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Lipophilic Hydroxytyrosol Esters: Fatty Acid Conjugates for Potential Topical Administration

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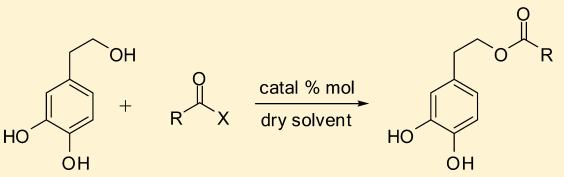
Supporting Information

ABSTRACT: Hydroxytyrosol is a potent antioxidant natural molecule isolated from olive leaves and fruits. The presence of three hydroxy groups in its structure poses a limit for the topical application of this lead compound. A set of hydroxytyrosol conjugates with fatty acids at different molecular weights were synthesized under mild conditions. The topical delivery features of this new set of antioxidant molecules were evaluated as a function of their permeation profiles through the human stratum corneum and viable epidermis membranes. A dependence on their partition coefficients, their molecular weights, and their isometric configurations was then postulated. Encouraging results prompt further investigations on the polyfunctional role that hydroxytyrosol conjugates could have as agents in both anti-inflammatory and antioxidant therapies.



The topical treatment of diseases has attracted great interest in the pharmaceutical sciences.[1,2] Natural and synthetic products are generally proposed for their therapeutic activities, and their effects are evaluated extensively through different formulations.[3,4] Even if a systemic pathway represents the best option for these drugs, side effects, frequently related to the formulation excipients or due to metabolic products, may occur after administration.[5] Recently, different groups have proposed the topical administration route as an alternative for drug activity.[6] Both natural and synthetic anti-inflammatory and antioxidant drugs have been applied topically to the skin, and their effects have been evaluated using "in vitro"[7] and "in vivo"[8] models. Natural products, as free or entrapped formulations, have been considered in these models as an efficient alternative to synthetic drugs. In particular, olive leaves (*Olea europea* L., Oleaceae) and olive oil extracts have been considered as therapeutic agents, and various examples of these derivatives are reported in the literature.[9] Experimental findings showed that, although a complex matrix effect is involved in the therapeutic activity of olive oil, phenolic derivatives are generally associated with its efficacy. To this purpose, our research group has described phenolic derivates, such secoiridoid derivatives of 3,4-dihydroxyphenylethanol (hydroxytyrosol), as potential cyclooxygenase inhibitors[7] and antitumor drugs.[10] In particular, peracetylated derivatives of hydroxytyrosol show an efficacy due to their antioxidant properties if applied both in "in vitro"[7,11] and "in vivo"[12] models. Furthermore, hydroxytyrosol exerts a protective effect on UVA-irradiated cells, thus preventing the overexpression of typical oxidative stress markers, and delays LDL (low-density lipoprotein),[13] prevents platelet aggregation,[14] inhibits several lipoxygenases,[15] and scavenges peroxyl radicals.[16] In addition, hydroxytyrosol alters L-iso-aspartine residues induced by UVA irradiation and counteracts the cytotoxic effects of reactive oxygen species (ROS) in various human cellular systems.[17] These findings have suggested that hydroxytyrosol may be proposed as a potent natural antioxidant and anti-inflammatory agent for several therapeutic applications,[10,18] and the topical route might represent an efficacious pathway for its administration.

These properties have prompted various procedures for chemical[19] and enzymatic synthesis[20] or extraction of hydroxytyrosol.[21] However, the physicochemical features of hydroxytyrosol suggest this compound will undergo a low topical permeation through the human stratum corneum and viable epidermis (SCE) membranes. In recent years, the conjugation of hydroxytyrosol with fatty acids of different molecular weights has been suggested to increase the lipophilicity of the molecule without modifying its antioxidant properties.[22−25] There is still a need for new methods for the synthesis of these derivatives since the few reported examples are in the patent literature[23−26] or involve the use of enzymes,[27] with the yields having been obtained for only two reported examples.[28] Our group has developed environmentally friendly catalytic methods for the strategic protection/deprotection steps of functional groups,[29−31] and some of them concern the acylation of alcohols and phenols using lanthanoid salts as

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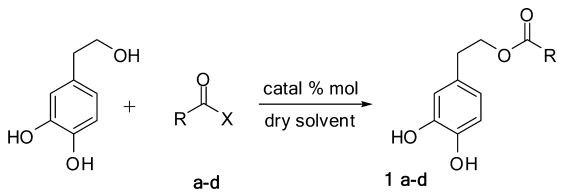
Lewis acid catalysts. Herein, a simple and efficient catalytic method to obtain acylated hydroxytyrosol derivatives with a long alkyl chain is reported. Butyryl, decanoyl, elaidyl, linoleyl, oleyl, stearyl, and palmitoyl moieties have been conjugated to hydroxytyrosol, and permeation studies carried out in "in vitro" modeling using Franz diffusion cells.

RESULTS AND DISCUSSION

Several derivatives of hydroxytyrosol were prepared by synthesis as shown in Scheme 1. Several methods of derivatization were attempted, as is summarized in Table S1, Supporting Information.

Scheme 1. Synthesis of Hydroxytyrosol Fatty Esters



a: R=$CH_3(CH_2)_{14}$; b: R=$CH_3(CH_2)_2$; c: R=$CH_3(CH_2)_8$; d: R=$CH_3(CH_2)_{16}$; e: R=$CH_3(CH_2)_7$=$(CH_2)_7$ (cis); f: R=$CH_3(CH_2)_7$=$(CH_2)_7$ (trans); g: R=$CH_3(CH_2)_4CH$=$CHCH_2CH$=$CH(CH_2)_7$ (cis); X = RCO or Cl

The biological activity of phenolic compounds depends on their antioxidant properties as much as their lipophilicity. As mentioned in a previous report, the inclusion of a lipophilic chain in the hydroxytyrosol molecule enhanced its antioxidant capacities in the biological model investigated.[22] Furthermore, lipophilic hydroxytyrosyl esters such as **1a**–**g** show a high free-radical-scavenging capacity, preventing protein oxidation and lipid peroxidation when ex vivo cells are exposed to active-oxygen substances and/or free radicals. The same hydroxytyrosol esters showed greater antioxidant activity than α-tocopherol or BHT to protect proteins and lipids against oxidation caused by peroxyl radicals in a brain homogenate.[22]

Conventionally, the standard measure of the hydrophobicity of a compound is its octanol–water partition coefficient, K_{ow}, which is the equilibrium ratio of the concentration of the compound in an octanol phase and its concentration in an aqueous phase. K_{ow} is frequently used to estimate drug partitioning between aqueous and lipid phases[32] and is closely related to aqueous solubility.[33] The experimental determination of the partition coefficient is based on a correlation of K_{ow} with reversed-phase (RP) HPLC retention times, which depends on a similarity of the retention mechanism to octanol–water partitioning but does not require that the reactions be identical. Klein et al.[34] validated and included a HPLC method as one of the standard K_{ow} determination methods. In a previous paper, the application of a modified HPLC method was used to evaluate the K_{ow} for hydroxytyrosol and an acetylated derivative.[6] Herein, we applied the same method to the K_{ow} determination of the hydroxytyrosol lipophilc esters **1a**–**g** (see Supporting Information). As shown in Table 1, all the hydroxytyrosol fatty esters with more than four carbon atoms in their chain were considerably more lipophilic than natural hydroxytyrosol (so that it was not practical to determine exactly their K_{ow} by this HPLC method). However, it was possible to conclude that hydroxytyrosol palmitate (**1a**), decanoate (**1c**), stearate (**1d**), oleate (**1e**), elaidate (**1f**), and linoleate (**1g**) have

Table 1. Experimental and Calculated log K_{ow} Values of Compounds 1a–g

compound	log K_{ow} [a]	cLog K_{ow} [b]	miLog K_{ow} [c]
hydroxytyrosol	0.809	1.1	0.516
hydroxytyrosol butanoate (**1b**)	1.64	2.52	2.645
hydroxytyrosol palmitate (**1a**)	>3.3 [d]	8.09	7.697
hydroxytyrosol decanoate (**1c**)	>3.3 [d]	5.35	5.35
hydroxytyrosol sterate (**1d**)	>3.3 [d]	9.02	8.976
hydroxytyrosol oleate (**1e**)	>3.3 [d]	9.12	9.75
hydroxytyrosol elaidate (**1f**)	>3.3 [d]	8.38	8.38
hydroxytyrosol linoleate (**1g**)	>3.3 [d]	8.668	8.668

[a]Experimental log K_{ow} values were determined by HPLC.[6] [b]Theoretical log K_{ow} values were determined by Actelion Property Explorer. [c]Theoretical log K_{ow} values were determined by Molinispiration Property Explorer. [d]The reported value of 3.3 is the higher log K_{ow} value determined by the RP-HPLC method, and it is referred to naphthalene used as standard in the calibration curve.

K_{ow} values much higher than hydroxytyrosol and hydroxytyrosol butanoate (**1b**).[35]

Permeation through the skin represents a very important prerequisite for the topical delivery of bioactive compounds, especially in the case of pro-drugs,[36] synthetic conjugates,[37] or colloidal devices[2] applied to the skin. As previously reported, the efficacy of new formulations containing natural or synthetic products is based on their ability to penetrate the SCE membrane without any modification.[38] Overcoming this barrier allows the drugs to be delivered into the site of action, thus improving their therapeutic activity. Another important aspect to be considered for the topical delivery of bioactive compounds is their repartition between the external surface of the skin and lipids of the SCE membrane.[39]

In order to test the topical application of the hydroxytyrosol fatty esters compared to free hydroxytyrosol, an "in vitro" permeation study using Franz diffusion cells was performed on all the conjugates previously synthesized. Figure 1 (panels A and B) shows the percutaneous permeation profile of hydroxytyrosol and conjugates **1a**–**g** through the SCE membranes. No lag time was observed during the experiments, and zero-order kinetics were observed for various formulations (Figure 1A). The permeation profiles of the hydroxytyrosol conjugates show that different compounds increased the percentage of hydroxytyrosol permeated through the SCE membranes (Figure 1A). Only elaidate conjugate **1f** showed a profile similar to that obtained for hydroxytyrosol, probably depending on the *trans* configuration of the double bond on the carbon chain.

These findings demonstrated also that the hydroxytyrosol butanoate (**1a**), decanoate and linoleate (**1c** and **1g**), oleate (**1e**), and stearate and palmitate (**1d** and **1e**) permeation profiles were increased 1.8-fold, 2.2-fold, 3.6-fold, and 1.7-fold, respectively, compared to hydroxytyrosol (Figure 1A). The differences recorded for the different hydroxytyrosol conjugates probably depend on their partition coefficients, their molecular weights, and the isomeric configuration of their double bonds. In fact, in spite of the behavior of the *cis*-unsaturated oleate **1e** and linoleate **1g**, no significant permeation increase regarding the simple hydroxytyrosol was obtained for the *trans* isomer elaidate **1f** (Figure 1A), despite this derivative containing 18 carbon atoms in its side chain. The *trans* chemical structure of elaidate conjugate **1f** is responsible for a side chain folding that could decrease the molecular radius, thus reducing its contact

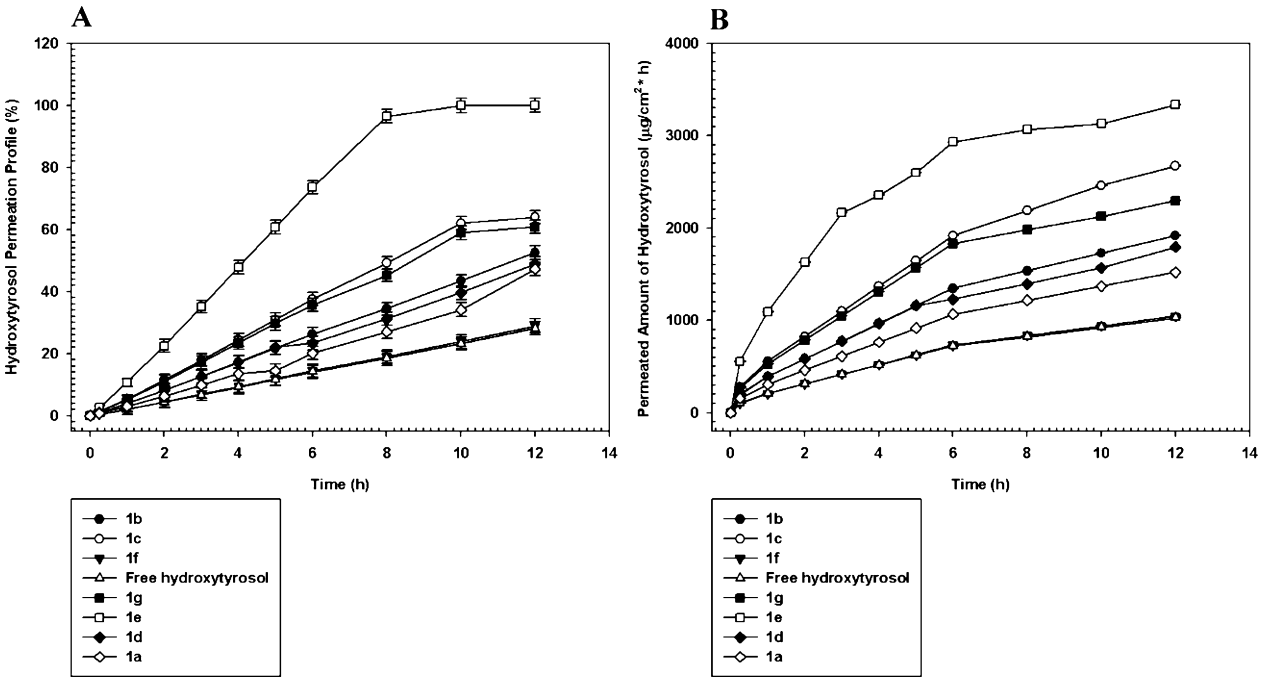


Figure 1. "In vitro" percutaneous permeation through an SCE membrane of various hydroxytyrosol conjugates **1a**–**g** as a function of percentage (A) and amount (B) of compounds delivered through the skin. The chemical structures of different compounds are reported in Scheme 1. Experimental findings are the average of three different measurements ± standard deviations.

surface with the SCE membrane (Figure 1A, B). This effect became more evident in comparison with the oleate derivative (**1e**). Although hydroxytyrosol oleate (**1e**) is characterized by the same number of atoms in the side carbon chain, the opposite double-bond geometry compared to hydroxytyrosol elaidate (**1f**) gives rise to a difference in their SCE permeation profiles (Figure 1A, B). In fact, the amount of hydroxytyrosol that permeated through the SCE membrane in the case of the oleate conjugate **1e** after 12 h was 3334.88 $\mu g/cm^2\ h^{-1}$, compared to 1045.82 $\mu g/cm^2\ h^{-1}$ obtained in the case of elaidate conjugate **1f** (Figure 1B).

The palmitate (**1a**), butanoate (**1b**), decanoate (**1c**), and stearate (**1d**) conjugates showed permeation profiles similar to one another (Figure 1A, B). These permeation profiles seem to be slightly dependent on the side carbon chain dimension and strongly influenced by the double-bond geometry. Percentages of 64.18% and 60.52% permeation (Figure 1A) were obtained for the decanoate (**1c**) and the linoleate (**1g**), respectively, while the amounts of compounds permeating through the skin were 2672.05 $\mu g/cm^2\ h^{-1}$ (**1c**) and 2297.39 $\mu g/cm^2\ h^{-1}$ (**1g**), respectively (Figure 1B).

In conclusion, a new mild catalytic system is proposed for the synthesis of a range of lipophilic hydroxytyrosol fatty esters. These conjugates offer the potential of being administered as topical therapeutic agents for the treatment of cutaneous diseases. Almost all the compounds produced permeated efficaciously through the SCE membrane when applied in an "in vitro" Franz cell model. The skin permeation of hydroxytyrosol conjugates generally correlated to their chemical structure and to the configuration of the unsaturated fatty acids conjugated to hydroxytyrosol.

EXPERIMENTAL SECTION

General Experimental Procedures. UV–vis quantification was performed using a Perkin-Elmer Lambda 20 instrument connected to Perkin-Elmer UV WinLab 2.8 acquisition software (Perkin-Elmer GmbH, Überlingen, Germany). ^1H and ^{13}C NMR spectra were recorded on a Bruker WM 300 NMR spectrometer on samples dissolved in $CDCl_3$. Chemical shifts are given in parts per million (ppm) from tetramethylsilane as the internal standard (0.0 ppm). Coupling constants (J) are given in hertz. ESIMS were performed on a Applied Biosistem hybrid Q-STAR XL spectrometer working in a positive mode. The RP-HPLC system used in the log K_{ow} determination was made by a JASCO PU 1580 unit that included an Intellgent HPLC pump with an external column heater and multiwavelength JASCO MD detector 1540. An Altech 4.6 × 150 mm Adsobosphere C_{18} column with 5 μm particles of bonded silica gel with a guard column (4.6 × 7.4 mm Adsobosphere C_{18}) was used. Absorbance chromatograms were obtained at 254 nm. TLC was performed using 60-F264 silica plates on alumina (Merk). Flash chromatography was performed on a Supelco Versa Flash HTFP station on silica gel cartridges. Fetal calf serum (FCS) was purchased from Gibco [Invitrogen Corporation, Giuliano Milanese (Mi), Italy]. Resorcinol, catechol, benzoic acid, and naphthalene (≥99% purity) were purchased from Sigma-Aldrich. Polysorbate 80 (Tween 80) was obtained from ACEF Spa (Piacenza, Italy). Double-distilled pyrogen-free water was from Sifra SpA (Verona, Italy). All other chemical reagents used in this investigation were of analytical grade (Carlo Erba, Milan, Italy).

Synthesis of Hydroxytyrosol. Hydroxytyrosol was synthesized using a procedure reported in the literature.[6] 3,4-Dihydroxyphenylacetic acid (28.73 mmol, 1 equiv) was reacted with ethanol (EtOH) (15.0 mL) in the presence of 10% v/v H_2SO_4 at reflux temperature for 3 h and reduced with a solution of $NaBH_4$ (7–10 equiv) in water (40 mL) at room temperature for 8 h, as reported in the literature.[6] ^1H NMR (300 MHz, $CDCl_3$) δ 2.53 (3H, t, J = 7.4 Hz, H-2), 3.17 (1H, s, OH), 3.49 (2H, t, J = 7.4 Hz, H-3), 6.66–6.41 (1H, m), 6.60–6.58 (2H, m); EIMS m/z 136 $[M - H_2O]^+$, 43 $[MeCO]^+$.

Synthesis of Hydroxytyrosol Fatty Esters. To a solution of hydroxytyrosol (1.62 mmol) in dry THF (8 mL) were added 1 equiv of each fatty acid chloride (1.62 mmol) and 1 mol % of $Er(OTf)_3$ (0.0162 mmol) under stirring. Each mixture was reacted for 12 h at room temperature under nitrogen. After completion the mixture was poured in water saturated with $NaHCO_3$ and extracted

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with $CHCl_3$ (3 × 10 mL). The organic layers were combined and dried on Na_2SO_4 and filtered, and the solvent was evaporated under vacuum. The crude product was purified by flash chromatography ($CHCl_3$/MeOH, 9.5/0.5, as eluent).

*Hydroxytyrosol palmitate (**1a**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.7 Hz), 1.28 (24H, m), 1.64 (2H, m), 2.32 (2H, t, J = 7.2 Hz), 2.88 (2H, t, J = 7.0 Hz), 4.38 (2H, t, J = 7.0 Hz), 6.73 (3H, m); HRMS (FAB) m/z 415.28 $[M + Na]^+$.

*Hydroxytyrosol butyrrate (**1b**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90 (3H, t, J = 6.8 Hz), 1.79 (2H, m), 2.32 (2H, t, J = 7.2 Hz), 2.96 (2H, t, J = 7.1 Hz), 4.41 (2H, t, J = 7.1 Hz), 7.05 (3H, m); HRMS (CI) m/z 224.10 $[M]^+$.

*Hydroxytyrosol decanoate (**1c**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (3H, t, J = 6.8 Hz), 1.26 (12H, m), 1.61 (2H, m), 2.28 (2H, t, J = 7.2 Hz), 2.92 (2H, t, J = 7.1 Hz), 4.28 (2H, t, J = 7.1 Hz), 7.05 (3H, m); ESIMS (positive mode) m/z 323.21 $[M + H]^+$.

*Hydroxytyrosol stearate (**1d**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.7 Hz), 1.28 (28H, m), 1.64 (2H, m), 2.32 (2H, t, J = 7.2 Hz), 2.88 (2H, t, J = 7.0 Hz), 4.38 (2H, t, J = 7.0 Hz), 6.73 (3H, m); HRMS (CI) m/z 420.32 $[M]^+$.

*Hydroxytyrosol oleate (**1e**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (3H, t, J = 7.0 Hz), 1.30 (14H, m), 1.61 (2H, m), 1.98 (4H, m), 2.32 (2H, t, J = 7.6 Hz), 2.80 (2H, t, J = 7.2 Hz), 4.28 (2H, t, J = 7.3 Hz), 5.35 (2H, m), 6.60 (1H, dd, J = 3.1, 1.9 Hz), 6.79 (2H, dd, J = 8.0, 1.9 Hz); HRMS (CI) m/z 419.21 $[M + H]^+$.

*Hydroxytyrosol elaidate (**1f**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (3H, t, J = 6.6 Hz), 1.30 (14H, m), 1.61 (2H, m), 1.98 (4H, m), 2.32 (2H, dt, J = 17.9, 6.4 Hz), 2.70 (2H, t, J = 7.1 Hz), 4.28 (2H, t), 5.35 (2H, m), 6.79 (3H, m); ^{13}C NMR (300 MHz, $CDCl_3$) δ 13.9, 22.6, 25.4, 28.9, 29.0, 29.1, 29.1, 29.2, 29.4, 29.6, 29.7, 31.8, 32.5, 32.5, 33.7, 115.5, 116.0, 121.3, 130.2, 130.5, 130.8, 142.4, 143.8, 174.1; ESIMS (positive mode) m/z 419.53 $[M + H]^+$, 441.52 $[M + Na]^+$.

*Hydroxytyrosol linoleate (**1g**):* ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.6 Hz), 1.30 (14H, m), 1.61 (2H, t, J = 7.1 Hz), 1.98 (4H, m, J = 7.5 Hz), 2.32 (2H, dt, J = 18.2, 7.4 Hz), 2.75 (2H, m), 2.77 (2H, t, J = 7.1 Hz), 4.25 (2H, t, J = 7.1 Hz), 5.35 (4H, m), 6.79 (3H, m); HRMS (CI) m/z 417.29 $[M]^+$.

HPLC Operations, Materials, Solutions, and Chromatographic Conditions. The HPLC system calibration and the log K_{ow} determination were performed as previously described.[6] The organic compounds with known log P used as standards were hydroquinone, vanillin, *p*-cresol, and *p*-chlorophenol (Supporting Information). All the standard substances were 99% pure and were obtained from Sigma-Aldrich. The mobile phase was chosen according to the literature[40] (30% water/CF_3COOH, pH 2.4, 70% methanol), in order to correlate linearly the known log K_{ow} values of the standards with their HPLC retention times and to estimate the log K_{ow} of the unknown compounds, **1a**–**g**, by linear regression analysis. For those compounds too lipophilic to be analyzed by this method, the assumption was made that their log K_{ow} is higher than the most lipophilic standard analyzed (naphthalene, log K_{ow} = 3.3) by the same method.

Percutaneous Permeation of Lipophilic Hydroxytyrosol Derivates. Franz diffusion cells were used to evaluate "in vitro" the percutaneous permeation of different liphophilic hydroxytyrosol derivates through SCE membranes, which were prepared using fresh abdominal human skin obtained from plastic surgery from a group of volunteers fully informed about the nature of the study and the procedures involved and who gave a written consent. The stratum corneum and viable epidermis were separated from subcutaneous fat tissue according to a method previously reported.[8] Experimental investigations were carried out within 24 h of surgical removal of the skin. Franz diffusion cells were characterized by a 0.75 cm^2 diffusion surface area and a nominal receptor volume of 4.75 mL. The receptor chamber was filled with a mixture of double-distilled pyrogen-free water/ethanol/FCS (4/1/5 v/v) and maintained under continuous stirring at 600 rpm. SCE membranes were placed between the donor and receptor compartment with the stratum corneum side up, and the system was equilibrated for 6 h before the experiment. The donor compartment was filled with 200 μL of each lipophilic hydroxytyrosol derivative dissolved in the receptor chamber solution (4 mg/mL), and a steady-state condition was maintained during experimental investigations. The experiments were carried out in nonocclusive conditions for 24 h at a thermostatted temperature of 36 ± 1 °C. A minimum of three diffusion cells were used simultaneously for each formulation, and 1 mL of each sample was withdrawn every 1 h up to 12 h of incubation using an FC 204 fraction collector [Gilson Italia S.r.l., Cinisello Balsamo (MI), Italy] connected to a Minipuls 3 peristaltic pump (Gilson Italia S.r.l.). The volume withdrawn was replaced by the same volume of fresh receptor phase. Samples collected from the receiving compartment were analyzed immediately using a UV–vis spectrophotometer as described below. Each formulation was analyzed in triplicate, and the results are expressed as mean values ± standard deviation.

UV–Vis Spectrophotometer Characteristics and Calibration. UV–vis quantification of the lipophilic hydroxytyrosol derivatives was performed with the zero-order spectrum, and the first derivative spectrum was recorded for each compound (see Supporting Information). The absorbances of different samples were recorded at the λ_{max} measured for each compound, and the measurements were carried out in triplicate as a function of their specific calibration curve (see Supporting Information).

Standard Sample Preparation for UV Calibration Curve. Calibration curves for UV–vis analysis were carried out using different lipophilic hydroxytyrosol derivates. Standard solutions were obtained by dissolving 1 mg of single derivatives in 1 mL of EtOH. A linear correlation was obtained in the concentration range between 25 and 0.5 μg/mL. Six different readings (25, 10, 5, 2.5, 1, and 0.5 μg/mL) were measured for each single agent by adding the standard solution to a blank fetal calf serum sample. The samples obtained were then extracted in the following manner and immediately submitted to UV–vis analysis. FCS solution was used as blank during the analysis.

Sample Preparation for UV–Vis Analysis. Hydroxytyrosol lipophilic derivatives (4 mg/mL) were collected from a receptor chamber and extracted using ethanol (100 μL). Organic solvent was added to 100 μL of sample and transferred into an Eppendorf tube of 1.5 mL. Each mixture was vortex-mixed at 700 rpm for 10 s (MS1 minishaker, Ika-Werke Gmbh, Staufen, Germany), combined with hexane (500 μL), and further vortex-mixed for additional 30 s to obtain a complete homogenization of samples. Organic phases were centrifuged in a Mini Spoin Eppendorf centrifugue for 15 min at 13.400 rpm and further separated through a gravimetric process. The supernatant-containing hexane solution (450 L) was transferred in a clean tube, dried under nitrogen (N_2) flux, and diluted with 1 mL of EtOH in a glass tube for UV–vis analysis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A description of the unsuccessful attempts made in preparing the hydroxytyrosol esters as well as the yields recorded with the optimized synthetic procedure (Table S1), the experimental details of bioassays and synthetic procedures, UV and MS, ESIMS, and NMR spectra of hydroxytyrosol conjugates, HPLC chromatogram of hydroxytyrosol and hydroxytyrosol butanoate (**1b**), and their UV calibration curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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