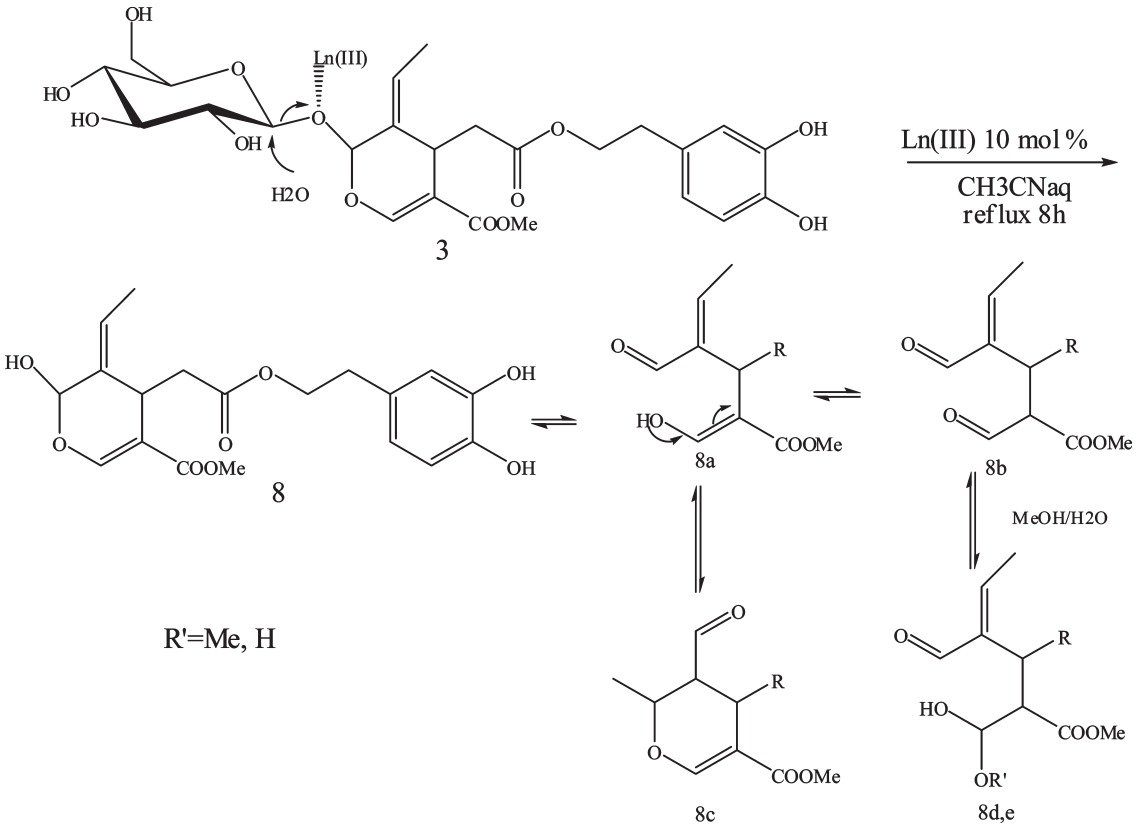

Figure 2. Synthesis of aglycone derivatives by acetal hydrolysis.

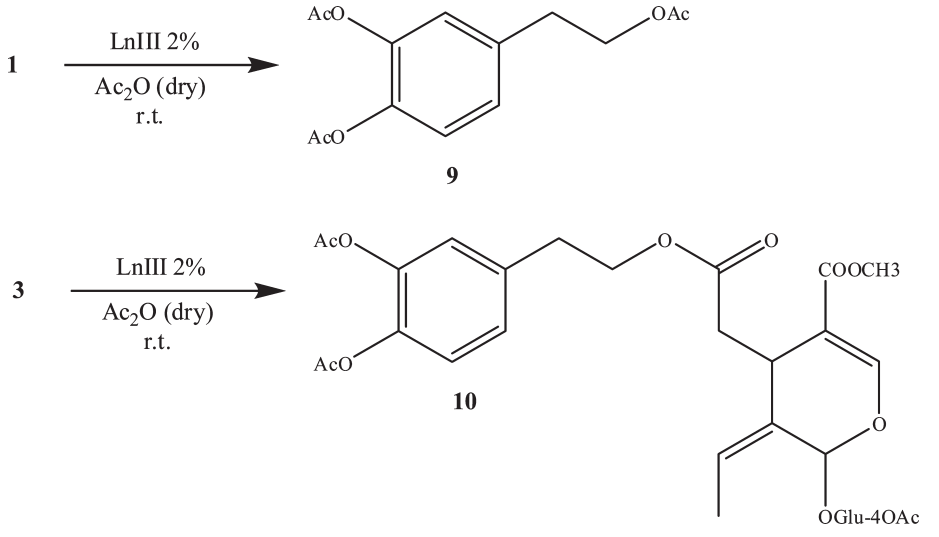

Figure 3. Patented acetylation protocol.

means of the evaluation of percentage inhibition in the production of their physiological mediators in the human whole blood (HWB) assay, respectively corresponding to thromboxane B_2 (TXB_2) and the prostaglandin E_2 (PGE_2) (*24*) (see the Supporting Information). In the preliminary in vitro screening, carried out at three different concentrations (see the Supporting Information), compounds **1** and **9** were shown to be the most active inhibitors. Thus, a concentration−response investigation was performed for peracetylated hydroxytyrosol **9** only (**Table 2**).

As reported earlier (*25*), the concentration for 50% inhibition (IC_{50}) values for ibuprofen are 5 and 223 μM for COX-1 and COX-2, respectively. The IC_{50} values for celecoxib (*26*) are 16 and 0.54 μM for COX-1 and COX-2, respectively. Therefore, it is worth noting that in the case of peracetylated hydroxytyrosol **9**, the inhibition data IC_{50} against COX-1 and COX-2 were in the range of 3−10 and 0.1−1 μM, respectively, quite lower than the values reported for (−)-oleocanthal **7** (IC_{50} of about 25 μM for both COX-1 and COX-2) (*7*).

To confirm the exciting in vitro results, we decided to screen in vivo all synthesized compounds for their anti-inflammatory activity using the well-known carrageenan-induced paw edema (*27*). The synthesized compounds (**1**, **3**, **8a**−**d**, **9**, **10**, and **11a**−**d**) and standard drugs such as ibuprofen and celecoxib were administered orally (50 μmol/kg po), and after

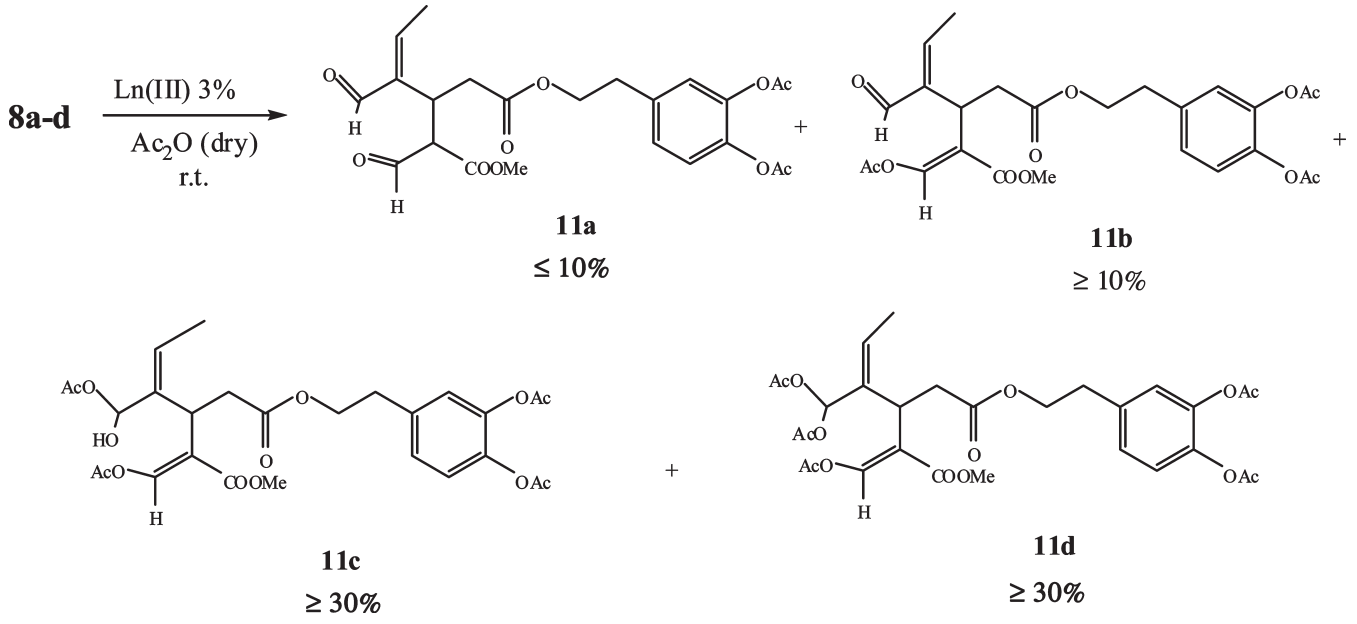

Figure 4. Patented acetylation protocol.

Table 2. Concentration−Response Table for the Inhibition of Human Whole Blood COX-1 and COX-2 Activities by Peracetylated Hydroxytyrosol **9**

	COX-1		COX-2	
concn (μM)	% inhibition	SD	% inhibition	SD
30	58.22	2.06	68.20	8.06
10	51.54	9.81	66.37	11.19
3	44.81	2.74	63.98	0.39
1	42.26	3.46	66.54	0.01
0.1	34.51	6.06	36.71	0.12

30 min, all animals were injected with 0.1 mL of 1% carrageenan solution (prepared in normal saline) in the subplantar aponeurosis of the left hind paw and the volume of paw was measured by using a plethysmometer at 1 and 3 h post carrageenan treatment. All derivatives (**1**, **3**, **8a**−**d**, **9**, **10**, and **11a**−**d**) showed significant activity (**Table 3**), and two of them, **1** and **9**, displayed more potent anti-inflamatory activity than the standard drugs ibuprofen and celecoxib, efficaciously reducing the increase in paw volume by 62.2 and 65.9%, respectively (**Table 3**).

To rationalize the recognition mechanism of compounds **1**, **3**, **9**, and **10** within the COX isoforms, we performed a molecular modeling study. We focused our attention on the above-reported molecules due to their experimentally confirmed anti-inflammatory activity and because they have demonstrated chemical stability and availability in pure form. Moreover, these compounds showed antioxidant properties, suggesting a concurrent second inhibitory mechanism of action. The Protein Data Bank (PDB) (*28*) crystallographic structures 1Q4G (*29*) and 1PXX (*30*) were considered after a preliminary pretreatment (see the Supporting Information), respectively, as COX-1 and COX-2 enzyme models. The crystallographic resolution, respectively equal to 2.0 and 2.9 Å, and the chemical nature of cocrystallized ligands, two nonsteroidal anti-inflammatory drugs (NSAIDs), were the choice criteria of these models within the large availability of similar ones in the PDB.

Interestingly, the 1Q4G structure describes for the first time a glycerol molecule interacting with the heme moiety, suggesting the cofactor as a new accessory target for COX inhibition. On the basis of this observation, we studied the recognition of our compounds with respect to both classic (site A) and tentative accessory (site B) clefts. According to the Glide methodology, in which the heme iron is considered at its maximum oxidation state (+3) (*30*), the docking binding site has been defined by means of a regular box centered onto the chain A tyrosine 385. Its volume was about 390,000 Å, widely covering classic COX binding site and the heme moiety (see the Supporting Information). Compounds **1**, **3**, **9**, and **10** showed, as appeared in the docking experiments, good affinity toward both COX isoforms. Interestingly, all ligands were able to be recognized, with some differences, at both sites A and B (see the Supporting Information). On the other hand, disagreement has been observed between experimental and theoretical affinity data. **9** and **10** have reported a more favorable interaction with respect to **1** and **3** in all cases (see the Supporting Information). Such a discrepancy can be explained by analyzing the binding modes of our compounds at the COX site A. Actually, only **1** and **3** deeply recognized site A, reporting quite similar configurations with respect to the other known NSAIDs. **9** and **10** have partially occupied binding site A, interfering only with the catalytic gorge entrance. Compounds **1** and **3** have reported productive contacts with site A amino acids, and **9** and **10** did not interact with known relevant COX-1 and -2 activity residues, excluding the cofactor heme (see the Supporting Information). Moreover, their configurations have been highly solvent exposed, suggesting a faster bound−unbound kinetic equilibrium with respect to **1** and **3**.

In conclusion, we applied some simple patented methods to mild chemical manipulation of complex natural substances. The entire proposed process accomplishes many green chemistry tasks and shows that it is possible develop sustainable complex protocol using some nonconventional techniques. All of the considered molecules have been shown to possess significant anticyclooxygenase activity, and the peracetylated hydroxytyrosol **9** seems to be a very interesting starting point to investigate the peculiar activity of this kind of derivatives. Particularly, in the molecular modeling, we have considered the interaction of the cofactor as a new interesting target to explain the different behavior of our ligands. However, in a cellular environment, it is not easily reachable and, consequently, the heme recognition could be

Table 3. Effects of the Compounds and Standard Drugs on Carrageenan-Induced Paw Edema

compound	edema vol, 1 h	edema vol, 3 h	inibition of paw edema, 1 h (%)	inibition of paw edema, 3 h (%)
control	0.82 ± 0.022	0.82 ± 0.029		
ibuprofen	0.29 ± 0.033	0.35 ± 0.009	64.6	57.3
celecoxib	0.19 ± 0.013	0.25 ± 0.003	76.3	69.5
1	0.31 ± 0.008	0.38 ± 0.011	62.2	53.7
3	0.42 ± 0.031	0.50 ± 0.009	48.8	39.0
8a−d	0.39 ± 0.012	0.48 ± 0.029	52.4	41.5
9	0.28 ± 0.014	0.33 ± 0.019	65.9	59.8
10	0.48 ± 0.023	0.51 ± 0.017	41.5	37.8
11a−d	0.34 ± 0.031	0.39 ± 0.006	58.5	52.4

considered as an unspecific COX secondary binding site. Perhaps our compounds could interact with site B, negatively modulating the activity of both COX isoforms.

ABBREVIATIONS USED

HTyr, hydroxytyrosol or 2-(3,4-dihydroxyphenyl)ethanol; Tyr, tyrosol or 2-(4-hydroxyphenyl)ethanol; Ole, oleuropein; LDL, low-density lipoprotein; TXB_2, thromboxane B_2; LTB_4, leukotriene B_4; ROS, reactive oxygen species; LPS, lipopolysaccharide; VHSV, hemorrhagic septicemia rhabdovirus; PGE_2, prostaglandin E_2; NSAD, nonsteroidal anti-inflammatory drug.

Supporting Information Available: General experimental procedures and spectroscopic characterization of new compounds, experimental log *P* determination, biological assay, and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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