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ScienceDirect
Bioorganic Chemistry 35 (2007) 137–152
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Hydroxytyrosol lipophilic analogues: Enzymatic synthesis, radical scavenging activity and DNA oxidative damage protection

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Received 14 July 2006
Available online 31 October 2006

Abstract

The olive oil phenol hydroxytyrosol (**3**), as well its metabolite homovanillic alcohol (**4**), were subjected to chemoselective lipase-catalysed acylations, affording with good yield 10 derivatives (**5**–**14**) bearing C_2, C_3, C_4, C_{10} and C_{18} acyl chains at C-1. Hydroxytyrosol (**3**) and its lipophilic derivatives showed very good DPPH$^{\cdot}$ radical scavenging activity. Compounds **3**, **4** and their lipophilic analogues **5**–**14** were subjected to the atypical Comet test on whole blood cells: **3** and its analogues **5** and **6**, with little hydrophobic character ($\log P \leqslant 1.20$), showed a good protective effect against H_2O_2 induced oxidative DNA damage. The homovanillic alcohol **4** and its analogues **10**–**14** resulted scarcely effective both as radical scavengers and antioxidant agents.

Keywords: Hydroxytyrosol; Homovanillic alcohol; Lipophilic analogues; Radical scavenging; DNA damage protection; Comet assay; Antioxidants

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† In honour of the 80th birthday of Professor Mario Piattelli.

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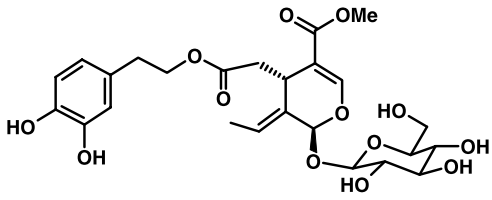
doi:10.1016/j.bioorg.2006.09.003

1. Introduction

The beneficial health effects of a Mediterranean diet have been claimed in a variety of studies [1,2], and are corroborated by epidemiological studies indicating a lower incidence of coronary heart diseases [3] and cancer [4] associated with the nutritional behaviour of people living in the Mediterranean area. Extra-virgin olive oil is the principal fat component of the Mediterranean diet, and its chemical constituents have been intensively studied with the aim of identifying the main protective agents [5–7]. A number of studies indicate that olive oil phenolic constituents have antioxidant and antimicrobial properties [8,9], contributing to the reduction of ROS-RNS [10] and so playing an important role in chemoprevention of colonrectal carcinogenesis [6]. The main olive oil phenols are oleuropein (**1**), tyrosol (**2**) and hydroxytyrosol (**3**) [9]. Hydroxytyrosol is incorporated in the aglycon of oleuropein and is thought to be released by hydrolysis from this glycoside during olive storage and pressing, due to the action of cellular esterases or acidic catalysis [11].

Many literature data indicate the potent 'in vitro' antioxidant activity of hydroxytyrosol (**3**) [12–14], in agreement with theoretical predictions on *ortho*-diphenols [15]. In addition, **3** has been proved to prevent oxidative damage in human erythrocytes [8] and is also considered an important anticancer component of virgin olive oil [10,16]. Further studies suggest that **3** has a high oral bioavailability and is largely absorbed, differently from oleuropein [9,17,18]. Some human metabolites of hydroxytyrosol have been identified, among them the homovanillic alcohol (**4**), a lipophilic metabolite reported as a radical-scavenger comparable to **3** and which is believed to have a role in the beneficial properties exerted by olive oil [19]. Some studies on olive phenols (among them hydroxytyrosol 1-acetate, **5**) [12,20–22] have pointed out the importance of the lipophilic character of the antioxidant with reference to the dispersion medium (bulk oil, emulsions), to the cell uptake and membrane crossing, and to the substrate to be protected (LDL or membrane constituents). In particular, Saija et al. had shown a higher antioxidant activity of **3** in unilamellar vesicles of dipalmitoylphopsphatydilcoline/linoleic acid [12]; similar results were obtained more recently by Paiva-Martins et al. [21]. Gordon et al. found a higher antioxidant activity of **3** (and a comparable activity of **5**) in bulk oil [20]. Morellò et al. found that **3** in oil is a more efficient antioxidant molecule than α-tocopherol [22].

In this scenario, we aimed at examining the effects elicited by the enhanced lipophilic characteristic on the antioxidant properties of hydroxytyrosol (**3**) and homovanillic alcohol (**4**). We planned to evaluate **3**, **4** and a series of their analogues, bearing lipophilic acyl chains of different length at C-1 both for radical scavenging activity and protection of the oxidative DNA damage. To this end, we applied an enzymatic methodology for the chemoselective acylation of the alcoholic hydroxyl, previously employed by other authors for phenolic alcohols [23] and similar to the method used in our previous study on resveratrol analogues [24]. Lipophilic derivatives of **3** and **4**, with an amphiphilic structure, are potentially important also for possible applications in nanotechnology, with specific reference to their self-organising properties. Reports in this field include lipophilic analogues of phenolic compounds [25,26].



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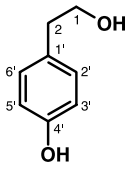

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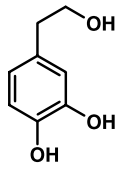

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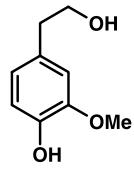

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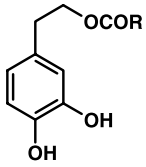
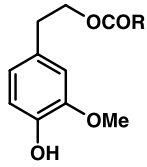




5	R = $-CH_3$	**10**	R = $-CH_3$
6	R = $-CH_2CH_3$	**11**	R = $-CH_2CH_3$
7	R = $n-(CH_2)_2CH_3$	**12**	R = $n-(CH_2)_2CH_3$
8	R = $n-(CH_2)_8CH_3$	**13**	R = $n-(CH_2)_8CH_3$
9	R = $n-(CH_2)_{16}CH_3$	**14**	R = $n-(CH_2)_{16}CH_3$

2. Materials and methods

2.1. General

All reagents were of commercial quality and were used as received (Merck and Sigma–Aldrich). Solvents were distilled using standard techniques. Ascorbic acid, vinyl acetate, propionate, butyrate, decanoate and stearate, lipases from *Aspergillus niger*, *Candida cylindracea*, *Mucor miehei*, *Mucor javanicus*, *Chromobacterium viscosum*, and wheat germ were purchased from Sigma; tyrosol, lipases from *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Rhizopus arrhizus*, *Rhizopus niveus*, porcine pancreas were purchased from Fluka,

homovanillic alcohol from Aldrich. The *Candida antarctica* lipase (Chirazyme L-2, cf., C2, Lyo) sample was a gift from Roche.

Mass spectra were recorded in ESI mode (both positive and negative) on a Micromass ZQ2000 spectrometer (Waters). The ^{1}H NMR spectra were recorded at constant temperature (27 °C) on a Varian Unity Inova spectrometer working at 500 MHz. UV spectra were recorded on a Double-ray Lambda 25 spectrophotometer (Perkin-Elmer).

Analytical thin-layer chromatography (TLC) was performed on silica gel (Merck 60 F_{254}) plates using cerium sulphate as developing reagent.

2.2. Synthesis of hydroxytyrosol (*3*)

Five hundred milligrams (3.62 mmol) of **2** were dissolved in sodium phosphate buffer (30 ml) containing 1.3 g (7.38 mmol) of ascorbic acid with a final pH of 6.5, and tyrosinase (50 mg) was added. The reaction mixture was constantly bubbled with air. The mixture was stirred for 16 h at r.t., then transferred to a separatory funnel and extracted three times with 300 ml portions of ethyl acetate; the extracts were dried over anhydrous Na_2SO_4. After removal of the solvent, the product was purified through flash-chromatography on silica Diol column with a gradient of increasing percentage of MeOH in CH_2Cl_2 (from 1% to 3.5%), and subsequently with 10% MeOH in CH_2Cl_2.

2.3. Preliminary screening: acetylation

The enzymatic acetylations were carried out in vials in which the enzyme of chosen (lipases from *A. niger*, *C. cylindracea*, *M. javanicus*, *P. cepacia*, *C. antarctica*, *M. miehei*, *C. viscosum*, *P. fluorescens*, *R. arrhizus*, *R. niveus*, porcine pancreas and wheat germ, 5 mg) was added to a solution of **3** (5 mg, 0.032 mmol) in *t*-butylmethyl ether (1 ml), containing vinyl acetate (0.64 mmol). Control reactions without enzyme were carried out under the same conditions. After the reactions were quenched, the enzyme was filtered off, and the filtrates were taken to dryness.

2.4. General procedure of preparative enzymatic esterifications

Candida antarctica lipase (100 mg) and the acyl donor (vinyl acetate, propionate, butyrate, decanoate and stearate) (14 mmol) were added to a solution of the substrate (**3** or **4**, 0.72 mmol) in *t*-butylmethyl ether (25 ml) and the mixture was shaken (400 rpm) at 40 °C for a convenient period of time, as reported below. The progress of each reaction was monitored, at regular time intervals, by ^{1}H NMR. The reactions were quenched by filtering off the enzyme and the filtrate was evaporated *in vacuo*. Solvents were removed under reduced pressure and the products were purified by flash chromatography on silica gel DIOL 40–63 μm (Merck) or Lichroprep Si 60. The elution system is reported below for each purified compound.

2.4.1. 3,4-Dihydroxyphenethyl acetate (*5*)

Compound **5** was prepared by a 35 min reaction and was purified on silica Diol using a gradient from 80% CH_2Cl_2 in *n*-hexane to 100% CH_2Cl_2; yield 95.0%. ESI-MS: mass calculated 196.20 for $C_{10}H_{12}O_4$, found *m*/*z* 195 $[M-H]^-$; ^{1}H NMR ($CDCl_3$) δ 6.78 (d, 1H,

$J = 8.0$ Hz, H-5′), 6.73 (s, 1H, H-2′), 6.62 (d, 1H, $J = 8.0$ Hz, H-6′), 5.68 and 5.46 (each bs, 1H, 3′ and 4′–O*H*), 4.23 (t, 2H, $J = 7.0$ Hz, H-1), 2.81 (t, 2H, $J = 7.0$ Hz, H-2), 2.05 (s, 3H, –OCOCH_3).

*2.4.2. 3,4-Dihydroxyphenethyl propionate (**6**)*

Compound **6** was prepared by a 35 min reaction and was purified on silica Diol using a gradient from 80% CH_2Cl_2 in *n*-hexane to 95% CH_2Cl_2 in *n*-hexane; yield 95.1%. ESI-MS: mass calculated 210.23 for $C_{11}H_{14}O_4$ found *m*/*z* 209 $[M-H]^-$; ^{1}H NMR ($CDCl_3$) δ 6.79 (d, 1H, $J = 8.0$ Hz, H-5′), 6.73 (d, 1H, $J = 2.0$ Hz, H-2′) 6.63 (dd, 1H, $J = 8.0$ Hz, 2.0 Hz, H-6′), 4.24 (t, 2H, $J = 7.0$ Hz, H-1), 2.82 (t, 2H, $J = 7.0$ Hz, H-2), 2.32 (q, 2H, $J = 7.5$ Hz, –OCOCH_2CH$_3$), 1.12 (t, 3H, $J = 7.5$ Hz, –OCOCH$_2$CH_3).

*2.4.3. 3,4-Dihydroxyphenethyl butyrate (**7**)*

Compound **7** was prepared by a 35 min reaction and was purified on silica Diol using a gradient from 70% CH_2Cl_2 in *n*-hexane to 90% CH_2Cl_2 in *n*-hexane; yield 96.5%. ESI-MS: mass calculated 224.26 for $C_{12}H_{16}O_4$ found *m*/*z* 223 $[M-H]^-$; ^{1}H NMR ($CDCl_3$) δ 6.78 (d, 1H, $J = 8.0$ Hz, H-5′), 6.73 (s, 1H, H-2′), 6.63 (d, 1H, $J = 8.0$ Hz, H-6′), 5.74 and 5.51 (each bs, 1H, 3′ and 4′–O*H*), 4.24 (t, 2H, $J = 7.0$ Hz, H-1), 2.81 (t, 2H, $J = 7.0$ Hz, H-2), 2.28 (t, 2H, $J = 7.5$ Hz, –OCOCH_2CH$_2$CH$_3$), 1.63 (sext, 2H, $J = 7.5$ Hz, –OCOCH$_2$CH_2CH$_3$), 0.92 [t, 3H, $J = 7.5$ Hz, –OCO(CH$_2$)$_2$CH_3].

*2.4.4. 3,4-Dihydroxyphenethyl decanoate (**8**)*

Compound **8** was prepared by a 75 min reaction and was purified on silica Diol using a gradient from 60% CH_2Cl_2 in *n*-hexane to 100% CH_2Cl_2; yield 93.3%. ESI-MS: mass calculated 308.42 for $C_{18}H_{28}O_4$ found *m*/*z* 307 $[M-H]^-$; ^{1}H NMR ($CDCl_3$) δ 6.78 (d, 1H, $J = 8.0$ Hz, H-5′), 6.73 (d, 1H, $J = 1.5$ Hz, H-2′), 6.63 (dd, 1H, $J = 8.0$, 1.5 Hz, H-6′), 5.71 and 5.48 (each bs, 1H, 3′ and 4′–OH), 4.24 (t, 2H, $J = 7.5$ Hz, H-1), 2.81 (t, 2H, $J = 7.5$ Hz, H-2), 2.29 [t, 2H, $J = 7.5$ Hz, –OCOCH_2(CH$_2$)$_7$CH$_3$], 1.59 [quin, 2H, $J = 7.5$ Hz, –OCOCH$_2$CH_2(CH$_2$)$_6$CH$_3$], 1.25 [s, 12H, –OCO(CH$_2$)$_2$(CH_2)$_6$CH$_3$], 0.88 [t, 3H, $J = 7.0$ Hz, –OCO(CH$_2$)$_8$CH_3].

*2.4.5. 3,4-Dihydroxyphenethyl stearate (**9**)*

Compound **9** was prepared by a 180 min reaction and was purified on silica Diol using a gradient from 55% CH_2Cl_2 in *n*-hexane to 95% CH_2Cl_2 in *n*-hexane; yield 92.3%. ESI-MS: mass calculated 420.64 for $C_{26}H_{44}O_4$ found *m*/*z* 419 $[M-H]^-$; ^{1}H NMR ($CDCl_3$) δ 6.78 (d, 1H, $J = 8.0$ Hz, H-5′), 6.73 (s, 1H, H-2′), 6.63 (d, 1H, $J = 8.0$ Hz, H-6′), 5.60 and 5.38 (each bs, 1H, 3′ and 4′ –O*H*), 4.24 (t, 2H, $J = 7.0$ Hz, H-1), 2.81 (t, 2H, $J = 7.0$ Hz, H-2), 2.29 [t, 2H, $J = 7.0$ Hz, –COCH_2(CH$_2$)$_{15}$CH$_3$], 1.59 [quin, 2H, $J = 7.0$ Hz, –OCOCH$_2$CH_2(CH$_2$)$_{14}$CH$_3$], 1.25 [s, 28H, –OCO(CH$_2$)$_2$(CH_2)$_{14}$CH$_3$], 0.88 [t, 3H, $J = 6.5$ Hz, –OCO(CH$_2$)$_{16}$CH_3].

*2.4.6. 4-Hydroxy-3-methoxyphenetyl acetate (**10**)*

Compound **10** was prepared by a 60 min reaction and was purified on Lichroprep Si 60 using 50% CH_2Cl_2 in *n*-hexane; yield 96.8%. ESI-MS: mass calculated 233.23 for $C_{11}H_{14}O_4Na$, found *m*/*z* 233 $[M+Na]^+$; ^{1}H NMR ($CDCl_3$) δ 6.83 (d, 1H, $J = 8.0$ Hz, H-5′), 6.70 (d, 1H, $J = 8.0$ Hz, H-6′), 6.70 (bs, 1H, H-2′), 5.66 (bs, 1H, 4′–O*H*), 4.24

(t, 2H, $J = 7.5$ Hz, H-1), 3.86 (s, 3H, 3′–OCH_3), 2.85 (t, 2H, $J = 7.5$ Hz, H-2), 2.03 (s, 3H, –OCOCH_3).

*2.4.7. 4-Hydroxy-3-methoxyphenetyl propionate (**11**)*

Compound **11** was prepared by a 90 min reaction and was purified on Lichroprep Si 60 using 45% CH_2Cl_2 in *n*-hexane; yield 98.1%. ESI-MS: mass calculated 247.26 for $C_{12}H_{16}O_4Na$, found *m/z* 247 $[M+Na]^+$; ^1H NMR ($CDCl_3$) δ 6.83 (d, 1H, $J = 8.0$ Hz, H-5′), 6.70 (d, 1H, $J = 2.0$ Hz, H-2′), 6.69 (dd, 1H, $J = 8.0$ Hz ; 2.0 Hz, H-6′), 5.72 (bs, 1H, 4′–*OH*), 4.24 (t, 2H, $J = 7.0$ Hz, H-1), 3.82 (s, 3H, 3′–OCH_3), 2.85 (t, 2H, $J = 7.0$ Hz, H-2), 2.31 (q, 2H, $J = 7.5$ Hz, –OCOCH_2CH$_3$), 1.12 (t, 3H, $J = 7.5$ Hz, –OCOCH$_2$CH_3).

*2.4.8. 4-Hydroxy-3-methoxyphenetyl butyrate (**12**)*

Compound **12** was prepared by a 90 min reaction and was purified on Lichroprep Si 60 using 35% CH_2Cl_2 in *n*-hexane; yield 90.9%. ESI-MS: mass calculated 261.29 for $C_{13}H_{18}O_4Na$, found *m/z* 261 $[M+Na]^+$; ^1H NMR ($CDCl_3$) δ 6.83 (d, 1H, $J = 8.0$ Hz, H-5′), 6.71 (s, 1H, H-2′), 6.70 (d, 1H, $J = 8.0$ Hz, H-6′), 5.65 (bs, 1H, 4′–O*H*), 4.24 (t, 2H, $J = 7.0$ Hz, H-1), 3.85 (s, 3H, 3′–OCH_3), 2.85 (t, 2H, $J = 7.0$ Hz, H-2), 2.26 (t, 2H, $J = 7.5$ Hz, –OCOCH_2CH$_2$CH$_3$), 1.63 (sext, 2H, $J = 7.5$ Hz, –OCOCH$_2$CH_2CH$_3$), 0.92 [t, 3H, $J = 7.5$ Hz, –OCO(CH$_2$)$_2$CH_3].

*2.4.9. 4-Hydroxy-3-methoxyphenetyl decanoate (**13**)*

Compound **13** was prepared by a 90 min reaction and was purified on Lichroprep Si 60 using 15% CH_2Cl_2 in *n*-hexane; yield 97.5%. ESI-MS: mass calculated 345.45 for $C_{19}H_{30}O_4Na$, found *m/z* 345 $[M+Na]^+$; ^1H NMR ($CDCl_3$) δ 6.83 (d, 1H, $J = 8.0$ Hz, H-5′), 6.71 (s, 1H, H-2′), 6.69 (d, 1H, $J = 8.0$ Hz, H-6′), 5.65 (bs, 1H, 4′ –O*H*), 4.25 (t, 2H, $J = 7.0$ Hz, H-1), 3.86 (s, 3H, 3′–OCH_3), 2.85 (t, 2H, $J = 7.0$ Hz, H-2), 2.28 [t, 2H, $J = 7.5$ Hz, –OCOCH_2(CH$_2$)$_7$CH$_3$], 1.59 [quin, 2H, $J = 7.5$ Hz, –OCOCH$_2$CH_2(CH$_2$)$_6$CH$_3$], 1.26 [s, 12H, –OCO(CH$_2$)$_2$(CH_2)$_6$CH$_3$], 0.88 [t, 3H, $J = 7.0$ Hz, –OCO(CH$_2$)$_8$CH_3].

*2.4.10. 4-Hydroxy-3-methoxyphenetyl stearate (**14**)*

Compound **14** was prepared by a 240 min reaction and was purified on Lichroprep Si 60 using a gradient from 100% *n*-hexane to 5% CH_2Cl_2 in *n*-hexane; yield 98.0%. ESI-MS: mass calculated 457.67 for $C_{27}H_{46}O_4Na$, found *m/z* 457 $[M+Na]^+$; ^1H NMR ($CDCl_3$) δ 6.84 (d, 1H, $J = 8.0$ Hz, H-5′), 6.71 (bs, 1H, H-2′), 6.70 (d, 1H, $J = 8.0$ Hz, H-6′), 5.63 (bs, 1H, 4′–O*H*), 4.25 (t, 2H, $J = 7.0$ Hz, H-1), 3.86 (s, 3H, 3′–OCH_3), 2.86 (t, 2H, $J = 7.0$ Hz, H-2), 2.28 [t, 2H, $J = 7.0$ Hz, –OCOCH_2(CH$_2$)$_{15}$CH$_3$], 1.60 [quin, 2H, $J = 7.0$ Hz, –OCOCH$_2$CH_2 (CH$_2$)$_{14}$CH$_3$], 1.26 [s, 28H, –OCO(CH$_2$)$_2$(CH_2)$_{14}$CH$_3$], 0.88 [t, 3H, $J = 6.5$ Hz, –OCO(CH$_2$)$_{16}$CH_3].

2.5. Measurement of the DPPH· scavenging activity

The radical scavenging activity of each compound was evaluated according to a modified version of the method of Brand-Williams et al. [27]. The initial concentration was 92 μM, (mAU = 1, $\varepsilon = 12{,}628$), of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) in cyclohexane, and was controlled for each experiment from a calibration curve made by measuring the absorbance at 515 nm of standard samples of DPPH· at different concentrations, in

a quartz cuvette (1 cm of light path). Typical procedure: to 2 ml of the freshly prepared solution of DPPH$^{\cdot}$, 10, 20 and 30 μl of freshly prepared solution in cyclohexane of compounds at various concentrations were added. The reaction mixtures were vigorously stirred and kept for 5 h in the dark at room temperature, in order to reach the steady state. Each measurement was acquired in triplicate. The results, expressed as SC_{50} (Scavenging Concentration), that is the concentration of analyte required to quench 50% of the initial DPPH$^{\cdot}$ radicals under the experimental conditions given, are reported in Table 2.

2.6. Measurement of partition coefficient (log P)

A solution (0.15 mM) of each compound in *n*-octanol was kept at 60 °C for an hour. An UV spectrum was then run, and the value of absorbance at the maximum was measured (A_0). Equal volumes of organic solution and phosphate buffer (0.1 M, pH 7.4) were vigorously mixed for 5 min with a magnetic stirrer, and the two layers left to separate (30 min). An UV spectrum of the organic layer was then run, in order to determine the value A_x. The partition coefficient ($\log P$) was calculated according to the following relationship: $P = A_x/(A_0 - A_x)$. A solution of *n*-octanol saturated with the buffer was used as blank. Experimental $\log P$ values are reported in Table 3. Calculated $\log P$ values, obtained with ACD/labs $\log P$ program version 8, are the following: 0.02 (**3**), 0.96 (**5**), 1.42 (**6**) 2.02 (**7**) 5.21 (**8**), 9.46 (**9**), correlation coefficient between experimental/calculated values (except **8** and **9**) was $R^2 = 0.99$; 0.33 (**4**), 1.27 (**10**), 1.80 (**11**), 2.33 (**12**), 5.52 (**13**), 9.77 (**14**), correlation coefficient between experimental/calculated values (except **13** and **14)** was $R^2 = 0.97$.

2.7. Measurement of the oxidative damage protection

2.7.1. Cellular system

Whole blood cells were chosen as test cells to avoid any DNA damage related to separation of cellular types. Blood samples (50 μl) taken from healthy human donors by finger pricks, were added to 950 μl of solution A (Ca^{2+}/Mg^{2+}-free HBSS, 20 mM EDTA, 10% DMSO, pH 7.5–7.7). The suspension was maintained at 4 °C for up to 1 h and centrifuged at 600*g* (10 min). The pellet was washed in PBS 1×, resuspended in a little volume of PBS 1× and aliquots of 10 μl were prepared to be used immediately for the different assays.

2.7.2. Atypical Comet assay

The measurement of DNA damage was performed by alkaline atypical version of Comet assay, by which the substances to be examined were added directly to agarose embedded cells (cellular version). Briefly, aliquots $1.5–2 \times 10^5$ cells, suspended in 10 μl of 1× PBS, were mixed with 65 μl of 0.5% low melting agarose (LMA), melted and stabilised in water bath at 37 °C. The cell suspension was loaded onto a 1% normal melting agarose (NMA) preloaded microscope slides and allowed to solidify at 4 °C for 5 min. Afterwards, the slides were covered with a third layer of 75 μl (LMA) and employed for the atypical Comet assay, as detailed below.

The sandwich gels, prepared on the microscope glass slides, were located in different homemade plastic bags containing 5 ml of PBS 1× pH 7.4 in which, according to the various treatments, was treated as following:

(i) kept for 20 min, in the dark, in a water bath at 37 °C (control untreated samples);
(ii) added with each one of the different test compounds taken singularly in the concentration of 50 μM and kept for 20 min, in the dark, in a water bath at 37 °C;
(iii) kept for 20 min, in the dark, in a water bath at 37 °C, added with 200 μM H_2O_2 as oxidative stress inducer and re-kept as previously for other 20 min;
(iv) added with each one of the different test compounds at the already mentioned concentrations and kept for 20 min, in the dark, in a water bath at 37 °C. Then the samples after washing with PBS 1×, were added with 200 μM H_2O_2 and re-kept as previously for other 20 min. This double treatment was performed in order to test the protective capability of the different compounds versus the induction of the oxidative stress.

At the end of the treatment time, all the slides were washed with PBS and immersed in lysis solution (1% *N*-laurosil-sarcosine, 2.5 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10) at 4 °C for 1 h. Afterwards, the samples were denatured in a high-pH buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 min to promote DNA unwinding, and finally electrophoresed in the same buffer at 0.7 V/cm for 40 min. After electrophoresis, the slides were gently removed, rinsed with neutralization buffer (0.4 M Tris–HCl, pH 7.5) at room temperature, stained with 100 μl of ethidium bromide (2 μg/ml) for 5 min and scored immediately or after storage for up 24 h in a humidified box in the dark at 4 °C, using a Leika fluorescence microscope (Leika, Wetzlar, Germany) interfaced with a computer. About 100 cells for each slide were examined and DNA damage was assessed with the image elaboration computer program Software (Leica-QWIN©) by measuring different parameter as (a) tail length (TL), tail intensity (TI) and area (TA); (b) head length (HL), head intensity (HI) and area (HA). The level of DNA damage expressed as (1) the percentage of the fragmented DNA (TDNA) and (2) tail moment (TMOM), the product of TD (distance between head and tail) and TDNA.

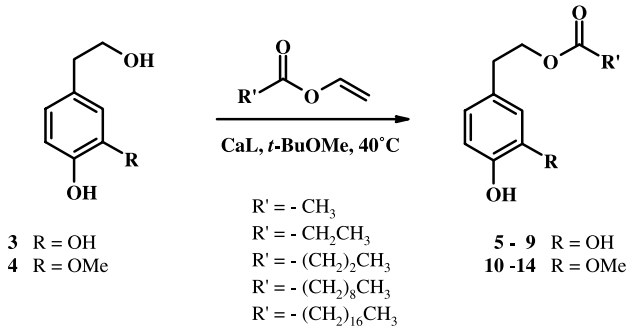
We chosen to represent the results as TDNA in order to thoroughly evaluate the scenario of the DNA status following the different treatments, also in agreement with recent literature data [28,29].

Each experiment, performed in duplicate, was repeated three times and the means ± SEM for each set of values was calculated. Negative control (untreated sample) and positive control (200 μM H_2O_2 treated sample) were included in each experiment. In addition to assure validation of the data a home-made quality control was considered. This, prepared at the beginning of the experiments as control untreated randomly selected pooled cells, was utilised as internal quality control according to Collins [30].

3. Results and discussion

3.1. Chemistry

Based on literature data, as well as the expected higher reactivity of the primary alcoholic function to the biocatalysed esterification, we used a chemoselective acylation methodology to obtain lipophilic analogues of the above cited phenolic compounds. Although chemoselective acylation of phenolic alcohols based on the use of classical chemical reagents has been reported with satisfactory yields [31], employing enzymes in organic solvents to this end avoids the use of noxious reagents, while at the same time offering the



Scheme 1. General procedure of enzymatic esterifications.

advantage of an easy purification of the product. Hydroxytyrosol (**3**, 3,4-dihydroxyphenethyl alcohol), was freshly prepared from the more stable and easily available tyrosol (**2**), based on a previously reported enzymatic procedure employing a tyrosinase-catalysed hydroxylation of the aromatic ring at controlled pH and in the presence of ascorbic acid [32]. A preliminary acetylation screening with 12 different lipases (see Section 2) was carried out on **3**. We employed vinyl acetate as reagent and *t*-butylmethyl ether as solvent, on the basis of the good solubility of the substrate and the satisfactory results obtained in our previous biocatalysed esterifications [24]. The best results were obtained with *C. antarctica* lipase, even if other lipases (from *M. miehei*, *P. fluorescens* and porcine pancreas) were able to catalyse the acetylation. In particular, *C. antarctica* lipase gave a more rapid reaction allowing almost complete conversion of the substrate in 35 min, and good yield (95%) of a single acetylation product. *C. antarctica* lipase (CAL) was selected for carrying out the reactions on a preparative scale, as detailed in the following.

According to Scheme 1, the reaction of **3** with vinyl acetate in the presence of CAL afforded after 35 min a single acetylation product, 1-acetylhydroxytyrosol (**5**, 3,4-dihydroxyphenethyl acetate), with 95% yield. The same acylation protocol was applied to **3** using in separate experiments vinyl propionate, vinyl butanoate, vinyl decanoate and vinyl stearate as acylation reagents. Results are summarised in Table 1. 1-propanoylhydroxytyrosol (**6**, 3,4-dihydroxyphenethyl propionate) and 1-butanoylhydroxytyrosol (**7**, 3,4-dihydroxyphenethyl butyrate) were obtained in approximately 95–96% yield within 35 min, whereas

Table 1
Preparative enzymatic esterifications of compounds **3** and **4**[*]

Phenol	Acylating agent	Product	Time (min)	Yield (%)
3	Vinyl acetate	**5**	35	95.0
3	Vinyl propionate	**6**	35	95.1
3	Vinyl butyrate	**7**	35	96.5
3	Vinyl decanoate	**8**	75	93.3
3	Vinyl stearate	**9**	180	92.3
4	Vinyl acetate	**10**	60	96.8
4	Vinyl propionate	**11**	90	98.1
4	Vinyl butyrate	**12**	90	90.9
4	Vinyl decanoate	**13**	90	97.5
4	Vinyl stearate	**14**	240	98.0

[*] Reaction conditions: phenol:acylating agent ratio 1:20, CAL, 25 ml *t*-BuOMe, 40 °C.

1-decanoylhydroxytyrosol (**8**, 3,4-dihydroxyphenethyl decanoate) was obtained in 93% yield after 75 min. A slower reactivity of the substrate was observed towards vinyl stearate, and 1-stearoylhydroxytyrosol (**9**, 3,4-dihydroxyphenethyl stearate) was obtained in 92% yield after 3 h. All the above cited reaction products were purified and characterised through spectroscopic analysis.

Following an analogous procedure, homovanillic alcohol (**4**, 3-methoxy-4-hydroxyphenethyl alcohol) was treated with the above cited acyl donors in the presence of CAL, thus affording the corresponding C-1 acetate (**10**, 4-hydroxy-3-methoxyphenetyl acetate), propionate (**11**, 4-hydroxy-3-methoxyphenetyl propionate), butanoate (**12**, 4-hydroxy-3-methoxyphenetyl butyrate), decanoate (**13**, 4-hydroxy-3-methoxyphenetyl decanoate) and stearate (**14**, 4-hydroxy-3-methoxyphenetyl stearate). Compounds **10**–**13** were obtained with yields in the range 91–97.5% and reaction times in the range 60–90 min; **14** was obtained with 98% yield after 4 h.

3.2. Radical scavenging activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) radical has been widely used to evaluate the radical scavenging activity of a variety of natural polyphenols, including tyrosol and hydroxytyrosol [14]. The basic methodology is to measure the decay of visible absorption of the DPPH$^{\cdot}$ radical, due to its conversion into a colourless hydrazine (DPPH-H) when an H-donor (for instance, a phenol) causes the H-atom transfer reaction. In a first step, the DPPH$^{\cdot}$ radical scavenging test was used to evaluate the radical scavenging properties of hydroxytyrosol (**3**) and homovanillic alcohol (**4**); tyrosol (**2**) was added for comparison. Results are reported in Table 2 as SC_{50}. As expected, the higher potency of **3** ($SC_{50} = 24.6$ μM) as DPPH$^{\cdot}$ radical scavenger was confirmed, in comparison with **2** ($SC_{50} = 33.2$ μM). Compound **4** ($SC_{50} = 46.1$ μM) resulted clearly less active than **3**, in contrast with previous literature data [19]. Although only the lipophilic analogues **5** and **6**–**9** appeared worthy of evaluation as radical scavengers, also we tested derivatives

Table 2
DPPH$^{\cdot}$ scavenging activity of compounds **2**–**14**

Compounds	SC_{50} (μM)[a] ± SD
2	33.2 ± 5.6
3	24.6 ± 6.5
4	46.1 ± 3.4
5	21.9 ± 1.4
6	22.9 ± 5.1
7	24.7 ± 1.0
8	24.8 ± 10.2
9	20.5 ± 6.6
10	42.9 ± 5.8
11	44.6 ± 1.8
12	41.8 ± 2.2
13	44.5 ± 4.2
14	40.5 ± 1.8

[a] SC_{50} (μM), Scavenging Capacity: phenol concentration, expressed in μM, able to quench 50% of DPPH radicals in a 92 μM solution (mAU = 1, solvent: cyclohexane; see text for details). Each reported value is the mean of three separate measurements.

10–**14** to have a more reliable evaluation of the radical scavenging properties for the homovanillic system. As reported in Table 2, the SC_{50} values of the lipophilic analogues of **3** and **4** are, respectively, in the range 20.5–24.8 μM and 40.5–44.6. These data confirm the lower radical scavenging activity of the homovanillic system and show that the antiradical activity of **3** is not notably influenced by the presence and length of an acyl chain at C-1.

3.3. log *P* determination

Lipophilicity plays an important role in cell-uptake, receptor binding and other properties influencing the biological activity of a drug candidate, and may be essential for dispersion of food antioxidants in bulk oils or fats. Lipophilicity of a molecule is currently evaluated by measurement of log *P*, a parameter related to the partition coefficient between *n*-octanol and water [33]. For a better comparison of the lipophilic properties of the hydroxytyrosol analogues and the reference compounds **2**–**4**, we planned to determine the experimental log *P* for compounds **2**–**14**. Previous literature data show that variations of log *P* with pH for hydroxytyrosol are negligible [20]. Thus, all our measurements were run in phosphate buffer (pH = 7.4) and results are reported in Table 3. Due to the high lipophilicity of compounds **8**, **9**, **13** and **14**, we could not obtain reliable experimental data for these compounds. Thus, we calculated log *P* values for compounds **3**–**14**: a satisfactory correlation with experimental log *P* values was observed for the series **3**–**7** and **10**–**12** (see Section 2). On this basis, we added for comparison the calculated log *P* values for compounds **8**, **9**, **13** and **14**. The lipophilic character increases from hydroxytyrosol (**3**, log *P* = 0.09) to homovanillic alcohol (**4**, log *P* = 0.50) and tyrosol (**2**, log *P* = 0.69). Within the two groups of hydroxytyrosol/homovanillic alcohol derivatives, log *P* values increase, as expected, with the increasing length of the acyl chain, in the range of approximately 1–10 values of log *P*.

Table 3
log $P_{7.4}$ of compounds **2**–**14**[*]

Compounds	log $P_{7.4}$ ± SD
2	0.69[a] ± 0.2
3	0.09[a] ± 0.02
4	0.50[a] ± 0.11
5	0.95[a] ± 0.56
6	1.20[a] ± 0.46
7	1.77[a] ± 0.42
8	5.20[b] ± 0.46
9	9.46[b] ± 0.48
10	1.50[a] ± 0.11
11	2.33[a] ± 0.33
12	2.54[a] ± 0.15
13	5.52[b] ± 0.25
14	9.77[b] ± 0.26

[a] Mean of triplicate determinations.
[b] Calculated values.
[*] Determined as *n*-octanol/water partition coefficient.

3.4. Oxidative DNA damage protection

The atypical version of Comet assay was utilised as valid, quick and effective methods to test the effects of different treatments directly on agarose embedded whole blood cells [29,34,35]. Electrophoresis was performed in alkaline buffer (pH > 13) for both versions of the test in order to detect all the different types of damage (SSBs, DSBs, APsite, adducts, cross-links etc.). The results are expressed as TDNA (percentage of fragmented DNA) or tail intensity, considering this value as more properly indicative of the number of DNA breaks [28,29,36] that, beyond some critical amount of damage, increase more linearly than tail length (TL), usually considered as the result of the length of relaxed DNA loops. Nevertheless, also as already reported by other authors [28,29], in our experimental conditions the data examined as TL (data not reported) or TDNA showed a very similar behaviour.

We planned to submit to the atypical Comet assay the reference compounds **2**–**4** and the lipophilic analogues of **3** and **4**, namely compounds **5**–**14**. These compounds were tested on whole blood cells (cellular version of atypical Comet assay) in order to evaluate both their possible basal DNA damaging properties and their capacity to counteract the H_2O_2 caused oxidative stress. Fig. 1a reports the TDNA values obtained by treatment of whole cells with compounds **2**–**4** and the hydroxytyrosol analogues **5**–**9**, compared with the values obtained for untreated control cells (**C**). In Fig. 1b are presented the TDNA values obtained when these compounds were added before the H_2O_2 insult in comparison with the data obtained for solely H_2O_2 treated cells (**H_2O_2**).

The data in Fig. 1a (basal DNA damage) show that among the reference compounds, **2** and **4** have a significant basal DNA damaging effect with respect to the control values, whereas no significant damage is caused by **3**. Interestingly, a DNA damaging activity is observed for the hydroxytyrosol lipophilic analogues as the length of the acyl chain increases: in fact, 1-acetylhydroxytyrosol (**5**) and 1-propanoylhydroxytyrosol (**6**) show a minimal, barely significant effect, while the damage significantly increases for compounds **7**, **8** and **9**, bearing, respectively, butanoyl, decanoyl and stearoyl acyl chain. Fig. 1b (oxidative DNA damage protection) clearly show that **3** is highly protective towards the oxidative induced DNA damage in whole blood cells, differently from **2** and **4**. Among the lipophilic analogues, **5** and **6** are comparable to **3** in counteracting oxidative stress, but the protective effect progressively decreases in the order $\mathbf{7} < \mathbf{8} \simeq \mathbf{9}$.

In separate experiments, the reference compounds **2**–**4** and the homovanillic alcohol analogues **10**–**14** were examined and the TDNA values thus obtained are reported in Fig. 2a (basal DNA damage) and Fig. 2b (oxidative DNA damage protection). The experiment on basal DNA damage confirm that **3** does cause any significant damage at basal level, whereas **4** and its lipophilic analogues **10**–**14** are comparable in causing a basal damage, without a clear correlation with the length of the acyl chain. The experiment reported in Fig. 2b confirms the good protective properties elicited by **3** versus H_2O_2 induced insult, as well as the poor protection given by **4**. The lipophilic analogues of this latter show similar level of TDNA among the different analogous tested and slightly higher than that of **4**.

Summarizing the above reported data, we point out an inverse correlation between basal DNA damaging properties and a protective effect towards H_2O_2 induced oxidative damage: among the reference compounds, **3** is, with respect to **2** and **4**, less damaging at basal level and clearly more effective in protecting against H_2O_2 induced damage. Within

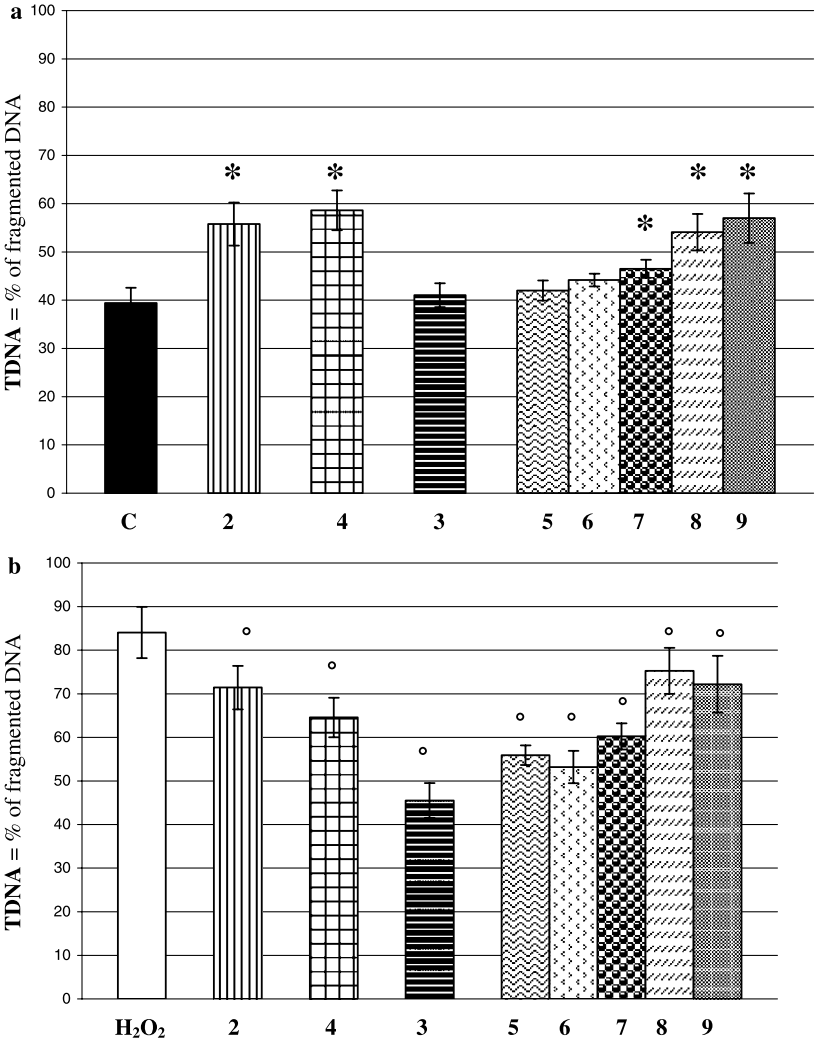


Fig. 1. Atypical Alkaline COMET assay for compounds **2**–**9**. *Significantly different from Control untreated whole blood cells ($p < 0.001$). °Significantly different from H_2O_2 alone treated cells ($p < 0.001$). The results are reported as TDNA = % of fragmentated DNA. (a) Basal DNA damage: whole blood cells were treated for 20 min at 37 °C with the tested compounds at 50 μM of concentration. (b) Oxidative DNA damage protection: whole blood cells were first treated for 20 min at 37 °C like (a) and second, after a wash with PBS 1×, for 20 min at 37 °C with H_2O_2 (200 μM). Each experiment, performed in duplicate, was repeated three times and the means ± SEM for each values was calculated.

the group of lipophilic analogues of hydroxytyrosol, DNA damaging properties and protective effects of the compounds are adversely proportional to the chain length. This trend was not observed for the lipophilic analogues of homovanillic alcohol, which appear scarcely protective, even less than the reference compound **4**, apart from the length of the acyl chain.

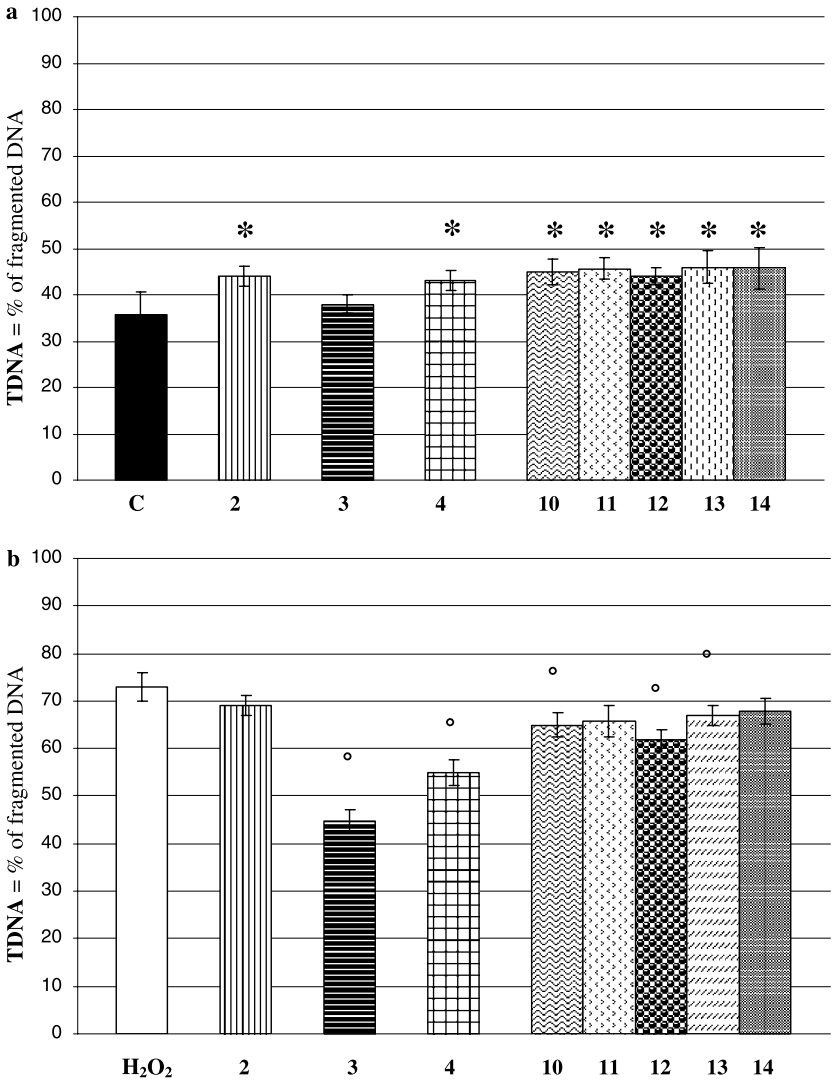


Fig. 2. Atypical Alkaline COMET assay for compounds **2**–**4** and **10**–**14**. *Significantly different from Control untreated whole blood cells ($p < 0.001$). °Significantly different from H_2O_2 alone treated cells ($p < 0.001$). The results are reported as TDNA = % of fragmentated DNA. (a) Basal DNA damage: whole blood cells were treated for 20 min at 37 °C with the tested compounds at 50 μM of concentration. (b) Oxidative DNA damage protection: blood cells were first treated for 20 min at 37 °C like (a) and second, after a wash with PBS 1×, for 20 min at 37 °C with H_2O_2 (200 μM). Each experiment, performed in duplicate, was repeated three times and the means ± SEM for each values was calculated.

4. Conclusions

The olive oil phenols hydroxytyrosol (**3**)—prepared from **2** through an enzymatic hydroxylation reaction—and homovanillic alcohol (**4**) were used as substrates for

chemoselective biocatalysed acylations, affording, with very good yields and short reaction times, ten derivatives (**5**–**14**) bearing C_2, C_3, C_4, C_{10} and C_{18} acyl chains at C-1. The reference compounds **2**–**4** and the lipophilic analogues **5**–**14** were subjected to the DPPH$^{\cdot}$ radical scavenging test: the *ortho*-diphenol hydroxytyrosol was confirmed as a potent radical scavenger, significantly more active than **2** and the hydroxytyrosol metabolite **4**. The H-donating properties of **3** were maintained for all its lipophilic derivatives showing $\log P$ ranging from 0.95 to 9.46. Data on **4** and its analogues **10**–**14** soundly demonstrate that the homovanillic system is significantly less active as DPPH$^{\cdot}$ radical scavenger than the hydroxytyrosol system, in contrast with a previous literature report [19]. Hydroxytyrosol (**3**) also showed very good properties in protecting against oxidative DNA damage, when compared with **2** and **4** in the measurement of DNA damage by atypical Comet assay. This is in agreement with literature reports indicating *ortho*-diphenols as more effective antioxidants than simple phenols [15], due to stabilisation of the phenoxy-radical through hydrogen bonding [37]. This effect is blocked by methylation of one hydroxyl group, as in **4**.

The effects elicited by compound **3** and its analogues **5** and **6**–**9**, added to whole blood cells both alone and before a successive H_2O_2 insult, were examined by atypical Comet test. Interestingly, the experimental results show a good protective effect for **3** and its analogues **5** and **6**, with $\log P \leqslant 1.20$, a moderate effect for **7** ($\log P = 1.77$) and negligible protection for **8** and **9** ($\log P > 5$), these latter exerting a significant DNA damage in basal conditions. Thus, it appears that a longer acyl chain than C_4 and a higher $\log P$ than 2 causes the loss of the protective properties of hydroxytyrosol towards oxidative insulted blood cells and should be avoided in the preparation of future lead candidates. In conclusion, the lipophilic analogues **5**–**9** may be profitably used, in principle, as food antioxidants in bulk lipids or emulsions. 1-Acetylhydroxytyrosol (**5**) and 1-propanoylhydroxytyrosol (**6**), in particular, could be further evaluated for possible applications in pharmaceutical, nutritional or cosmetic fields. The experiments carried out on compound **4** and its analogues **10**–**14** showed a different result, indicating that the homovanillic system has poor antioxidant activity, without correlation with increased lipophilicity and length of acyl groups in C-1. This is in agreement with previous observations showing that polyphenols with a catechol moiety display an higher antioxidative and DNA-protective activity with respect to those lacking of a free *ortho*-dihydroxy group [38]. If **4** would be confirmed as a main product of hydroxytyrosol metabolism in humans, these results should be considered in future studies about the bioavailability of hydroxytyrosol as a beneficial component of extra-virgin olive oil. In addition, all the C-1 acylated phenols here discussed (**5**–**14**) may be of potential interest in the study of self-organising systems promoting innovative nanopatterning or for other applications in nanotechnology.

Acknowledgments

Authors are indebted with Dr. Mario Foti and Dr. Carmelo Daquino (Istituto di Chimica Biomolecolare del CNR, Sezione di Catania, Italy) for helpful suggestions on DPPH$^{\cdot}$ scavenging activity determination. This work was financially supported by Ministero della Pubblica Istruzione, Università e Ricerca Scientifica (PRIN, Rome, Italy) as well as by a grant from the Università degli Studi di Catania (Progetti di Ricerca di Ateneo, Catania, Italy).

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