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Synthesis and Structure/Antioxidant Activity Relationship of Novel Catecholic Antioxidant Structural Analogues to Hydroxytyrosol and Its Lipophilic Esters

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Supporting Information

ABSTRACT: A large panel of novel catecholic antioxidants and their fatty acid or methyl carbonate esters has been synthesized in satisfactory to good yields through a 2-iodoxybenzoic acid (IBX)-mediated aromatic hydroxylation as the key step. The new catechols are structural analogues of naturally occurring hydroxytyrosol (3,4-DHE). To evaluate structure/activity relationships, the antioxidant properties of all catecholic compounds were evaluated in vitro by ABTS assay and on whole cells by DCF fluorometric assay and compared with that of the corresponding already known hydroxytyrosyl derivatives. Results outline that all of the new catechols show antioxidant capacity in vitro higher than that of the corresponding hydroxytyrosyl derivatives. Less evident positive effects have been detected in whole cells experiments. Cytotoxicity experiments, using MTT assay, on a representative set of compounds evidenced no influence in cell survival.

KEYWORDS: *catechols, aromatic hydroxylation, lipophilic antioxidants, ABTS assay, cell culture DCF assays, structure/antioxidant activity relationship*

INTRODUCTION

In the human body, reactive oxygen species (ROS) are continuously produced during metabolic processes, and their action is controlled by endogenous antioxidants that suppress or minimize free radicals and their chain reactions. When there is an imbalance in the redox equilibrium, oxidative stress is activated. These conditions stimulate aging pathogenesis and degenerative or chronic diseases and favor cell apoptosis and cancer.[1] Several epidemiological studies have provided support for a protective effect of the consumption of fresh fruits, vegetables, and virgin olive oil, the integral ingredients of the Mediterranean diet. In particular, the protective effect of virgin olive oil has been attributed to the presence of bioactive polyphenols[2] and their antioxidant activity, notably high in catechols because of their lower O−H bond dissociation enthalpy (77.7−80.1 kcal/mol) when compared to phenols (85.1−88.0 kcal/mol).[3] Among the polyphenols present in virgin olive oil, hydroxytyrosol [2-(3,4-dihydroxyphenyl)-ethanol, referred to as 3,4-DHE, Figure 1] has been shown to scavenge hydrogen peroxide[4] and to inhibit the hypochlorous acid-derived radicals. In addition, it exhibits several biological activities reducing the risk of coronary heart disease and atherosclerosis[5] and showing antimicrobial,[6] antiproliferative, and apoptotic activities.[7−9]

Having amphiphilic character (log P = 0.08), 3,4-DHE has only limited applications in hydrophobic/lipidic media or as a preservative in food technology and the cosmetic industry.

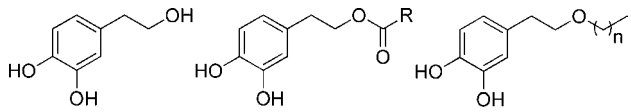

Figure 1. Hydroxytyrosol and its lipophilic derivatives.

Therefore, in view of the above remarkable biological properties, many 3,4-DHE derivatives have been prepared in the past few years, and their structure/antioxidant activity relationships (SAR) have been studied. Namely, SAR studies on 3,4-DHE derivatives have evidenced the key role of the free catechol function as stated by the low level of antioxidant activity shown by 3,4-DHE derivatives carrying an ester function at the catechol hydroxyls.[10] On the contrary, chemoselective acylation or alkylation at the 3,4-DHE alcohol function with long saturated and unsaturated alkyl chains gives rise to lipophilic 3,4-DHE esters[10−16] or ethers[17] (Figure 1). As a consequence, the importance of the chemoselective esterification/etherification at the alcoholic hydroxyl has been outlined. Moreover, lipophilic 3,4-DHE derivatives overcome

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the above solubility limitations and are easily incorporated in cosmetic and food preparations.[18] In the literature, most of the studies on lipophilic antioxidants reported that the longer the alkyl chain length, the more active the antioxidant; some other contributions showed, in contrast, a nonlinear trend of the chain length in emulsified,[19−21] liposomal,[22,23] and cellular systems.[14,24,25] In these works, the antioxidant activity increases more or less when the hydrophobicity is increased, whereas after a threshold is reached for medium chain length, the antioxidant activity collapses to a near zero value.

As to the effect on the antioxidant activity of the relative position of the two phenolic hydroxyls in these systems, previous SAR studies have shown that the best results are obtained when the two hydroxyls are adjacent (catechols). This is related both to the above-mentioned lower dissociation enthalpy[3] and to the formation of an intramolecular H-bond. However, nothing is known about the effect of moving the catechol function to the 2,3-position with respect to the alcohol side chain. Furthermore, a previous study, carried out on a few 3-(3,4-dihydroxyphenyl)propyl fatty acid esters (3,4-DHP esters), reported an increase of antioxidant activity with respect to the corresponding 3,4-DHE derivatives.[10]

On these bases and as a part of our research devoted to the preparation of biologically active compounds inspired by 3,4-DHE,[14,26,27] it seemed interesting to explore SAR in novel catechols. Therefore, we planned a study focused on the synthesis and evaluation of the antioxidant activities of a large panel of novel catecholic compounds and their lipophilic derivatives (Figure 2). Namely, with respect to 3,4-DHE **1**, the

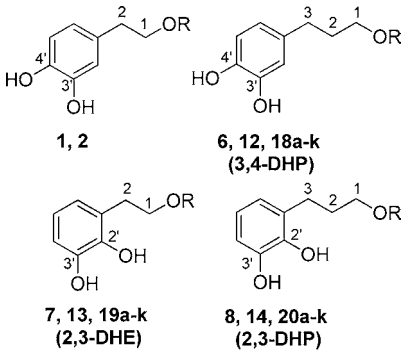

Figure 2. Catechol derivatives projected as antioxidants together with hydroxytyrosol **1** and its methyl carbonate **2**.

3-(3,4-dihydroxyphenyl)propanol (3,4-DHP) derivatives **6**, **12**, and **18** are higher homologues; 2-(2,3-dihydroxyphenyl)-ethanol (2,3-DHE) derivatives **7**, **13**, and **19** show a different substitution pattern of the catechol moiety; and 3-(2,3-dihydroxyphenyl)propanol (2,3-DHP) derivatives **8**, **14**, and **20** exhibit a combination of the two previous structural modifications.

We describe here a convenient and practical procedure useful to synthesize the novel catechols reported in Figure 2 from commercially inexpensive phenols. The antioxidant properties of all new catechols were evaluated in vitro by 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic)diammonium salt (ABTS) assay[28] and on whole cells by dichlorodihydrofluorescein (DCF) fluorometric assay.[29] Structure/antioxidant activity relationships of all new compounds are discussed by comparison with that of hydroxytyrosol **1**, methyl carbonate **2**,[30] and its already known esters.[14]

MATERIALS AND METHODS

Safety. IBX was reported to be explosive under impact or heating to >200 °C.[31] Dess and Martin suggested that the explosive properties of some samples of IBX were due to the presence of impurities of potassium bromate utilized during its preparation, that is, the oxidation of 2-iodobenzoic acid in acidic medium.[32] A safe and convenient preparation of IBX involves the utilization of oxone in water.[33]

Reagents. All chemicals used were of analytical grade. Solvents and starting phenols **3** and **4** (Figure 3) were purchased from Sigma Aldrich (Milan, Italy). 2-Iodoxybenzoic acid (IBX) was prepared according to the safe procedure reported in the literature.[33] IBX−polystyrene was furnished by Novabiochem (loading factor = 1.1 mmol/g). 3,4-DHE **1**, hydroxytyrosyl methyl carbonate **2**,[26,34] and 3-(2-hydroxyphenyl)propanol **5**[35] were synthesized as already described in the literature. Silica gel 60 F254 plates and silica gel 60 were purchased from Merck (Milan, Italy). 2′,7′-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was obtained from Molecular Probes (Eugene, OR, USA). L6 cells from rat skeletal muscle were from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), antibiotics, and sterile plastic ware for cell culture were from Flow Laboratory (Irvine, CA, USA). Fetal bovine serum was from GIBCO (Grand Island, NY, USA).

Instrumental Analyses. HPLC analyses were performed on a Thermo Spectra series P200 apparatus equipped with a Thermo Hypersil BDS C18 column (250 × 4.6 mm, 5 μm) and an UV detector selected at λ = 280 nm. Elutions were carried out at 1 mL/min flow rate by using a 10 min gradient from an H_2O/CH_3CN mixture (90:10, v/v) to pure CH_3CN. GC-MS analyses were performed on a Shimadzu VG 70/250S apparatus equipped with a Supelco SLB-5 ms column (30 m, 0.25 mm, and 0.25 μm film thickness). The analyses were performed using an isothermal temperature profile of 100 °C for 2 min, followed by a 10 °C/min temperature gradient until 280 °C for 15 min. The injector temperature was 280 °C. High-resolution mass spectrometry (HRMS) analyses were recorded with Micromass Q-TOF micro mass spectrometer (Waters). ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ (99.8% in deuterium) and in CD_3OD (99.8% in deuterium) using a 200 MHz nuclear resonance spectrometer from Bruker. All chemical shifts are expressed in parts per million (δ scale) and referenced to either the residual protons or carbon of the solvent. Spectral data of new compounds are reported in the Supporting Information.

Synthesis of Catechols 6−8. To a solution of the appropriate substrate **3**, **4**, or **5** (0.5 mmol) in dimethyl carbonate (DMC, 5.0 mL) was added IBX−polystyrene (1.2 mmol). The mixture was kept at room temperature for 1−2 h under magnetic stirring. The reaction was monitored by thin layer chromatography (TLC) and by GC-MS analysis. When the substrate disappeared, the heterogeneous reagent was recovered by filtration and the resulting solution was treated with sodium dithionite for several minutes. After evaporation of DMC under reduced pressure, the corresponding catecholic products were recovered as colorless oils (**6**, 80%; **7**, 68%; **8**, 65%).

Synthesis of Methyl Carbonate Catecholic Derivatives 12−14. *Step a.* The appropriate substrate **3**, **4**, or **5** (0.5 mmol) was dissolved in DMC (5.0 mL); then, 1,8-diazabicicylo[5.4.0]undec-7-ene (DBU, 0.6 mmol) was added, and the mixture was heated to reflux temperature for 6−8 h. The reaction was monitored by TLC and GC-MS. After the disappearance of the substrate, the reaction mixture was cooled to room temperature and DMC was evaporated under vacuum. The residue was dissolved in ethyl acetate and washed with 1 N HCl and saturated NaCl solution. The organic phase was dried over Na_2SO_4 and then filtered and concentrated under vacuum. Methyl

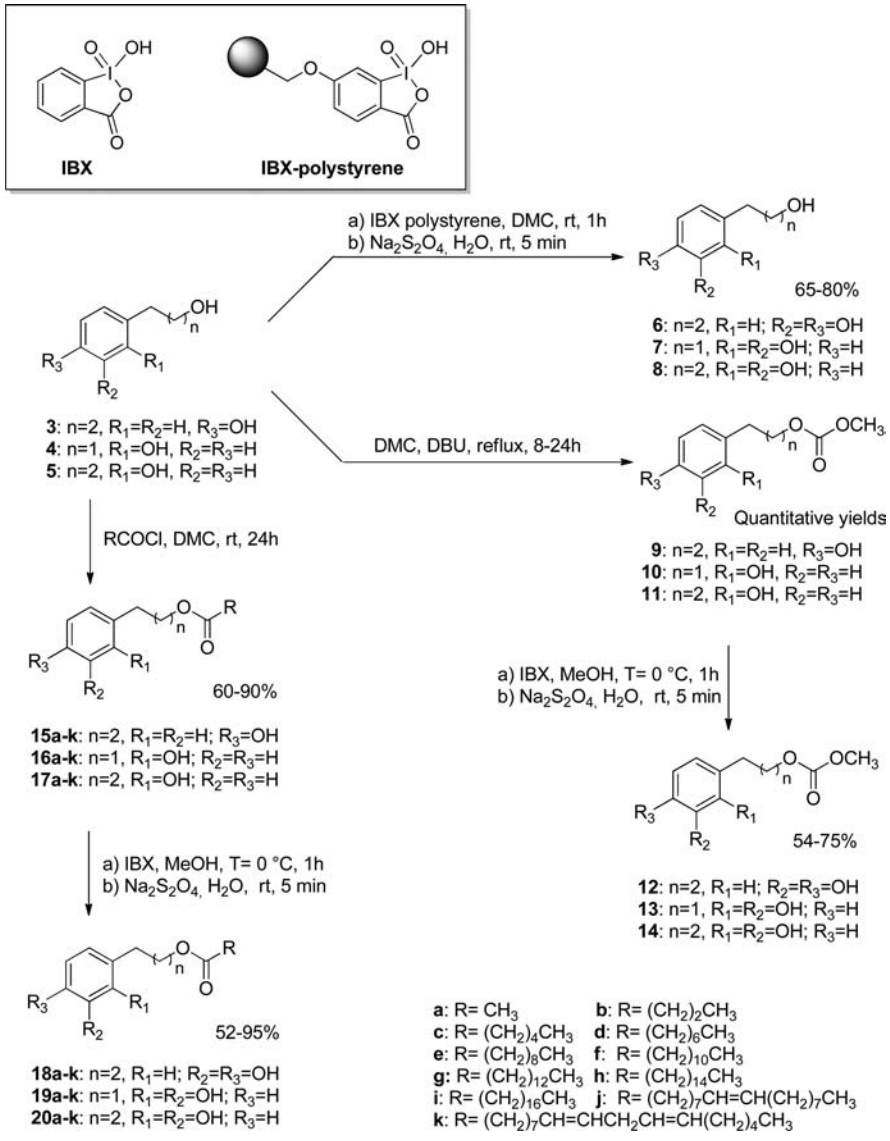

Figure 3. Synthesis of catechols **6**, **7**, and **8**; the corresponding methyl carbonates **12**, **13**, and **14**; and fatty acid esters **18a−k**, **19a−k**, and **20a−k**.

carbonates **9**, **10**, and **11** were isolated as colorless oils in quantitative yield.

Step b. The appropriate methyl carbonate **9**, **10**, or **11** (0.5 mmol) was dissolved in methanol (2.5 mL), and then IBX (0.6 mmol) was added. The solution was stirred at 0 °C until disappearance of the substrate. At the end, water and sodium dithionite were added. After the workup, catecholic compounds **12**, **13**, and **14** were obtained in 75, 64, and 54% yields.

Synthesis of Lipophilic Esters 18a−k, 19a−k, and 20a−k. *Step a.* The appropriate catechol **3**, **4**, or **5** (0.5 mmol) was dissolved in DMC (5.0 mL), and then the suitable acyl chloride (0.6 mmol) was added. The solution was stirred at room temperature for 24 h until disappearance of the substrate. DMC was evaporated under vacuum to afford a mixture that was solubilized with ethyl acetate, washed with saturated NaCl solution, and dried over Na_2SO_4. Purification on silica gel of the mixture by eluting with hexane/ethyl acetate gave the corresponding esters **15a−k**, **16a−k**, and **17a−k** in satisfactory to good yields (60−90%).

Step b. The appropriate esters **15a−k**, **16a−k**, or **17a−k** (0.1 mmol) were dissolved in CH_3OH (2 mL), and then IBX (0.1 mmol) was added. The solution was stirred at 0 °C until disappearance of the substrate. At the end, water and $Na_2S_2O_4$ were added and the solution was stirred for 5 min at room temperature. After evaporation of the solvent under vacuum, the residue was solubilized with ethyl acetate and treated with a saturated solution of $NaHCO_3$. The aqueous phase was extracted with ethyl acetate. The organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. After the workup, esters **18a−k**, **19a−k**, and **20a−k** were obtained in 52−95% yields.

Partition Coefficients Values (Log *P*). Log *P* values, simulating partitioning of catechol derivatives in an *n*-octanol/water system, have been calculated by Advance Chemistry Development Chem Sketch Software V 12.01 (1994−2009 ACD/Lab).

Hydrophilic−Lipophilic Balance (HLB). HLB has been calculated using Griffin's method: HLB = 20 × Mh/MW (where Mh = molecular mass of hydrophilic portion and MW = molecular weight).

Evaluation of Antioxidant Capacity by ABTS Assay. The antioxidant capacity of all compounds was measured according to the method of Pellegrini et al.[17] as the quenching capacity toward the ABTS radical cation, but the analyses were run at room temperature instead of 30 °C. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was used as reference antioxidant. The analyses were performed in ethanol (0.2% of water) by measuring the absorbance at λ = 734 nm using a Perkin-Elmer Lambda 14P spectrophotometer. Each sample (1 mL) was obtained by dilution of the $ABTS^{\bullet+}$ aqueous mother solution with ethanol to an absorbance of 0.70 ± 0.20 and then adding 10 μL aliquots of the ethanol solutions of antioxidants. All catecholic derivatives were analyzed at four different final concentrations ranging from 1 to 10 μM. The extent of color fading was

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measured after 2 min, and four measures were recorded for each concentration. Solvent blanks were also run. Collected data showed standard deviation below 5%. 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 capacities were extrapolated at 10 μM concentration and reported as trolox equivalent antioxidant capacity (TEAC), defined as the concentration (mmol/L) of trolox having the antioxidant capacity equivalent to that of a 1.0 mmol/L solution of the substance under investigation. Results are expressed as the mean ± standard deviation and reported as numerical data in the Supporting Information and plotted in Figures 4 and 5. Statistical analyses were performed by applying Student's test. The level of significance was $p < 0.005$ for all data.

Interatomic Distance Calculations. The distances between the acyl oxygen and the nearest catechol hydroxyl in hydroxytyrosyl acetate and acetates **18a**, **19a**, and **20a** were calculated after MM2 minimizations using *CS* Chem3D Ultra, 2001 Cambridge Soft Corp., and are reported in Figure 6.

Evaluation of Antioxidant Activity in L6 Myoblast Cells by DCF Assay. The antioxidant activities in cell culture of all catecholic derivatives were analyzed using L6 cells derived from rat skeletal muscle, following the procedure described by Pedersen at al.[29] Trolox was used as reference antioxidant. 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.[36] 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 using a Perkin-Elmer (Norwalk, CT, USA) LS 50B luminescence spectrometer. Excitation and emission wavelengths were set at λ = 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 = 300 μM); DMSO at the concentrations used did not affect the fluorescence signal. Cells were incubated with compounds at the final concentration of 10 μM for 10 min at 37 °C before the addition of cumene hydroperoxide; none of the tested compounds gave rise to fluorescence by itself. The antioxidant activities of all catechol derivatives were determined by the decrease in the intracellular DCF fluorescence, reported as ΔF/10 min, and were calculated relative to the fluorescence change induced by 300 μM cumene hydroperoxide alone (100%). Data are reported as the mean ± standard deviation of at least $n = 4$ different experiments. Statistical analyses were carried out by applying Student's test. Statistical significance with respect to cumene hydroperoxide (considered 100%) is always below 0.005% except for compounds **18i−k**, **19g**, **19j**, **19k**, and **20i−k** ($p < 0.05$). Results are expressed as the mean ± standard deviation and reported as numerical data in the Supporting Information and plotted in Figures 7 and 8.

Cell Survival Assessment. Cell survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[37] Five millimolar stock solutions of catechols **1**, **2**, **6−8**, **12−14**, **18d**, **19d**, **20d**, **18h**, **19h**, and **20h** were prepared and subsequently diluted in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin to final concentrations of 50, 20, and 10 μM. L6 cells were plated at 5000/well in a 96-well plate in 200 μL of complete medium. After 24 h, DMEM was discarded from the wells, and 100 μL of the test solutions was added. After 24 h of incubation, 10 μL of MTT solution (5 mg/mL) was added to each well and further incubated for 4 h. Lysis buffer was prepared by dissolving 40% (w/v) sodium dodecyl sulfate (SDS) in deionized water, mixing an equal volume of *N*,*N*-dimethylformamide with the SDS solution, and adjusting the pH to 4.7. After incubation with MTT, 100 μL of lysis buffer was added to each well and the absorbance read at λ = 570 nm, using 630 nm as the reference wavelength, on a microplate reader (Camberra Packard, Schwadorf, Austria). Each compound was tested in triplicate; medium without antioxidant and medium with 3% EtOH were added in control wells. Standard deviations were always below 5% except for control (10%) and ethanol (6%). Student's t test evidenced that none of the data were statistically different from control. Results are reported in Figure 9.

RESULTS AND DISCUSSION

Synthesis of Catecholic Compounds. The synthesis of hydroxytyrosol-like catechols and their lipophilic esters suffers from two difficulties. The first problem arises from the intrinsic high antioxidant activity of the catechol function that results in quick oxidation in the air, particularly on silica gel and in alkaline medium, to afford black polymeric matter. Therefore, the usual synthetic approaches to hydroxytyrosol-like catechols rule out the oxidative introduction of hydroxyls into the aromatic ring while favoring the reduction of an appropriate acyl side chain in a preformed catechol.[38] The second problem concerns the simultaneous presence of alcoholic and catecholic groups that gives rise to chemoselectivity problems during the esterification reactions. Reported solutions for chemoselective acylation at the alcohol function stand either on trans-esterification,[12] lipase-catalyzed,[11,13] or catecholic hydroxyl protection/deprotection procedures.[14]

However, we have recently reported that both 2-iodoxybenzoic acid (1-hydroxy-1-oxo-1*H*-1λ^5-benz[*d*][1,2]-iodoxol-3-one, IBX)[39−41] and its supported recyclable modification (IBX−polystyrene)[42] are able to chemo- and regioselectively introduce a second hydroxyl on tyrosyl esters [2-(4-hydroxyphenyl)ethanol esters], giving rise to 3,4-DHE esters.[26,27,34] This efficient aromatic hydroxylation occurs without any further oxidation of the produced catechol function and takes place under mild and not dry conditions. In addition, we have also found that the use of DMC as solvent in the acylation of tyrosol derivatives emphasizes the higher nucleophilicity of the alcoholic hydroxyl with respect to the phenolic ones and results in chemoselective esterification at the alcoholic side chain.[26,30]

On these bases and in view of the environmentally benign properties of both IBX and DMC, we planned the synthesis of the novel catechols **6−8** and their respective methyl carbonates **12−14** and fatty acid esters **18−20** (Figure 2) as depicted in Figure 3.

Namely, 3-(3,4-dihydroxyphenyl)-1-propanol **6** (3,4-DHP), 2-(2,3-dihydroxyphenyl)-1-ethanol **7** (2,3-DHE), and 3-(2,3-dihydroxyphenyl)-1-propanol **8** (2,3-DHP) were prepared by chemoselective IBX−polystyrene aromatic hydroxylation in DMC from the parent commercial phenols **3**, **4**, and **5**. Methyl carbonate derivatives **12**, **13**, and **14** were prepared according to a two-step procedure (Figure 3). A selective protection of the alcoholic group of **3**, **4**, and **5** by DMC/DBU system gave the intermediate methyl carbonates **9**, **10**, and **11**.[30] The subsequent IBX-mediated hydroxylation afforded the corresponding catecholic methyl carbonates **12**, **13**, and **14**. In the same manner, acylation of phenols **3**, **4**, and **5**, carried out with the appropriate acyl chloride in DMC, was highly chemoselective at the alcoholic side chain and gave esters **15a−k**, **16a−k**, and **17a−k**. Finally, esters **15a−k**, **16a−k**, and **17a−k** underwent IBX-mediated hydroxylation to afford the lipophilic catecholic derivatives **18a−k**, **19a−k**, and **20a−k**. To the best of our knowledge only compounds **18b**, **18h**, and **18i** are reported in the literature.[10,11] Spectroscopic properties of all new products are described in the Supporting Information.

Lipophilicity Measurement. To evaluate the effect of structural modifications on the lipophilicity of all catecholic compounds, partition coefficients (log *P*) and HLB values were

Table 1. Partition Coefficient Values (Log *P*) and Hydrophilic−Lipophilic Balance (HLB) of Catechols 6, 7, and 8, Methyl Carbonates 12, 13, and 14, and Fatty Acid Esters 18a−k, 19a−k, and 20a−k

compd	log P^a	HLB^b	compd	log P^a	HLB^b	compd	log P^a	HLB^b
6	1.2500		**7**	0.7940		**8**	1.2500	
12	2.1927	13.4	**13**	1.7580	12.9	**14**	2.1927	13.4
18a	1.8430	14.4	**19a**	1.3870	14.0	**20a**	1.8430	14.4
18b	2.7550	12.7	**19b**	2.2990	12.2	**20b**	2.7550	12.7
18c	3.6670	11.4	**19c**	3.2110	10.9	**20c**	3.6670	11.4
18d	4.5790	10.3	**19d**	4.1230	9.8	**20d**	4.5790	10.3
18e	5.4910	9.7	**19e**	5.0350	8.9	**20e**	5.4910	9.7
18f	6.0166	8.6	**19f**	5.9470	8.2	**20f**	6.0166	8.6
18g	6.8512	8.0	**19g**	6.4339	7.5	**20g**	6.8512	8.0
18h	7.6858	7.4	**19h**	7.2685	6.9	**20h**	7.6858	7.4
18i	8.5204	7.0	**19i**	8.1031	6.5	**20i**	8.5204	7.0
18j	8.2012	7.0	**19j**	7.7839	6.6	**20j**	8.2012	7.0
18k	7.8820	7.0	**19k**	7.4647	6.6	**20k**	7.8820	7.0

[a]Calculated by ACD Chem Sketch Software V12.01 (1994−2009 ACD/Lab). [b]Griffin's method: HLB = 20 × Mh/MW, where Mh = molecular mass of hydrophilic portion and MW = molecular weight.

calculated. As shown in Table 1 and as expected, methyl carbonate derivatives **12**, **13**, and **14** exhibit a higher lipophilicity than the corresponding catechols **6**, **7**, and **8** and even slightly higher than the acetates **18a**, **19a**, and **20a**, confirming that the introduction of the methyl carbonate moiety induces an increase of the lipophilicity. A similar trend is observed for hydroxytyrosol **1** (log *P* = 0.7940, see Materials and Methods) and its methyl carbonate derivative **2** (log *P* = 1.758, HLB = 12.9). Finally, the lipophilicity of catechol derivatives **18**, **19**, and **20** increases as the alkyl chain length increases as well, and this is in agreement with previous data about hydroxytyrosyl esters.

Antioxidant Capacity by ABTS Assay. The antioxidant capacity of polyphenolic antioxidants having different degrees of lipophilicity has been deeply investigated in the past few years.[19,24,43] Porter's polar paradox hypothesis, which claims that "polar antioxidants are more effective in non-polar lipids whereas non-polar antioxidants are more active in water/oil emulsions",[44] has been proved not to be always valid to explain the complex behavior of antioxidants having amphiphilic properties, such as fatty acid esters of catechols and related compounds. Recent works have demonstrated that the antioxidant activity of lipophilic catechols is not linearly correlated with hydrophobicity and is often connected with the antioxidant location in the biphasic environment.[19,25,43,45−47] The ABTS assay is a widely applied method for measuring the radical-scavenging ability of antioxidants in a polar environment. In this context, this assay, performed in ethanol, in which all catecholic compounds are soluble in the range of concentrations used, can give interesting insight into the real effect of growing lipophilicity, avoiding the problems connected with solubility or micelle formation. The antioxidant capacities of all synthesized compounds have been measured in ethanol solution within a concentration range of 1−10 μM. Linear regression calculations of dose−response curves of the antioxidant capacity versus concentration of the sample were performed. Trolox was utilized as reference. The antioxidant capacity of catechol derivatives was measured and used to calculate the TEAC. Experimental values for catechols **6**−**8**, the corresponding methyl carbonates **2** and **12**−**14**, and fatty acid esters **18**−**20** are listed in the Supporting Information and are graphically reported in Figures 4 and 5, respectively.

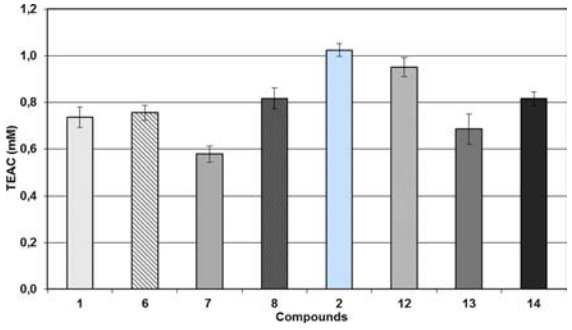

Figure 4. Radical-scavenging capacity of hydroxytyrosol **1**, catechols **6**, **7**, **8**, and the corresponding methyl carbonates **2**, **12**, **13**, and **14** evaluated by the ABTS assay in EtOH. Results are expressed as millimoles of trolox equivalents. Reported data show standard deviation below 5%. Statistical significance has *p* values always below 0.005%.

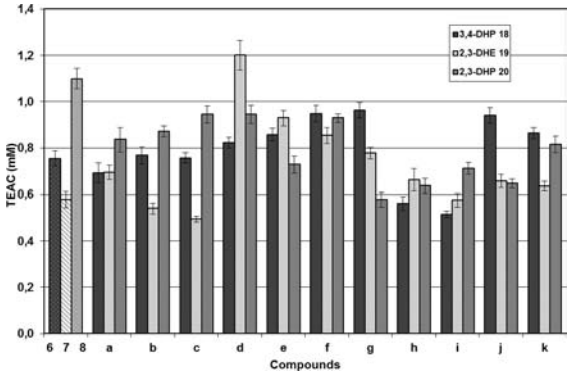

Figure 5. Radical-scavenging capacity evaluated by the ABTS assay in EtOH for catechols **6**, **7**, **8** and the corresponding fatty acid esters **18a**−**k**, **19a**−**k**, and **20a**−**k**. Results are expressed as millimoles of trolox equivalents. Reported data show standard deviation below 5%. Statistical significance has *p* values always below 0.005%.

As depicted in Figure 4, both catechols **6**, **7**, and **8** and their methyl carbonates **12**, **13**, and **14** show a good antioxidant capacity with TEAC values ranging between 0.6 and 1.1 mM. As above-discussed, methyl carbonates **12**−**14** display a higher lipophilicity than the corresponding catechols **6**−**8**, and the antioxidant properties are maintained or slightly enhanced by

the presence of the methyl carbonate moiety. In particular, the relative position of the two phenolic groups (2,3 or 3,4) does not affect the antioxidant activities, whereas side-chain homologation, irrespective of the phenolic position, results in a small increase of activity.

Antioxidant data of fatty acids esters **18a−k**, **19a−k**, and **20a−k** (Figure 5) can be discussed by taking into account three parameters: (1) the length (C2−C18) of the acyl chain; (2) the length (C2 or C3) of the alcohol chain; and (3) the position (2,3- or 3,4-) of the phenolic hydroxyls.

As to the first point, irrespective of both the acyl chain length and the catechol position, all tested esters show good antioxidant capacity, comparable to or higher than that of the parent catechols **6−8** (TEAC ranging between 0.5 and 1.2 vs between 0.6 and 0.8). The highest values are shown by medium-sized chain esters (C8−C14), whereas C16−C18 saturated fatty acid ester derivatives exhibit the lowest values. Furthermore, esters of unsaturated fatty acids (**18j−k**, **19j−k**, and **20j−k**) display a better radical-scavenging activity than the corresponding saturated esters **18i**, **19i**, and **20i**.

Any attempt to correlate antioxidant capacity with lipophilicity (see log *P* and HLB calculation, Table 1) failed, confirming that antioxidant properties do not depend only on this parameter. These findings are generally coherent with recent literature,[19,20,46] where the antioxidant activity of catecholic esters or ethers seems to follow a general parabolic trend with a maximum for medium-chain esters. This behavior, named "cutoff effect" and previously known only in a biological environment,[48] has been only recently observed in in vitro antioxidant capacity experiments of amphiphilic antioxidants in heterogeneous systems such as emulsions[19−21] and liposomes.[22,23] The qualified hypothesis is that the surfactant effect of polyphenolic fatty acid esters, which depends on the length of the fatty acyl chain, could affect the antioxidant capacity through a different placement of the antioxidant at the solution/oil droplet interface.[19,47]

However, this hypothesis cannot be taken into account in the case of the ABTS assay carried out in a monophasic system, where the formation of micelles can be ruled out at the used concentrations. In this case, the drop of antioxidant capacity for longer acyl chains could be explained in terms of increased conformational freedom of the ester chain resulting in folded structures in which catechol hydroxyls are shielded. A similar behavior has been already reported for hydroxytyrosyl esters, and the hypothesis of long-chain folding has been supported by molecular dynamics simulations for hydroxytyrosyl stearate.[14] Accordingly, the higher antioxidant capacity measured for oleic and linoleic esters (**18j−k**,**19j−k**, and **20j−k**) can derive from lowering of the acyl chain conformational freedom as a consequence of the double-bond rigidity.

In addition to the above acyl chain-dependent effects, the consequences of both the alcoholic chain length and the position of catechol hydroxyls lead to some interesting structure/activity evaluation. Elongation of the alcoholic side chain from two to three carbons seems to positively affect the antioxidant capacity. Indeed, excluding the case of the 2,3-DHE octanoate **19d**, TEAC values of 3,4-DHP esters **18a−k** and 2,3-DHP esters **20a−k** are always comparable or higher than those of both the corresponding previously reported hydroxytyrosyl esters (3,4-DHE esters)[14] and 2,3-DHE esters **19a−k**.

The position of the phenolic groups seems to positively affect the radical-scavenging activity as well. When 2,3-DHP esters **20a−k** are compared with 3,4-DHP esters **18a−k**, the former generally show better TEAC values than the latter. In analogy, 2,3-DHE esters **19a−k** exhibit a better antioxidant capacity than the previously reported hydroxytyrosyl analogues (3,4-DHE esters). Therefore, both the homologation of the alcoholic chain and the 2,3-position of catecholic hydroxyls produce an increase of TEAC values by acting either alone or together.

The increased antioxidant activity in 2,3-dihydroxy derivatives could be related to the easier formation of intramolecular hydrogen bonds between the C(2′)−OH and the acyl oxygen of the ester chain. Indeed, molecular mechanics MM2 minimizations showed (Figure 6) that only in 2,3-dihydroxy

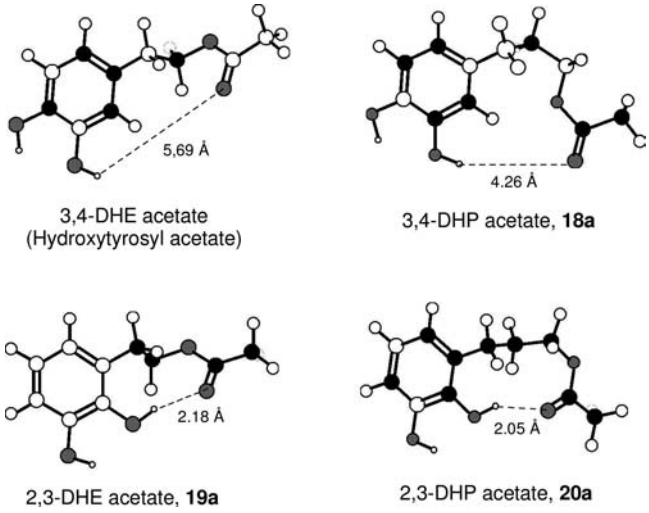

Figure 6. Calculated distances between the acyl oxygen and the nearest catechol hydroxyl after MM2 minimizations in 3,4-DHE acetate **18a**, **19a**, and **20a**.

derivatives **19a** and **20a** is the distance between the C(2′)−OH and the acyl oxygen of the ester chain suitable for intramolecular H-bonding, whereas, due to the presence of the proton at C(2′), this is impossible in both 3,4-DHE and 3,4-DHP acetates.

Therefore, both homologation and hydroxyl position cooperate to make effective intramolecular H-bonding in either 2,3-DHE esters **19a−k** or 2,3-DHP esters **20a−k**. Intramolecular H-bonding could weaken the phenolic O−H bond, thus resulting in both an easier hydrogen extraction and better radical-scavenging properties. On the other hand, these positive effects can be reduced by the above-mentioned shielding effect of folded conformations. This could account for the lower antioxidant activity observed in longer chain esters (>C14).

Antioxidant Activity in Cell Culture. The determination of the radical-scavenging activity of all catechol derivatives was performed using a DCF standard assay, based on the production of dichlorofluorescein radical (DCF), as fluorescent probe, by oxidation of dichlorofluorescin ($DCFH_2$) in cells. L6 rat muscle cells were used due to their sensitivity to ROS level.[49] Cumene hydroperoxide was used as H_2O_2 generator to produce oxidative stress. The experimental data for catechols **6−8**, the corresponding methyl carbonates **2**, **12−14**, and fatty acid esters **18−20** are available in the Supporting Information and graphically summarized in Figures 7 and 8, respectively. The percentage of DCF fluorescence inhibition is reported versus each tested antioxidant at 10 μM final concentration.

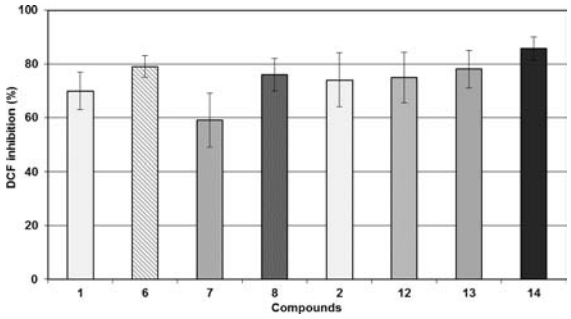

Figure 7. Percentage of DCF fluorescence inhibition in cell culture experiments for hydroxytyrosol **1**, catechols **6**, **7**, **8**, and the corresponding methyl carbonates **2**, **12**, **13**, and **14**. Final concentration is 10 μM. Standard deviations are below 14%. Statistical significance with respect to cumene hydroperoxide (considered 100%) is always below 0.005%.

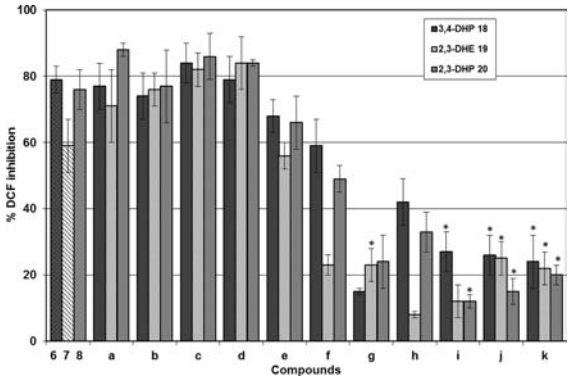

Figure 8. Percentage of DCF fluorescence inhibition in cell culture experiments for catechols **6**, **7**, **8** and fatty acid esters **18a**−**k**, **19a**−**k**, and **20a**−**k**. Final concentration of esters is 10 μM. Standard deviations are always below 14%. Statistical significance with respect to cumene hydroperoxide (considered 100%) is below 0.005%. *, compounds **18i**−**k**, **19g**, **19j**, **19k**, and **20i**−**k** have $p < 0.05$. Under the same conditions, trolox gives a value of DCF inhibition of 84%.

Hydroxytyrosol **1**, catechols **6**, **7**, and **8**, and the corresponding derivatives **2**, **12**, **13**, and **14** (Figure 7) show a decrease of fluorescence in comparison with the experiments with cumene hydroperoxide alone. These data suggest the penetration of all compounds into cells and the subsequent quenching of peroxide radicals. The antioxidant activity trend is similar to that obtained in in vitro experiments (see Figures 4 and 5). Both in catechols **1**, **6**, **7**, and **8** and in the corresponding carboxymethylated derivatives **2**, **12**, **13**, and **14**, the relative position of the catechol hydroxyls seems not to be related to the antioxidant activity, whereas a small favorable role is played by elongation of the alcohol chain.

Esters **18a**−**k**, **19a**−**k**, and **20a**−**k** penetrate into the cells as shown by a sharp decrease of measured fluorescence (Figure 8), but the structural changes in the different series of esters seem to have a lower influence in cell culture experiments if compared with the corresponding in vitro ones. However, also in the biological environment, 2,3-DHP derivatives **20a**−**k** show an antioxidant activity similar to or better than that of both their shorter homologues 2,3-DHE derivatives **19a**−**k** and the 3,4-DHP derivatives **18a**−**k**.

On the contrary, the relationship between acyl chain length and antioxidant activity for compounds **18**−**20** follows a general and distinctive sigmoid curve, already noted for ascorbyl,[24] hydroxytyrosyl,[14] and chlorogenate esters.[25] For short to medium (C2−C8) acyl chains (esters **18a**−**d**, **19a**−**d**, and **20a**−**d**) the antioxidant activity displays values generally higher than or comparable with that of free catechols **6**−**8**, with a maximum for C6−C8 derivatives. However, irrespective of both the alcohol chain length and the phenol hydroxyl position, esters with chains longer than eight carbons show a cutoff effect that results in a progressive activity drop from C10 to C12 acyl chains (esters **18e**,**f**, **19e**,**f**, and **20e**,**f**) and a sharp decrease for longer chains. At variance with the TEAC measurements, the decreasing trend is not reverted in the esters of unsaturated fatty acids (**18j**,**k**, **19j**,**k**, and **20j**,**k**).

The causes of the biological "cutoff effect" are still a matter of study. As already suggested for hydroxytyrosyl esters, 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 the entrapment into the plasma membrane caused by the higher affinity of long acyl chains with the phospholipids or hydrophobic proteins inside the bilayer. This hypothesis might be confirmed by recent experiments on membrane fluidity in daidzein alkoxy derivatives.[23]

Cell Survival Assessment. Considering the novelty of the compounds synthesized, to both rule out any influence of their cytotoxicity on the antioxidant activity observed and make possible their use as preservatives in food technology and the cosmetic industry, MTT measurements were carried out. Catechols **6**, **7**, **8**, the corresponding methyl carbonates **12**, **13**, **14**, and fatty acid esters **18d**, **19d**, and **20d** (C8 acyl chain) or **18h**, **19h**, and **20h** (C18 acyl chain) were chosen as representative compounds to measure cell survival in L6 rat skeletal muscle cells. Results were compared with those of 3,4-DHE **1** and its methyl carbonate **2**. Cells were treated with different doses of compounds (10, 20, and 50 μM) for 48 h. The extent of cell survival was measured by MTT assay (Figure 9). The percentage of cell survival remained high (>90%) at all doses analyzed, confirming the low cytotoxicity of all of the compounds examined. Furthermore, the similarity of data obtained for medium and long acyl chain esters rules out any effect of cytotoxicity on the decrease of antioxidant activity observed in long-chain fatty acid esters.

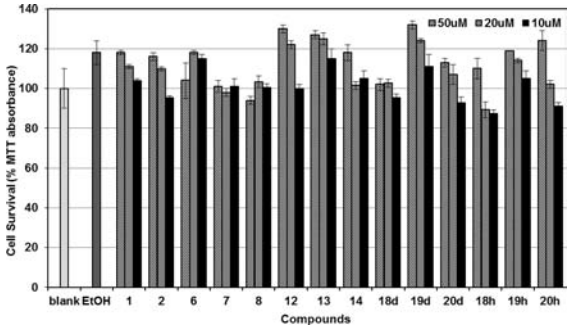

Figure 9. Effect of hydroxytyrosol **1**, catechols **6**, **7**, and **8**, and the corresponding methyl carbonate esters **2**, **12**, **13**, and **14** and fatty acid ester derivatives **18d**, **19d**, **20d** (C8 acyl chain) or **18h**, **19h**, and **20h** (C18 acyl chain) on L6 skeletal muscle cell survival. Cells were subjected to MTT assay, as detailed under Materials and Methods. The data are the averages of three experiments. Standard deviations are always below 5% except for control (10%) and ethanol (6%). None of the data were statistically different from control.

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In conclusion, a convenient and efficient procedure has been developed to synthesize a large panel of novel catechol-based compounds, structurally related to naturally occurring hydroxytyrosol and its fatty acid esters. The procedure is practical and efficient and requires neither harsh reaction conditions nor anhydrous solvents.

All compounds have been analyzed for their antioxidant effect by ABTS and DCF assay. Our results, both in vitro and in cell culture, evidence interesting structure/activity relationships. Catechols **6**, **7**, and **8** and the corresponding methyl carbonates **12**, **13**, and **14** show a quite flattened antioxidant activity. Namely, their antioxidant activities are not affected by the position of the catechol hydroxyls, whereas the alcohol chain length plays a small favorable role. This could be related to their high hydrophilicity, resulting in H-bonding with solvent. On the contrary, both the alcohol chain length and the catechol hydroxyl position show an influence on the antioxidant power of the corresponding and more lipophilic fatty acid esters. In particular, both the hydroxyl 2,3-position and the larger length of the alcohol chain seem to positively affect the antioxidant activity, and these experimental results can be explained in terms of the possible easier formation of intramolecular H-bonds.

Furthermore, the experimental data confirm the already reported[14] relationship between the length of the ester chain and the relative antioxidant effect. In both in vitro and in cell experiments, medium-sized fatty acid and carbonate esters of all three sets of compounds have shown an antioxidant activity higher than that of the corresponding catechols **6**–**8** and the 3,4-DHE **1** itself, with a maximum in the range of C4–C8. Acyl chain lengthening over 10/12 carbons results in lowering of the antioxidant activity, this behavior being strongly stressed in cell experiments. Accordingly, partition coefficients values (log *P*) and HLB data have excluded any correlation between antioxidant properties and lipophilicity, whereas cytotoxicity experiments for the tested compounds confirm the absence of any effect on cell survival. This behavior may be rationalized in terms of a balance between the favorable role played by the growing lipophilicity and the adverse effect arising in long-chain esters from the increasing amount of folded conformations, which can shield the catechol moiety. This effect, noticeable in TEAC experiments, is further stressed in cell experiments by trapping into the cell membrane bilayer.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR data and analytical data of all new compounds; TEAC and DCF assay values. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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