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ARTICLE

Fatty Acid Hydroxytyrosyl Esters: Structure/Antioxidant Activity Relationship by ABTS and in Cell-Culture DCF Assays

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A large series of hydroxytyrosyl esters of C2−C18 fatty acids with increasing lipophilicity was prepared by a new highly efficient method based on acylation of methylorthoformate-protected hydroxytyrosol. All products were tested for relative antioxidant effect using ABTS assays in ethanolic medium and DCF assays in L6 cells. No linear correlation between lipophilicity and antioxidant effect was found. ABTS assays showed a growing antioxidant capacity, with respect to hydroxytyrosol, only for medium-sized ester chains (C4−C10) and a nearly constant capacity for the higher homologues. This has been rationalized by molecular dynamics experiments in terms of partial shielding of the catecholic hydroxyls by long-chain esters. A similar and dose-dependent pattern was observed in DCF assays in L6 cells, but a sharp antioxidant activity drop resulted for long-chain esters, probably due to membrane entrapment.

KEYWORDS: Hydroxytyrosyl esters; hydroxytyrosol; antioxidant activity; cell-culture measurements

INTRODUCTION

Hydroxytyrosol (**1**, or 2-(3,4-dihydroxyphenyl)ethanol) is a well-known natural antioxidant (*1*−*3*) derived from chemical (*4*) or enzymatic (*5*) hydrolysis of glycoside oleuropein (**Figure 1**) and present in large amount in all parts of the olive tree. **1** has been found to protect cells against oxidative stress (*6*), to reduce risks connected with aging pathogenesis (*7*), and also to be active against microbial attack (*8*), cancer proliferation (*9*−*11*), and HIV-1 viral fusion and integration (*12*). Despite these remarkable properties and although various procedures for its chemical (*13*) or enzymatic synthesis (*14*) and extraction (*15*, *16*) are available, compound **1** is still seldom used as a dietary supplement or as a stabilizer in foods, cosmetic, or industrial preparations. This may be due to (i) high instability in the air (particularly on silica gel and in alkaline medium) (*13*) and (ii) its amphiphilic character (log P = 0.09) (*17*), which make difficult both its extraction from aqueous solutions and its solubilization in lipid environment.

In the past decade, growing attention has been devoted to hydroxytyrosyl esters, particularly those derived from fatty acids. Due to their higher solubility in an oily matrix (*18*), these derivatives can solve the problems related to both the extraction from aqueous solution and the use of **1** as an additive in foods and cosmetics. Therefore, many chemical (*19*−*21*) or enzymatic (*17*, *22*) approaches to synthesize hydroxytyrosyl esters have been reported. The main synthetic issue comes from the simultaneous presence of the alcoholic and catecholic groups that give rise to chemoselectivity problems (*23*), especially when acyl chlorides are used as acylating agents. Many processes are under patent (*24*) and others are not selective (*13*), providing a mixture of derivatives difficult to separate or requiring expensive catalysts (*20*), whereas the highly selective enzymatic syntheses are difficult to apply for large-scale processes.

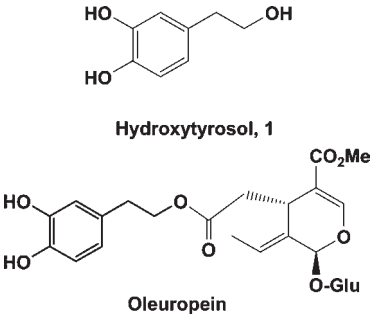

Figure 1. Structures of hydroxytyrosol (**1**) and oleuropein.

The evaluation of the antioxidant activity of hydroxytyrosyl esters shows a rather complex picture as well. The literature data for in vitro experiments (*17*, *18*, *22*, *25*) are difficult to compare to each other, due to the variety of methods (DPPH, ORAC, Rancimat) and reference antioxidants used. Moreover, only in a few cases have large series of fatty acid esters been tested (*19*, *25*), whereas comparable information for medium-chain derivatives is limited for C2, C4, C8, and C10 residues (*17*−*19*) or unavailable at all for C5−C7 and C9 fragments. Likewise, few data regarding the antioxidant activity of hydroxytyrosyl esters through biological experiments are reported. Namely, the prevention of lipid and protein oxidation (*18*, *19*) or DNA damage (*17*) has been

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studied, but additional experiments are needed on whole cells, tissues, or animals to analyze entirely their role as dietary supplements.

In this work a large series of hydroxytyrosyl esters (C2, C4, C6, C7, C8, C9, C10, C12, C14, C16, C18, and C18Δ9) have been synthesized and their antioxidant effects assessed by 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic)diammonium salt (ABTS) assay (*26*) and dichlorodihydrofluorescein (DCF) fluorometric assay on whole cells (*27*), comparing activity with Trolox (**25**) and **1**.

MATERIALS AND METHODS

Materials. All chemicals used were of analytical grade. Unless otherwise stated, all solvents and reagents were purchased from Sigma-Aldrich (Germany). Refined olive oil, stripped of endogenous natural antioxidants, was purchased from Sigma-Aldrich. **1** was obtained from oleuropein (Extrasynthese, France) following a previous protocol (*28*). L6 cells from rat skeletal muscle were from the American Type Culture Collection (Rockville, MD). 2′,7′-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was obtained from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM), antibiotics, and sterile plastic ware for cell culture were from Flow Laboratory (Irvine, U.K.). Fetal bovine serum was from GIBCO (Grand Island, NY). 2,2-(2-Methoxy-1,3-benzodioxol-5-yl)ethanol (**2**) and 2-(3,4-dihydroxyphenyl)ethyl acetate (**4**) were obtained as described elsewhere (*29*). Acyl chlorides of the various acids were used directly after distillation. When specified, solvents were dried over opportune drying agents and distilled. Silica gel 60 F254 plates and silica gel 60 were purchased from Fluka. Prewashed silica gel refers to silica gel washed with 0.1 N HCl and rinsed with hot distilled water until a negative test for chlorides. Petroleum ether used for chromatographic separations is the 40−60 °C fraction.

HPLC Analyses. Chromatographic analyses were performed on a TSP Spectra Series P200 apparatus equipped with a Thermo Hypersil BDS C18 column (250 × 4.6 mm, 5 μ) at λ = 280 nm. Elutions were carried out at 1 mL/min flow rate by using a 10 min gradient from H_2O/MeCN mixture (90:10, v/v) to pure MeCN.

Spectroscopic Data. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ (99.8% in deuterium) using a Varian Gemini 200 spectrometer. All chemical shifts are expressed in parts per million (δ scale) and are referenced to either the residual protons or carbon of the solvent. FT-IR spectra were recorded in $CHCl_3$ on a Bruker Vector 22 spectrometer. HRMS were recorded with a Micromass Q-TOF mass spectrometer (Waters). Only the spectral data of new compounds are reported here. Numbering of protons and carbons in compounds **5**−**14** is according to 1,3-benzodioxyl derivatives as shown in **Figure 2**.

Synthesis of 2-(2-Methoxybenzo[1,3]dioxol-5-yl)ethyl Esters 5−14. A solution of 2,2-(2-methoxy-1,3-benzodioxol-5-yl)ethanol (**2**) (*29*) (157 mg, 0.80 mmol) in dry tetrahydrofuran (THF, 4 mL) was transferred by a syringe into a flask equipped with a rubber injection septum, magnetic stirring, and an argon atmosphere. Dry pyridine (1.5 equiv, 95 μL) and the appropriate freshly distilled acyl chloride (1.5 equiv) were added, and the mixture was stirred at room temperature for 16 h. Short- and medium-chain esters **5**−**9** were isolated using synthesis route 1, whereas synthesis route 2 was used for long-chain esters **10**−**14**.

Synthesis Route 1. Excess acyl chloride was destroyed by adding methanol (1 mL), and the solution was neutralized with $NaHCO_3$ (saturated solution). Organic solvents were removed under reduced pressure, the resulting aqueous suspension was extracted three times with ethyl acetate, and the collected organic phases were dried over dry Na_2SO_4 and evaporated in vacuo. The resulting crude residue was purified over prewashed silica gel (40:1) by elution with petroleum ether/AcOEt (95:5) to afford pure products **5**−**9** with the yields reported.

Synthesis Route 2. Excess acyl chloride was destroyed by adding water (1 mL), and the solution was treated as above-described in synthesis route 1. Pure esters **10**−**14** were obtained with the yields reported.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl butyrate ***5*** (*oil, 189 mg, 0.71 mmol, yield 89%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.83 (s, 1H, H2); 6.80 (d, 1H, J = 8.0 Hz, H7); 6.77 (d, 1H, J = 1.7 Hz, H4); 6.71 (dd, 1H, J = 8.0, 1.7 Hz, H6); 4.23 (t, 2H, J = 4.0 Hz, CH_2O); 3.40 (s, 3H, OCH_3); 2.86 (t, 2H, J = 4.0 Hz, CH_2Ph); 2.27 (t, 2H, J = 7.4 Hz, H2″); 1.63 (tq, 2H, J = 7.4, 7.4 Hz, C3″); 0.92 (t, 3H, J = 7.4 Hz, H4″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.9 (C1″); 146.3 (C3′); 144.9 (C7′); 132.0 (C2); 122.2 (C5); 119.3 (C6); 109.2 (C7); 108.1 (C4); 65.1 (CH_2O); 50.2 (OCH_3); 36.4 (C2″); 35.1 (CH_2Ph); 18.6 (C3″); 13.9 (C4″); IR (cm^{-1}, $CHCl_3$) 3034, 2967, 2880, 2845 (ν_{C-H}); 1731 ($\nu_{C=O}$); 1610, 1498, 1444, 1386, 1355 ($\nu_{C=C}$); 1245, 1197, 1190, 1039 (ν_{C-O}). HRMS: found, 266.1155, $C_{14}H_{18}O_5$ requires 266.1154.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl hexanoate ***6*** (*oil, 212 mg, 0.72 mmol, yield 90%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.82 (s, 1H, H2); 6.79 (d, 1H, J = 7.9 Hz, H7); 6.76 (d, 1H, J = 1.6 Hz, H4); 6.69 (dd, 1H, J = 7.9, 1.6 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.39 (s, 3H, OCH_3); 2.85 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.27 (t, 2H, J = 7.5 Hz, H2″); 1.70−1.50 (m, 2H, H3″); 1.35−1.19 (m, 4H, H4″−H5″); 0.88 (t, 3H, J = 6.8 Hz, H6″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7 (C1″); 146.2 (C3′); 144.8 (C7′); 131.8 (C5); 121.9 (C6); 119.2 (C2); 108.9 (C7); 107.9 (C4); 64.8 (CH_2O); 49.9 (OCH_3); 34.9 (CH_2Ph); 34.3 (C2″); 31.3 (C4″); 24.6 (C3″); 22.3 (C5″); 13.8 (C6″); IR (cm^{-1}, $CHCl_3$) 3034, 3011, 2960, 2862 (ν_{C-H}); 1725 ($\nu_{C=O}$); 1633, 1610, 1493, 1459, 1443 ($\nu_{C=C}$); 1386, 1354. HRMS: found, 294.1469, $C_{16}H_{22}O_5$ requires 294.1467.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl heptanoate ***7*** (*oil, 231 mg, 0.75 mmol, yield 94%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.83 (s, 1H, H2); 6.79 (d, 1H, J = 7.9 Hz, H7); 6.76 (d, 1H, J = 1.6 Hz, H4); 6.71 (dd, 1H, J = 7.9, 1.6 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.40 (s, 3H, OCH_3); 2.86 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.4 Hz, H2″); 1.66−1.53 (m, 2H, H3″); 1.32−1.23 (m, 6H, H4″−H6″); 0.87 (t, 3H, J = 7.0 Hz, H7″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7 (C1″); 146.1 (C3′); 144.7 (C7′); 131.7 (C5); 121.9 (C6); 119.1 (C2); 108.8 (C7); 107.8 (C4); 64.8 (CH_2O); 49.9 (OCH_3); 34.9 (CH_2Ph); 34.3 (C2″); 31.4, 28.7 (C4″−C5″); 24.9 (C3″); 22.4 (C6″); 14.0 (C7″); IR (cm^{-1}, $CHCl_3$) 3030, 2927, 2860 (ν_{C-H}); 1725 ($\nu_{C=O}$); 1498, 1460, 1443 ($\nu_{C=C}$); 1385, 1250, 1042 (ν_{C-O}). HRMS: found, 308.1625, $C_{17}H_{24}O_5$ requires 308.1624.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl octanoate ***8*** (*oil, 251 mg, 0.78 mmol, yield 97%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.83 (s, 1H, H2); 6.79 (d, 1H, J = 7.9 Hz, H7); 6.76 (d, 1H, J = 1.6 Hz, H4); 6.71 (dd, 1H, J = 7.9, 1.6 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.40 (s, 3H, OCH_3); 2.86 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.4 Hz, H2″); 1.68−1.50 (m, 2H, H3″); 1.40−1.20 (m, 8H, H4″−H7″); 0.87 (t, 3H, J = 6.8 Hz, H8″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.8 (C1″); 146.1 (C3′); 144.7 (C7′); 131.7 (C5); 121.9 (C6); 119.2 (C2); 108.9 (C7); 107.9 (C4); 64.8 (CH_2O); 49.9 (OCH_3); 34.9 (CH_2Ph); 34.3 (C2″); 31.6, 29.1, 28.9 (3 C, C4″−C6″); 24.9 (C3″); 22.6 (C7″); 14.0 (C8″); IR (cm^{-1}, $CHCl_3$): 3032, 2961, 2855 (ν_{C-H}); 1722 ($\nu_{C=O}$); 1499, 1389 ($\nu_{C=C}$); 1252 (ν_{C-O}). HRMS: found, 322.1780, $C_{18}H_{26}O_5$ requires 322.1780.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl nonanoate ***9*** (*oil, 261 mg, 0.78 mmol, yield 97%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.83 (s, 1H, H2); 6.79 (d, 1H, J = 8.0 Hz, H7); 6.76 (d, 1H, J = 1.7 Hz, H4); 6.71 (dd, 1H, J = 8.0, 1.7 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.40 (s, 3H, OCH_3); 2.86 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.4 Hz, H2″); 1.66−1.56 (m, 2H, H3″); 1.31−1.26 (m, 10H, H4″−H8″); 0.87 (t, 3H, J = 7.0 Hz, H9″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.8 (C1″); 146.1 (C3′); 144.7 (C7′); 131.7 (C5); 121.9 (C6); 119.1 (C2); 108.9 (C7); 107.9 (C4); 64.8 (CH_2O); 49.9 (OCH_3); 34.9 (CH_2Ph); 34.3 (C2″); 33.9 (C7″); 31.8, 29.2, 29.1 (3 C, C4″-C6″); 24.7 (C3″); 22.6 (C8″); 14.1 (C9″); IR (cm^{-1}, $CHCl_3$) 2953, 2927, 2855 (ν_{C-H}); 1720 ($\nu_{C=O}$); 1498, 1463, 1444 ($\nu_{C=C}$); 1252, 1197, 1170, 1094, 1039 (ν_{C-O}). HRMS: found, 336.1938, $C_{19}H_{28}O_5$ requires 336.1937.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl decanoate ***10*** (*oil, 263 mg, 0.75 mm, yield 94%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.82 (s, 1H, H2); 6.79 (dd, 1H, J = 7.9, 0.5 Hz, H7); 6.76 (dd, 1H, J = 1.7, 0.5 Hz, H4); 6.70 (dd, 1H, J = 7.9, 1.7 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.40 (s, 3H, CH_3O); 2.86 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.2 Hz, H2″); 1.58 (m, 2H, H3″); 1.26 (m, 12H, C4″−C9″ protons); 0.88 (t, 3H, J = 6.8 Hz, H10″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7 (C1″); 146.2 (C3′);

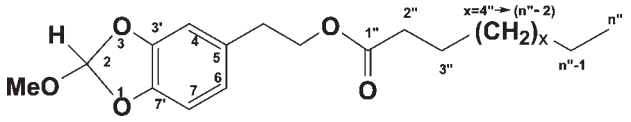


Figure 2. Carbon numbering of the hydroxytyrosyl ester skeleton used in NMR spectra.

144.7 (C7′); 131.8 (C5); 121.9 (C6); 119.2 (C2); 108.9 (C7); 107.9 (C4); 64.8 (CH_2O); 50.0 (OCH_3); 34.9 (CH_2Ph); 34.3 (C2″); 31.8 (C8″); 29.4 (C6″); 29.2 (C5″ and C7″); 29.1 (C4″); 24.9 (C3″); 22.6 (C9″); 14.0 (C10″); IR (cm^{-1}, $CHCl_3$) 3030, 2934, 2854 (ν_{C-H}); 1721 ($\nu_{C=O}$); 1630, 1499, 1444 ($\nu_{C=C}$); 1245 (ν_{C-O}). HRMS: found, 350.2092, $C_{20}H_{30}O_5$ requires 350.2093.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl dodecanoate ***11*** *(oil, 284 mg, 0.75 mm, yield 94%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.82 (s, 1H, H2); 6.79 (dd, 1H, J = 7.9, 0.5 Hz, H7); 6.76 (dd, 1H, J = 1.7, 0.5 Hz, H4); 6.70 (dd, 1H, J = 7.9, 1.7 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.40 (s, 3H, CH_3O); 2.86 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.1 Hz, H2″); 1.59 (m, 2H, H3″); 1.26 (m, 16H, H4″−H11″); 0.88 (t, 3H, J = 6.2 Hz, H12″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7 (C1″); 146.2 (C3′); 144.8 (C7′); 131.8 (C5); 121.9 (C6); 119.2 (C2); 108.87 (C7); 107.9 (C4); 64.8 (CH_2O); 50.0 (OCH_3); 35.0 (CH_2Ph); 34.3 (C2″); 31.9 (C10″); 29.6, 29.4, 29.3, 29.2, 29.1 (6 C, C4″−C9″); 25.0 (C3″); 22.7 (C11″); 14.0 (C12″); IR (cm^{-1}, $CHCl_3$): 3034, 3008, 2934, 2854 (ν_{C-H}); 2675, 1723 ($\nu_{C=O}$); 1633, 1610, 1499, 1467, 1444 ($\nu_{C=C}$); 1386, 1354 (δ_{OH}), 1247 (ν_{C-O}). HRMS: found, 378.2408, $C_{22}H_{34}O_5$ requires 378.2406.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl palmitate ***12*** *(oil, 319 mg, 0.74 mmol, yield 92%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.82 (s, 1H, H2); 6.81 (d, 1H, J = 7.9 Hz, H7); 6.76 (d, 1H, J = 1.9 Hz, H4); 6.70 (dd, 1H, J = 7.9, 1.9 Hz, H6); 4.23 (t, 2H, J = 7.0 Hz, CH_2O); 3.40 (s, 3H, CH_3O); 2.87 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.5 Hz, H2″); 1.58 (m, 2H, H3″); 1.27 (m, 24H, H4″−H15″); 0.87 (t, 3H, J = 7.0 Hz, H16″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.8 (C1″); 146.1 (C3′); 144.7 (C7′); 131.7 (C5); 121.9 (C6); 119.1 (C2); 108.9 (C7); 107.9 (C4); 64.8 (CH_2O); 49.9 (OCH_3); 34.9 (CH_2Ph); 34.3 (C2″); 31.9 (C14″); 29.7, 29.6, 29.4, 29.3, 29.1 (10 C, C4″-C13″); 24.9 (C3″); 22.7 (C15″); 14.1 (C16″); IR (cm^{-1}, $CHCl_3$) 3030, 2927, 2855 (ν_{C-H}); 1728 ($\nu_{C=O}$); 1498, 1470, 1444 ($\nu_{C=C}$); 1252, 1197, 1170, 1150, 1098, 1039 (ν_{C-O}). HRMS: found, 434.3030, $C_{26}H_{42}O_5$ requires 434.3032.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl stearate ***13*** *(oil, 333 mg, 0.72 mmol, yield 90%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.82 (s, 1H, H2); 6.79 (d, 1H, J = 8.0 Hz, H7); 6.76 (d, 1H, J = 1.5 Hz, H4); 6.70 (dd, 1H, J = 8.0, 1.5 Hz, H6); 4.23 (t, 2H, J = 7.0, CH_2O); 3.39 (s, 3H, CH_3O); 2.86 (t, 2H, J = 7.0 Hz, CH_2Ph); 2.28 (t, 2H, J = 7.5 Hz, H2″); 1.70−1.50 (m, 2H, H3″); 1.35−1.20 (m, 28H, H4″−H17″); 0.88 (t, 3H, J = 6.4 Hz, H18″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.6 (C1″); 146.2 (C3′); 144.8 (C7′); 131.8 (C5); 121.9 (C6); 119.2 (C2); 108.8 (C7); 107.9 (C4); 64.8 (CH_2O); 49.9 (CH_3O); 34.9 (CH_2Ph); 34.3 (C2″); 31.9 (C16″); 29.7, 29.6, 29.4, 29.3, 29.2, 29.1 (12 C, C4″−C15″); 24.9 (C3″); 22.7 (C17″); 14.0 (C18″); IR (cm^{-1}, $CHCl_3$) 3029, 3005, 2916, 2848 (ν_{C-H}); 1724 ($\nu_{C=O}$); 1632, 1607, 1495, 1463 1442 ($\nu_{C=C}$); 1387, 1354. HRMS: found, 462.3347, $C_{28}H_{46}O_5$ requires 462.3345.

2-(2-Methoxy-1,3-benzodioxol-5-yl)ethyl oleate ***14*** *(oil, 357 mg, 0.78 mmol, yield 97%):* ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.87 (s, 1H, H2); 6.83 (d, 1H, J = 7.9 Hz, H4); 6.79 (d, 1H, J = 1.6 Hz, H7); 6.72 (dd, 1H, J = 7.9, 1.6 Hz, H6); 5.37, 5.31(2 dtt, 2H, J = 10.8, 5.5, 1.6 Hz, H9″ and H10″); 4.23 (t, 2H, J = 7.0 Hz, CH_2Ph); 3.40 (s, 3H, OCH_3); 2.86 (t, 2H, J = 7.0 Hz, CH_2O); 2.30 (t, 2H, J = 7.5 Hz, H2″); 2.10−1.90 (m, 4H, H8″ and H11″); 1.70−1.50 (m, 2H, H3″); 1.40−1.20 (m, 20H, H4″−H7″ and H12″−H17″); 0.88 (t, 3H, J = 6.1 Hz, H18″); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.7 (C1″); 146.2 (C3′); 144.7 (C7′); 131.7 (C5); 130.0, 129.8 (C9″ and C10″); 121.9 (C6); 119.2 (C2); 108.9 (C7); 107.9 (C4); 64.8 (CH_2O); 49.9 (CH_3O); 34.9 (CH_2Ph); 34.1 (C2″); 31.9 (C16″); 29.8, 29.7, 29.5, 29.3, 29.1, (8 C, C4″−C7″ and C12″−C15″); 27.2 (C8″ and C11″), 24.9 (C3″); 22.6 (C17″); 14.1 (C18″); IR (cm^{-1}, $CHCl_3$) 3034, 3016, 3010, 2931 (ν_{C-H}); 1731 ($\nu_{C=O}$); 1628 ($\nu_{C=C}$); 1520, 1464, 1440, 1234, 1196, 1170, 1110, 1016 (ν_{C-O});. HRMS: found, 460.3191, $C_{28}H_{44}O_5$ requires 460.3189.

2-(3,4-Dihydroxyphenyl)ethyl Esters 15−24. The appropriate protected ester (0.32 mmol) was added to a suspension of Amberlist 15 (284 mg), K_2HPO_4 (56 mg, 0.32 mmol), and KH_2PO_4 (44 mg, 0.32 mmol) in 4 mL of dry THF and MeOH (10 equiv). The mixture was refluxed under argon atmosphere in the dark for 2 h until HPLC analysis showed complete conversion of the substrate. The suspension was filtered, and the liquid phase was evaporated in vacuo to leave a crude mixture that was purified over prewashed silica gel (20:1) by elution with petroleum ether/AcOEt (80:20) to afford the pure already known hydroxytyrosyl esters **15−24** with the reported yields. All spectroscopic data were coherent with those reported in the literature.

Table 1. Log *P* Values of Hydroxytyrosol (**1**) and Its Esters **4** and **15−24**

compd	calcd[a]	exptl
Htyr 1	0.021 ± 0.218	0.09 ± 0.02[b]
		−0.08 ± 0.09[c]
HtyrAc 4	0.961 ± 0.234	0.95 ± 0.56[b]
		0.78 ± 0.21[c]
HtyrBu 15	2.023 ± 0.235	1.77 ± 0.42[b]
		2.03 ± 0.44[c]
HtyrHex 16	3.086 ± 0.235	
Htyr Hep 17	3.617 ± 0.235	
Htyr Oct 18	4.149 ± 0.235	
Htyr Non 19	4.680 ± 0.235	
Htyr Dec 20	4.985 ± 0.235	5.20 ± 0.46[b]
Htyr Dod 21	6.275 ± 0.235	
Htyr Pal 22	8.400 ± 0.235	
Htyr Ste 23	9.462 ± 0.235	9.46 ± 0.48[b]
Htyr Ole 24	8.944 ± 0.242	

[a] Calculated by Advance Chemistry Development Chem Sketch software v. 12.01 (1994−2009 ACD/Lab). [b] Reference *17*. [c] Reference *30*.

2-(3,4-Dihydroxyphenyl)ethyl butyrate ***15*** *(63 mg, 0.28 mmol, yield 87%):* CAS Registry No. 644985-85-3 (*17*). *2-(3,4-Dihydroxyphenyl)ethyl hexanoate* ***16*** *(71 mg, 0.28 mmol, yield 88%):* CAS Registry No. 1064639-73-1 (*21*). *2-(3,4-Dihydroxyphenyl)ethyl heptanoate* ***17*** *(81 mg, 0.30 mmol, yield 95%):* CAS Registry No. 644985-86-4 (*24*). *2-(3,4-Dihydroxyphenyl)-ethyl octanoate* ***18*** *(83 mg, 0.30 mmol, yield 93%):* CAS Registry No. 205241-38-9 (*30*). *2-(3,4-Dihydroxyphenyl)ethyl nonanoate* ***19*** *(86 mg, 0.29 mmol, yield 91%):* CAS Registry No. 850786-51-5 (*20*). *2-(3,4-Dihydroxyphenyl)ethyl decanoate* ***20*** *(104 mg, 0.31 mmol, yield 97%):* CAS Registry No. 934749-52-7 (*17*). *2-(3,4-Dihydroxyphenyl)ethyl dodecanoate* ***21*** *(97 mg, 0.29 mmol, yield 91%):* CAS Registry No. 896100-00-8 (*19*). *2-(3,4-Dihydroxyphenyl)ethyl palmitate* ***22*** *(114 mg, 0.29 mmol, yield 91%):* CAS Registry No. 644985-87-5 (*22*). *2-(3,4-Dihydroxyphenyl)ethyl stearate* ***23*** *(126 mg, 0.30 mmol, yield 94%):* CAS Registry No. 609360-23-8 (*22*). *2-(3,4-Dihydroxyphenyl)ethyl oleate* ***24*** *(125 mg, 0.30 mmol, yield 94%):* CAS Registry No. 611237-25-3 (*22*).

Partition Coefficient Values (Log *P*). Log *P* values have been calculated by Advance Chemistry Development Chem Sketch software v. 12.01 (1994−2009 ACD/Lab) and compared with experimental data derived by the literature. All data are reported in **Table 1**.

Evaluation of Antioxidant Capacity by ABTS Assay. The antioxidant capacities of **1**, **4**, and **15−24** were measured according to the method of Pellegrini et al. (*26*), as their quenching capacity toward the ABTS radical cation. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, **25**) was used as reference antioxidant. The analyses were performed either in alcoholic (0.2% of water) or in EtOH/refined olive oil (2%, see Materials), measuring the absorbance at 734 nm using a Perkin-Elmer Lambda 14P spectrophotometer.

Compounds **4** and **15−24** were used directly after their deprotection and purification to avoid oxidation of the samples. Each sample for analysis (1 mL) was obtained by dilution of the $ABTS^{\bullet+}$ aqueous mother solution (*26*) with ethanol to an absorbance of 0.70 ± 0.20 and then adding 10 μL aliquots of the ethanol or ethanol/refined olive oil solutions of antioxidants. Hydroxytyrosol (**1**), Trolox (**25**), and esters **4** and **15−24** were analyzed in both conditions at four different final concentrations ranging from 1 to 15 μM. At variance with Pellegrini et al. (*26*) the analyses were run at room temperature. The extent of color fading was measured after 2 min, and four measures were recorded for each concentration. Solvent blanks were also run. Collected data showed standard deviation always below 3%. The dose−response curves were expressed as the percentage of absorbance decrease (% ABTS inhibition) against the amount of antioxidant concentration. Linear regression was elaborated using Microcal Origin 5.0 software. The antioxidant capacity either in ethanol or in ethanol/refined olive oil solution was reported as Trolox equivalent antioxidant capacity (TEAC), defined as the concentration (mmol/L) of Trolox having the antioxidant activity equivalent to that of a 1 mmol/L solution of the substance under investigation (*26*). Results are expressed as means ± standard deviation and are reported in **Figure 3**. Statistical analyses were performed by applying Student's test. The level of significance was $p < 0.05$ for all data.

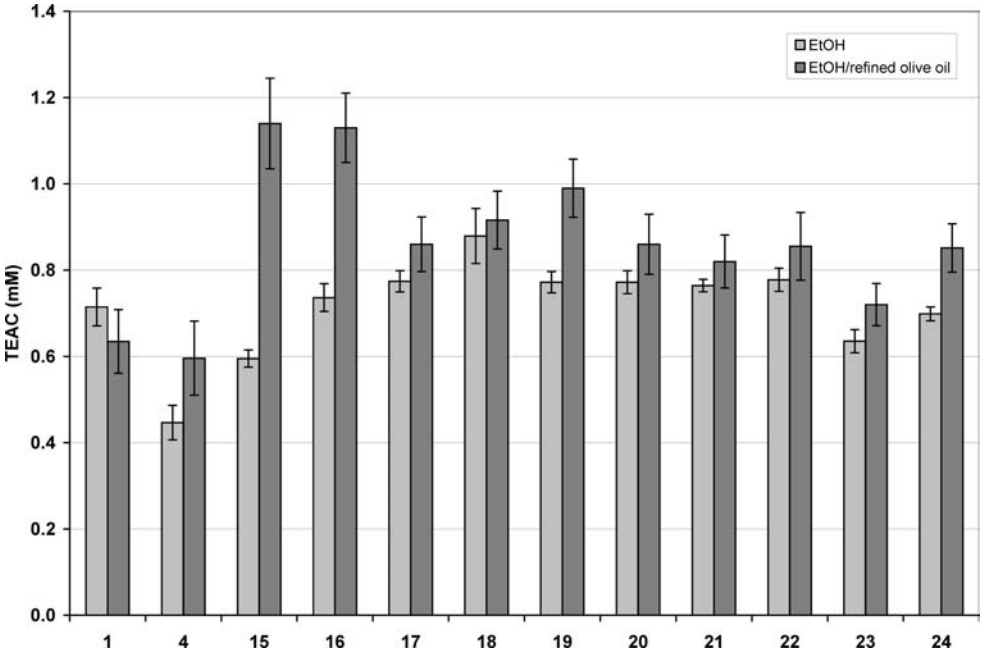


Figure 3. Radical scavenging capacity evaluated by ABTS assay in EtOH and EtOH/refined olive oil for hydroxytyrosol (**1**) and esters **4** and **15**−**24**. Results are expressed as millimoles of Trolox equivalents. Reported data show standard deviation always below 3%. Statistical significance has *p* values always below 0.05%.

Molecular Dynamics Simulations. GROMACS v. 4.0.2 package (*31*) was utilized for calculations. General AMBER force field (GAFF) (*32*) parameters were assigned for the analyzed molecule and ethanol. To obtain atomic partial charges, quantum chemical calculations were performed with the Gaussian 03 set of programs (Gaussian 03, revision C.02, Gaussian, Inc., Wallingford, CT, 2004). The ab initio geometry optimizations were performed with the B3LYP functional method and 6-31G* basis set. The Hartree−Fock level of theory with the 6-31G* basis set was used for electrostatic potential (ESP) calculations. The ANTECHAMBER v 1.27 set of programs was used for the restrained electrostatic potential charge fitting (RESP) and topology generation. The conversion to Gromacs topology files was performed by a script obtained from Dr. Pande's group Website (www.gromacs.org/).

The optimized conformation of the analyzed molecule obtained from quantum chemical calculations was centered in a rhombic dodecahedron box of size such that the distances between the molecule and the box boundaries were >0.5 nm. The volume of the box was 25.5 nm^3. The box was filled with 229 ethanol molecules. Position restraints with the force constant of 1000 kJ mol^{-1} nm^{-2} were applied on the heavy atoms of the molecule to prevent conformational changes during the solvent equilibration phase. A 500 ps simulation at constant number−volume−temperature (NVT) conditions and a 2 ns simulation at constant number−pressure−temperature (NPT) conditions with the position restraints on the molecule atoms were performed. Next, the restraints were released, and 30 ns molecular dynamics simulations under constant pressure and temperature conditions were conducted. A V-rescale algorithm with the coupling time constant of 0.1 ps was used to maintain a constant temperature value of 298 K. The constant pressure conditions were obtained by applying Berendsen barostat with the coupling constant of 1.0 ps. The time step of the simulations was 2 fs. Periodic boundary conditions were applied in all directions. Lennard-Jones and short-range electrostatic interactions were cut off at 1.0 nm. The particle-mesh Ewald method (PME) was used for long-range electrostatic interactions. All bonds were constrained with the LINCS algorithm. Analysis of the main conformational populations observed during the production run was performed using the g_cluster program of the Gromacs package. Results are reported in **Figure 4**.

Evaluation of Antioxidant Activity in L6 Myoblast Cells by DCF Assay. The antioxidant activities of **1**, **25**, **4**, and **15**−**24** in cell culture were analyzed using L6 cells derived from rat skeletal muscle, following the procedure described by Pedersen at al. (*27*). Incubation with the probe $DCFH_2$-DA at a final concentration of 10 μM (from a stock solution of 10 mM in DMSO) was carried out for 30 min in the dark at 37 °C, as reported by Pallottini et al. (*33*). The assay was carried out in 3 mL of final buffer containing 200 μL of cell suspension. Intracellular fluorescence was measured under continuous gentle magnetic stirring at 37 °C in a Perkin-Elmer (Norwalk, CT) LS 50B luminescence spectrometer. Excitation and emission wavelengths were set at 498 and 530 nm, respectively, using 5 and 10 nm slits for the two light paths. Cumene hydroperoxide in DMSO was used as radical generator (final concentration of 300 μM); DMSO at the concentrations used did not affect the fluorescence signal. Cells were incubated with compounds **1**, **25**, **4**, and **15**−**24** at the final concentration of 10 μM for 10 min at 37 °C before the addition of cumene hydroperoxide; none of the tested hydroxytyrosyl esters gave rise to fluorescence by itself. The antioxidant activities of **1**, **25**, **4**, and **15**−**24** were determined by the decrease in the intracellular DCF fluorescence,

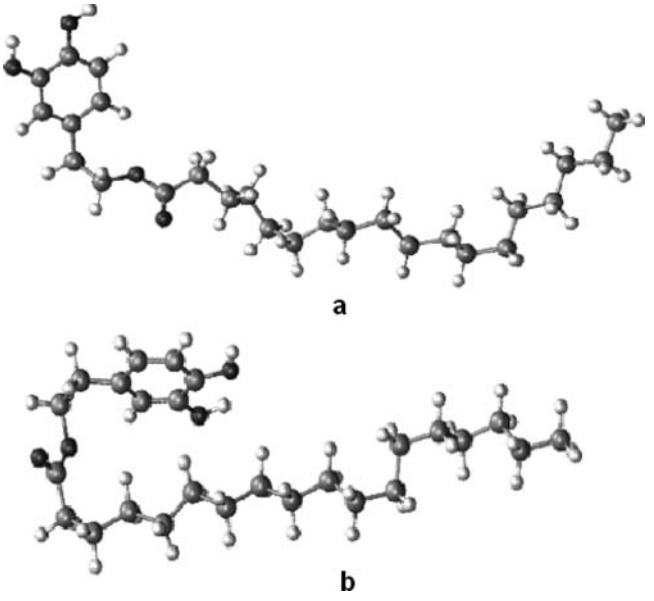


Figure 4. Molecular dynamics of hydroxytyrosyl stearate in EtOH solution. The two most representative clusters of conformations containing 2373 (**a**) and 463 (**b**) structures are shown.

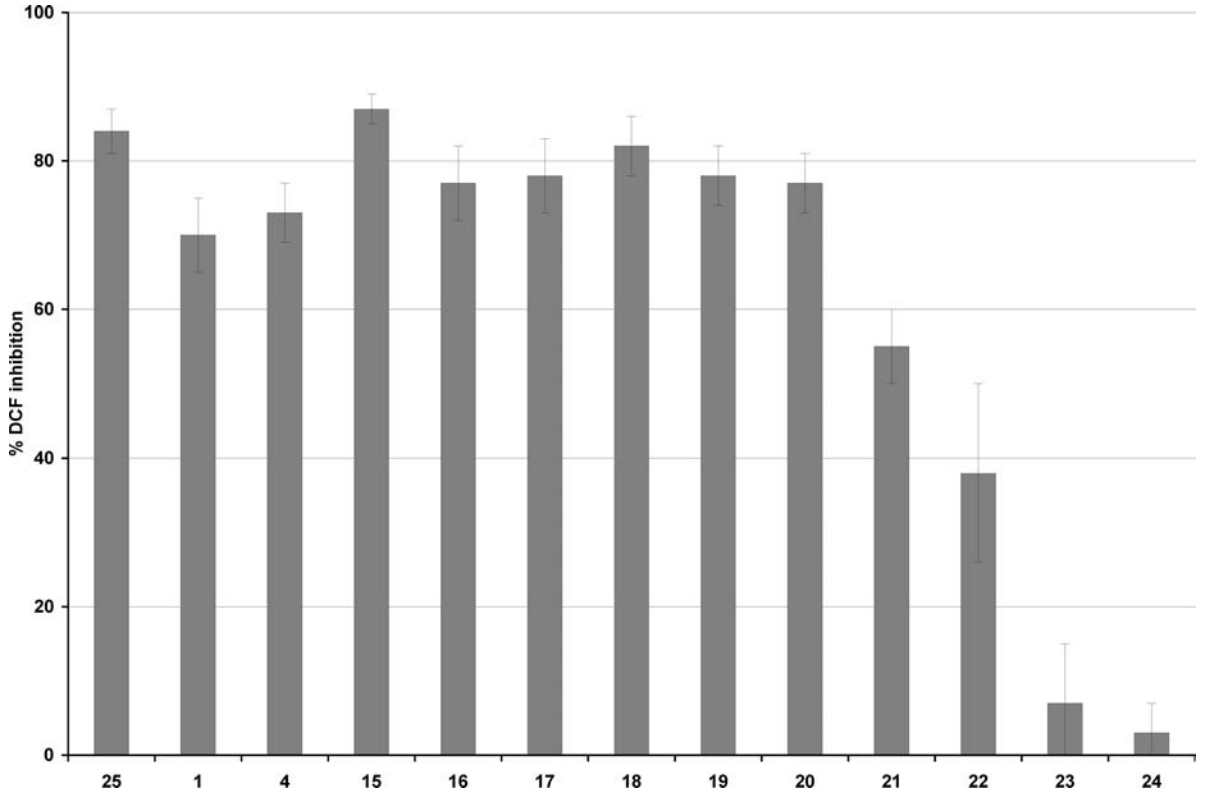


Figure 5. Percentage of DCF fluorescence inhibition in cell-culture experiments for Trolox (**25**), hydroxytyrosol (**1**), and esters **4** and **15**−**24**. Final concentration of esters is 10 μM. Standard deviations are always below 14%. Statistical significance with respect to cumene hydroperoxide (considered 100%) is always below 0.005% except for compounds **23** and **24** ($p < 0.25$).

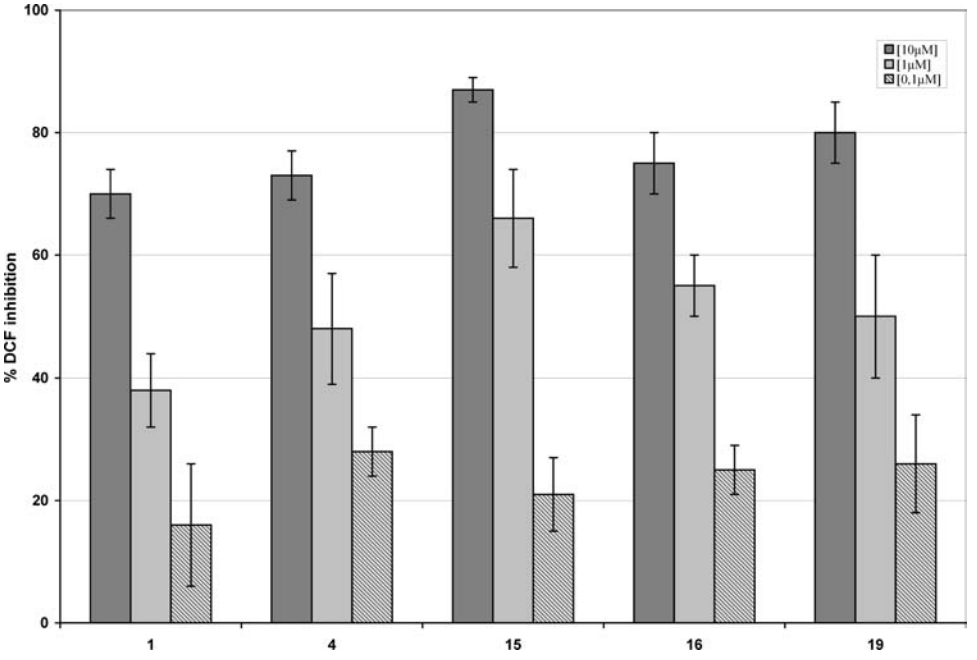
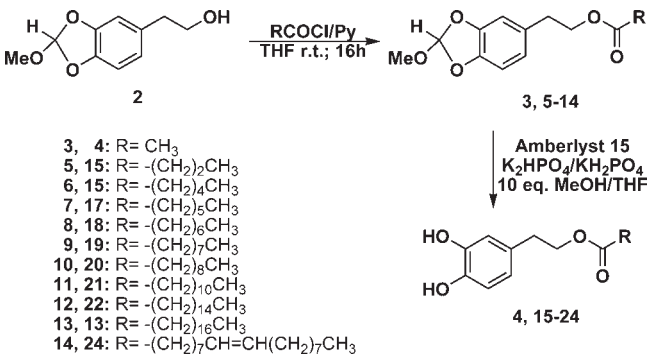


Figure 6. Dose−response experiments in cell culture. DCF fluorescence inhibition was performed at 10, 1.0, and 0.1 μM final concentrations of hydorxytyrosol (**1**) and esters **4**, **15**, **16**, and **19**. Standard deviations are always below 10%. With respect to cumene hydroperoxide (considered 100%), statistical significance is always below 0.005% except for analysis at 0.1 μM concentration ($p < 0.025$).

reported as $\Delta F/10$ min, and were calculated relative to the fluorescence change induced by 300 μM cumene hydroperoxide alone (100%). Data are reported as mean ± SD of at least $n = 4$ different experiments. Statistical analyses was carried out by applying Student's test. Statistical significance with respect to cumene hydroperoxide (considered to be 100%) is always below 0.005% except for compounds **23** and **24** ($p < 0.25$). Results are reported in **Figure 5**.

Dose−Response Activity in L6 Myoblast Cells by DCF Assay. Measurements of dose−response activity of compounds **1**, **4**, **15**, **16**, and **19** were carried out in the same conditions of the DCF assays only by

Scheme 1. Preparation of Hydroxytyrosyl Esters **4** and **15**−**24**



varying the amount of antioxidant to the final concentrations of 1.0 and 0.1 μM. Analogously, data are reported as mean ± SD of at least $n = 4$ different experiments. Statistical analyses was carried out by applying Student's test. With respect to cumene hydroperoxide (considered 100%), statistical significance is always below 0.005% except for analysis at 0.1 μM concentration ($p < 0.025$). Results are reported in **Figure 6**.

RESULTS AND DISCUSSION

Preparation of Hydroxytyrosyl Esters. Hydroxytyrosyl esters **15**−**24** have been prepared according to a procedure (**Scheme 1**) previously applied to the synthesis of hydroxytyrosyl acetate **4** (*29*), based on the preparation of the corresponding methyl orthoformates **5**−**14** by direct acylation with acyl chlorides of methyl orthoformate-protected hydroxytyrosol **2**. Subsequent protection removal gave esters **15**−**24**. This new protocol, which overcomes chemoselectivity problems, turned out to be highly efficient with respect to the previous protocol employed and suitable for the selective esterification of both short- and long-chain esters.

Recovery of derivatives **5**−**9** or **10**−**14**, at the end of the first step, required two different workups, respectively. As a matter of fact, the short-chain methyl esters, from the previously described (*29*) quenching with methanol of the excess acyl chloride, were easily separable by chromatography from the desired ortho esters **5**−**9** (see Synthesis Route 1 under Materials and Methods). On the contrary, the corresponding long-chain methyl esters were inseparable from the ortho esters **10**−**14**. Therefore, an aqueous workup (see Synthesis Route 2 under Materials and Methods) was used for the latter. With this modification, acylations of **2** were easily carried out and afforded compounds **5**−**14** in good to excellent isolated yields (89−97%). The new compounds **5**−**14** were fully characterized by spectroscopic analysis.

In a similar manner, removal of the orthoformate protection from **5**−**14** to obtain the desired hydroxytyrosyl esters **15**−**24** (**Scheme 1**) required a modification of the protocol reported for the acetate **4** (*29*). In fact, the simple acid-catalyzed methanolysis of **5**−**14** resulted in a competitive transesterification reaction with production, again, of the corresponding inseparable methyl esters in amounts increasing as the length of the carbon chain increased as well. Therefore, to minimize the competing methanol-promoted transesterifications, compounds **5**−**14** were all deprotected using a THF solution containing only 10 equiv of methanol. This resulted in nearly quantitative (86−95%) recovery of the pure esters **15**−**24** (**Scheme 1**), after simple filtration and evaporation of the solvent. Spectroscopic properties of all the already known esters **15**−**24** are consistent with the data reported in the literature.

Compounds **4** and **15**−**24** were used immediately after their purification to avoid the observed ester hydrolysis, probably catalyzed by the acidic catechol moiety. Indeed, pure esters, after 1 month of storage at −20 °C, gave mixtures of their hydrolysis products.

To evaluate the increasing lipophilicity, the partition coefficients (log *P*, see Materials and Methods) for **1** and all esters **4** and **15**−**24** were calculated and are reported in **Table 1** in comparison with experimental data from the literature. As expected, lipophilicity increases as the number of carbon atoms increases as well.

Antioxidant Capacity by ABTS Assay. As previously mentioned, many papers (*17*, *19*, *22*) are concerned with the determination of the antioxidant capacity in vitro using various methods. However, the Rancimat (*19*, *22*) and DPPH (*17*, *18*) data are performed in lipophilic conditions (bulk oil or cyclohexane solution) except for a recent publication presenting DPPH analysis performed in MeOH (*30*). The ABTS assay is a widely applied method for measuring the radical scavenging ability of antioxidants, especially those present in foods (*22*, *26*, *30*, *34*), in a hydrophilic environment.

The antioxidant capacity of hydroxytyrosol (**1**), its esters **4** and **15**−**24**, and Trolox (**25**) have been measured both in ethanol and in EtOH/refined olive oil (2%) solution, and linear regression calculations of dose−response curves of antioxidant capacity versus concentration of the sample have been determined. The relative values, expressed as TEAC (*26*) for each compound either in ethanol or in ethanol/refined olive oil solution, are reported in **Figure 3**.

All tested esters show a nearly constant antioxidant capacity, comparable with or higher than that of hydroxytyrosol (**1**) itself. The better values are shown by medium-sized acyl chains (esters **17**−**20** in ethanol solution and esters **15**, **16**, and **19** in EtOH/refined olive oil). Attempts to correlate the increase of lipophilicity, expressed as log *P* (**Table 1**), of all hydroxytyrosyl esters with their antioxidant capacity failed. It is worth noting that all hydroxytyrosyl esters show a slightly better antioxidant capacity when analyzed in the presence of very small amounts (around 2%) of refined olive oil in the ABTS solution.

These findings are coherent with recent literature (*30*, *34*) where, at variance with previous papers (*17*, *19*, *22*), an increased antioxidant capacity is reported for medium-sized (C4−C9) hydroxytyrosyl esters or ethers in comparison with hydroxytyrosol, whereas the elongation of the acyl chain does not enhance the antioxidant activity. This confirms that antioxidant capacity does not depend only on lipophilicity. A possible explanation could be related to a competition between two contemporary increasing but opposite effects. As the acyl chain length and lipophilicity increase (see log *P* data, **Table 1**), the conformational freedom of the ester chain increases as well, and this could result in folded structures in which catechol hydroxyls are shielded (*30*, *34*).

Molecular Dynamics Data. To confirm the above hypothesis we have analyzed by molecular dynamics the behavior of hydroxytyrosyl stearate (**23**), as the longest chain example. Snapshots of the molecular dynamics trajectory were sampled every 10 ps for a total of 3000 structures analyzed. The GROMOS method (*35*), which utilizes an rmsd cutoff to count the number of neighbors in a cluster, was applied. A rmsd cutoff of 0.3 nm was used. Six clusters of conformations were obtained with the first two most representative clusters containing 2373 and 463 structures. For the latter, the structures with the smallest average distance to the other members of the cluster are shown in **Figure 4**. As shown, almost 15% of conformers show a structure in which the ester acyl chain half covers the aromatic ring (**Figure 4b**) and could interfere with the approach of $ABTS^{\bullet+}$ to the catechol moiety. The low percentage of these conformations could partially account for the lower antioxidant capacity of long-chain esters. From this point of view, the best antioxidant activity of the

medium-sized esters could be ascribed to their increased lipophilicity not balanced by hindered conformations.

Antioxidant Activity in Cell Culture. It is well-known that muscle cells are particularly sensitive to the level of ROS (*36*). Therefore, L6 rat muscle cells have been chosen to evaluate the capacity of hydroxytyrosyl esters to penetrate cell membrane and act as radical scavengers inside the cells. A DCF standard assay (*27*) was carried out, based on the production of dichlorofluorescein radical (DCF), as fluorescent probe, by oxidation of dichlorofluorescein ($DCFH_2$). As described under Materials and Methods, cumene hydroperoxide was used as a H_2O_2 generator to produce an oxidative stress inside the L6 rat muscle cells (*33*), and the antioxidant activity of each sample was measured as a decrease of fluorescence. Results for hydroxytyrosol (**1**), Trolox (**25**), and hydroxytyrosyl esters **4** and **15**−**24** are reported in **Figure 5** as percent DCF fluorescence inhibition versus each tested antioxidant at 10 μM final concentration. All tested compounds show a decrease of fluorescence in comparison with the experiments with cumene hydroperoxide alone. This confirms the penetration of the hydroxytyrosyl esters into the cells and subsequent quenching of superoxide or other peroxide-forming radicals. Hydroxytyrosyl oleate (**24**) evidenced, in some experiments, a low unexplained pro-oxidant activity at the beginning of the measures.

Interestingly, the structure/antioxidant activity of esters **4** and **15**−**24** in cell-culture experiments follows a general sigmoid curve in a direct relationship with the length of the alkylic chain. For short to medium acyl chains (C2−C10) the antioxidant activity rises as the lipophilicity increases as well, giving values always higher than that of hydroxytyrosol (**1**) itself, with a maximum for the butyrate **15**. However, the elongation over 12 carbons does not play a favorable role. Indeed, a constant activity drop is observed for esters carrying C12−C18 acyl chains. A similar behavior has already been reported for prodrug esters (*37*) in which both diffusion across cell membranes and bioavailability increase as the lipophilicity increases as well, until a maximum for esters with C4−C9 acyl chains. It is possible to suppose that, at a certain level of lipophilicity, the easy diffusion of esters into the cells could be balanced (C10) or even made unproductive (C12−C18) by entrapment into the plasma membrane caused by the higher affinity of long acyl chains with the phospholipids or hydrophobic proteins inside the bilayer.

Dose−Response Tests. It is well-known that ROS produce a biphasic effect on muscle contraction: a high level of ROS inhibits contraction, whereas a low level activates it. After the first positive evaluation of antioxidants at 10 μM concentration, considering that the antioxidant plasma reserve is 1 μM (*38*), we wanted to assess the capability of the more efficient compounds at lower concentration. Therefore, dose−response experiments were carried out also at the concentrations of 1.0 and 0.1 μM on hydroxytyrosol (**1**) and the more active hydroxytyrosyl esters **4**, **15**, **16**, and **19**. Results are shown in **Figure 6**. **1** and all tested esters maintained a good antioxidant activity at 1.0 μM concentration with a DCF fluorescence inhibition ranging from 38% of hydroxytyrosol (**1**) to 66% of hexanoate **16**. Even at 0.1 μM concentration the antioxidant activity of esters against cumene hydroperoxide is not negligible (21−28%) and better than that of hydroxytyrosol itself (16%). This confirms the high potentiality of these compounds for possible application in nutraceutical and pharmacological preparations.

In conclusion, the protocol (*29*) previously reported for the synthesis of the hydroxytyrosyl acetate (**4**) from stabilized hydroxytyrosyl orthoformate **2** has been modified and successfully applied to the preparation of higher acyl homologues, namely, hydroxytyrosyl esters **15**−**24**. This easy and mild procedure overcomes chemoselectivity problems and allows the high-yielding acylation of hydroxytyrosol with fatty acid acyl chlorides. Furthermore, the protection of the catechol moiety avoids oxidation processes and allows the study of their antioxidant properties on the freshly deprotected substrates. Therefore, this protocol represents a very efficient alternative with respect to previously published methodologies (*19*−*21*).

All of the hydroxytyrosyl esters synthesized have been analyzed for their antioxidant effect both by ABTS and by DCF assay. Log *P* values and molecular dynamics were also performed to support the obtained results. Furthermore, dose−response tests on cells were carried out on hydroxytyrosol and the more active derivatives. Our results, both by ABTS assay and in cell-culture experiments, evidence the absence of a linear relationship between lipophilicity and antioxidant effect, and, at the same time, they suggest a complex relationship between the length of the ester chain and the relative antioxidant effect. In both cases, medium-sized esters have shown an antioxidant effect higher than that of hydroxytyrosol itself with a maximum in the range for C6−C10. Furthermore, dose−response tests on cells, for the more active derivatives, have shown a not negligible antioxidant activity, even at 0.1 μM concentration. However, in the ABTS assay, longer chain esters (C12−C18) show an antioxidant capacity similar to that of hydroxytyrosol (**1**), whereas in cells a sharp decrease of activity is observed.

As a possible explanation, the favorable role played by the growing lipophilicity, in medium-sized esters, could be balanced, or even reverted, by the chain length-dependent problems arising in long-chain esters. As shown by molecular dynamics data, folded conformations and the consequent shielding of the catechol moiety can partly account for the lower performance in alcoholic medium of long-chain esters with respect to the medium-sized ones. Strikingly, a small percentage of refined olive oil in the ABTS solutions seems to increase the capacity, even if the meaning of this behavior is still to be understood.

In cells, the complex balance between the capacity of permeation through cell membrane and diffusion into the intracellular fluid, where the DCF fluorescent probe is dispersed, can give rise to trapping of long-chain esters in the cell membrane bilayer.

Finally, as in the carried out experiments the fluorescent probe was confined in cell cytosol, these data do not account for the antioxidant activity that the long-chain hydroxytyrosyl esters could perform inside the membrane bilayer against external ROS attack. Further work is in progress to answer this question.

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