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4-Hydroxycinnamic Ethyl Ester Derivatives and Related Dehydrodimers: Relationship between Oxidation Potential and Protective Effects against Oxidation of Low-Density Lipoproteins

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The electrochemical oxidation potential of a series of monomeric and dimeric 4-hydroxycinnamic ethyl ester derivatives has been compared with their antioxidant activity toward copper-catalyzed human low-density lipoproteins (LDL) oxidation. Within the series of monomeric hydroxycinnamate derivatives, both oxidation potential and IC_{50} values decreased in the following order: sinapate > ferulate > *p*-coumarate. Among the 4-hydroxycinnamate dehydrodimer derivatives, noncyclized 8−8 diphenol dehydrodimers followed the same aforementioned sequence order and were found to be better antioxidants than their monomer counterparts. A good correlation between the inhibitory concentration and the oxidation potential was established among all these derivatives. However, a significantly deviating behavior was observed with the 8−5 dihydrobenzofuran and the 8−8 dihydronaphthol cyclic dehydrodimers, which showed lower activities toward copper-catalyzed human LDL, although their oxidation potentials remained very close to those of the noncyclized 8−8 dehydrodimers. Conversely, in the Trolox equivalent antioxidant capacity (TEAC) assay system, the 8−8 dihydronaphthol dehydrodimers were found to be the most efficient free-radical scavengers. Finally, in the series of dehydrodimers studied, it could be concluded that, whatever the in vitro test system used, (a) all dehydrodimer derivatives tested could contribute efficiently to the overall intake of antioxidants in the diet and (b) a low oxidation potential value was in favor of a satisfactory antioxidant activity.

KEYWORDS: 4-Hydroxycinnamic ethyl ester derivatives; low-density lipoproteins; dehydrodimers; free radical-scavenging activity; electrochemical oxidation potential−antioxidant activity relationship

INTRODUCTION

Low-density lipoprotein (LDL) is the major cholesterol carrier in the blood, and it is well established that an elevated plasma level of LDL is correlated with an increased risk of atherosclerosis. Oxidative modification of LDL is recognized to be implicated in atherogenic plaque formation, which is one of the main causes of coronary heart disease (*1*, *2*). Consequently, dietary antioxidants that protect LDL from oxidation may help to reduce atherogenesis and prevent coronary heart disease.

4-Hydroxycinnamic acid derivatives, which occur widely in food plants (fruits, vegetables, cereals, coffee, tea, etc.) (*3*, *4*), are known to be potent antioxidants, probably through their radical scavenging activity, although other mechanisms may be involved (*5*). In plants, these derivatives can also form dehydrodimers through oxidative cross-coupling (*6*, *7*). The main dehydrodimers identified in native lignin possess 8-O-ether, 8−5 dihydrobenzofuran, 8−8 diphenol, and 8−8 dihydronaphthol skeletons, whereas the 5−5 units are less abundant. During the past few years, particular attention has been paid to the determination of ferulic acid dehydrodimers abundance and function in several cereal dietary fibers. From these studies, it appeared that the content of dehydrodimers varied considerably among the different cereal species investigated (*4*, *7*−*13*). For example, in all cereal insoluble dietary fibers, the 8−5 dehydrodimers predominated, whereas in cereal soluble dietary fibers, the 8−8 coupled dimers often became the major dimers (*13*) (**Table 1**). Similarly, substantial amounts of 8−8 sinapic acid dehydrodimers have been recently detected in wild rice insoluble

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Table 1. Distribution of Ferulic Acid Dehydrodimers from Insoluble (IDF) and Soluble (SDF) Dietary Fiber of Some Cereal Grains[a]

		8–5[b]	8–8[b]	5–5[b]	8–O–4[b]
IDF	maize	37.7	16.5	24.9	20.6
	wheat	46.8	16.8	16.1	19.8
	spelt	49.9	16.7	15.8	20.0
	barley	50.7	16.1	14.9	18.4
	rye	54.0	18.4	13.4	14.2
	millet	46.9	25.0	12.9	15.2
SDF	maize	36.5	39.8	11.9	11.8
	wheat	37.4	36.4	16.0	10.2
	spelt	40.4	46.0	7.1	6.5
	barley	38.8	34.7	16.7	9.8
	rye	33.1	43.5	15.5	7.9
	millet	44.0	41.9	8.5	5.7

[a] Data previously reported in ref **13**. [b] Expressed as the percentage of each dimer over the total ferulic acid dehydrodimers.

cereal fiber, the 8−8 noncyclized dehydrodimer being the most abundant one, followed by the 8−8 dihydronaphthol (*14*).

To date, the ability of monomeric 4-hydroxycinnamate derivatives to inhibit in vitro oxidative modification of human LDL is well established (*5*, *15*−*19*), while little is known about the antioxidant activity of the related dehydrodimers. Recently, it has been reported that the dehydrodimers of ferulate, 8-O-ether, 8−5 benzofuran, 8−8 diphenol, and 5−5 biphenyl, exerted radical scavenging properties, but only the 8-O-ether and 8−5 noncyclic dehydroferulate inhibited the copper-catalyzed oxidation of human LDL (*20*−*22*). Conversely, there is no report on the antioxidant effects of the 8−8 diphenol and 8−8 dihydronaphthol dehydrodimers of sinapate.

In vitro, a wide range of enzymatic, chemical, and electrochemical methods has been used to mimic the oxidative coupling of 4-hydroxycinnamic acid derivatives (*23*−*27*). Recently, we have shown that anodic controlled-potential electrolysis of 4-hydroxycinnamic ethyl ester analogues, in acetonitrile solution, turned out to be a straightforward access to lignans, precursors of lignins, producing all natural coupling products simultaneously, in a one-pot experiment (*28*). As part of our continuing research efforts to find safe and efficient antioxidants (*29*−*31*), we report herein the relationship between the oxidation potential of a series of monomeric 4-hydroxycinnamic ethyl ester derivatives, together with related dehydrodimers (**Table 2**), and their antioxidant ability to inhibit copper-catalyzed oxidation of human LDL. Since the antioxidation of LDL may take place through several mechanisms such as metal chelation or protein binding, the antioxidant activity of these compounds was also evaluated by the Trolox equivalent antioxidant capacity (TEAC) assay, which strictly measured the free-radical scavenging properties.

MATERIALS AND METHODS

Chemicals. Acetonitrile (SDS-anhydrous analytical grade) was freshly distilled before use. Tetraethylammonium perchlorate was obtained from Fluka (purum purity grade). Tetramethylammonium hydroxide (TMAOH), 25 wt % solution in methanol, was supplied by Aldrich. Probucol, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and potassium persulfate were obtained from Sigma−Aldrich. ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] was supplied by WWR International S.A.S. Compounds **1**−**4** were prepared by a classic esterification procedure (*32*), with ethanolic solutions of commercially available acids and concentrated sulfuric acid. Dehydrodimers **5** and **7**−**10** were synthesized by the electrochemical procedure previously reported (*28*).

Electrochemical Synthesis of 8−8 Noncyclized Sinapic Ester Dehydrodimer 6. The apparatus and cells were identical with those described previously (*28*). Controlled-potential electrolysis was carried out in a cylindrical, three-electrode divided cell, with an electronic potentiostat. A deaerated solution of sinapic ethyl ester **3** (126 mg, 0.5 mmol), tetraethylammonium perchlorate (1.150 g, 5.0 mmol), and tetramethylammonium hydroxyde (210 μL, 0.5 mmol) in acetonitrile (250 mL) was oxidized in the main compartment, at a cylindrical carbon graphite working electrode (E = 0 mV vs SCE), under nitrogen. A platinum sheet, placed in the concentric cathodic compartment, served as the counter electrode, while the reference electrode was an aqueous saturated calomel electrode (SCE), isolated from the bulk solution in a glass tube with a fine-porosity frit. The electrolyte solution (0.02 M tetraethylammonium perchlorate in acetonitrile) was poured into the cathodic compartment, as well as into the glass tube, that contained the SCE electrode. After exhaustive oxidation, that is, when a steady-state minimum value of the current was recorded, the solution was acidified with a 0.5 M citrate-buffered aqueous solution of pH 6.0 (100 mL). The resulting mixture was concentrated to 150 mL, under reduced pressure at 50 °C, and extracted with ethyl acetate (200 mL). The organic phase was dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure, at 50 °C. Flash chromatography on silica gel, with a toluene−acetone (80:20) mixture as the eluent, afforded the 8−8 noncyclized sinapate dehydrodimer **6** (100 mg, 80%).

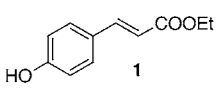
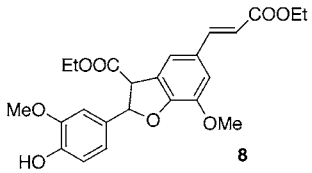
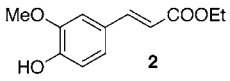
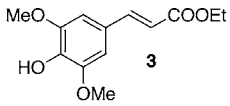
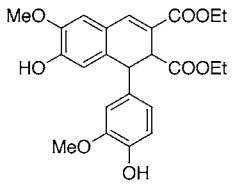
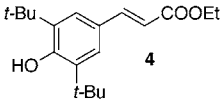
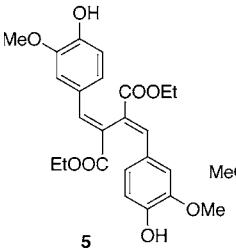
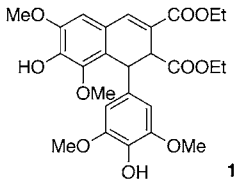
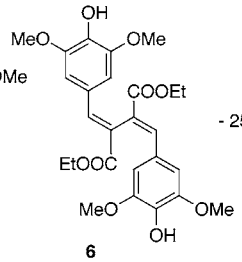
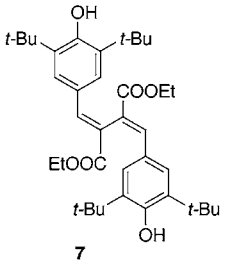
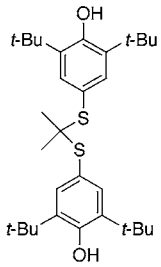
Diethyl (*E*,*E*)*-2,3-Bis*(*3,5-dimethoxy-4-hydroxybenzylidene*)*succinate* **6**. White solid recrystallized from a toluene−diethyl ether mixture, mp 90 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.10 (6H, t, J = 7.1, 2 × OCH_2CH_3), 3.85 (12H, s, 4 × OCH_3), 4.15 (4H, m, J = 7.1, 2 × OCH_2-CH_3), 5.70 (2H, br s, D_2O exchanged, 2 × HO), 6.85 (4H, s, 2 × H-2 and 2 × H-6), 7.85 (2H, s, H ethylenic). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.1 (2 × OCH_2CH_3), 56.2 (4 × OCH_3), 61.0 (2 × OCH_2CH_3), 107.0 (2 × CH-2 and 2 × CH-6), 125.5 (2 × C_q ethylenic), 126.3 (2 × C_q-1), 136.5 (2 × C_q-4), 142.2 (2 × CH ethylenic), 146.9 (2 × C_q-3 and 2 × C_q-5). MS (DCI): m/z = 503 [MH^+], 520 [$M - NH_4^+$].

Electrochemical Determination of Oxidation Peak Potential Values E_{P_a}. Cyclic voltammetric measurements were performed on a Radiometer-Tacussel PRG 5 multipurpose polarograph as a rapid response potentiostat, and a Radiometer-Tacussel GSTP 4 triangular wave forms generator. Current−potential curves were recorded on a Schlumberger SI 8312 instrument. The Radiometer-Tacussel CPRA water-jacketed cell was equipped with a platinum electrode Tacussel Pt 11 as the counter electrode, the reference electrode being similar to that mentioned above. The working electrode was a glassy carbon disk, carefully polished before each voltammogram with an aqueous alumina suspension. E_{P_a} values were determined, at a temperature of 25 °C, from 0.5 mM solutions of compounds **1**−**10**, in deaerated acetonitrile containing 0.01 M TEAP and 0.5 mM TMAOH (scan rate ν = 0.2 V s^{-1}).

Preparation of Human LDL. LDL were isolated from the pooled plasma of healthy normolipidemic human subjects and collected in the presence of EDTA (1.08 mM) by sequential ultracentrifugation (1.019 < d <1.050), according to the method of Havel et al. (*33*). For oxidation experiments, LDL was dialyzed in the dark, for 18 h at 4 °C, against 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM sodium chloride (PBS). After the determination of protein concentration by a pyrogallol technique (Elitech Diagnostics, Sees, France) (*34*), LDL were diluted to a final protein concentration of 0.1 or 0.4 g L^{-1}, depending on the antioxidant assay used.

Kinetics of Copper-Induced LDL Oxidation. The effects of antioxidants on kinetics of lipid oxidation of human LDL were assessed by spectrophotometric monitoring, at 234 nm, of conjugated diene lipid hydroperoxide formation (*35*), during copper-induced oxidation (5 μM copper, 10 mM PBS pH 7.4, 37 °C), according to the method of Esterbauer et al. (*36*). The tested compounds, dissolved in DMSO (10 μL), were added to dialyzed LDL (final volume 1 mL, 0.1 g of protein·L^{-1}) in final concentrations of 50 and 5 μM, just before the introduction of the cupric solution. LDL oxidized in the presence of DMSO constituted the solvent control. Absorbance at 234 nm was recorded every 5 min during 300 min, on a Uvikon Kontron spectrophotometer (reference, 5 μM $CuSO_4$ in 10 mM PBS, pH 7.4) and the differential absorbance ($\Delta A = A_t - A_{t=0}$) was calculated. Three phases could be distinguished from the absorbance change pattern, i.e., lag

Table 2. Oxidation Potential and Antioxidant Activity toward Cu(II)-Catalyzed LDL Oxidation of Compounds **1**–**10**

Compound	E_{Pa} (mV vs SCE) [a]	10^5 IC_{50} (M) [b]	Compound	E_{Pa} (mV vs SCE) [a]	10^5 IC_{50} (M) [b]
 **1**	+ 200	100.0 +/- 10.0	 **8**	- 150	8.0 +/- 0.6
 **2**	0	15.0 +/- 2.0			
 **3**	- 130	8.0 +/- 0.6	 **9**	- 220	7.5 +/- 0.5
 **4**	- 60	7.0 +/- 0.5			
 **5**	- 130	6.0 +/- 0.5	 **10**	- 230	10.0 +/- 1.0
 **6**	- 250	2.0 +/- 0.3			
 **7**	- 270	2.5 +/ -0.3	 **Probucol** [c]	- 160	10.0 +/- 1.0

[a] Oxidation peak potential values (E_{Pa}) measured by cyclic voltammetry at a glassy carbon electrode; 0.5 mM solution of compounds **1**–**10** containing 0.01 M tetraethylammonium perchlorate (TEAP) and 0.5 mM tetramethylammonium hydroxide (TMAOH). [b] Concentration of compounds **1**–**10** leading to 50% decrease of the amount of thiobarbituric acid-reactive substances (TBARS) concentration produced after 24 h of incubation. LDL (0.4 g of protein·L^{-1}) was incubated with 5 μM $CuSO_4$ at 37 °C in the presence of 10^{-7}–10^{-3} M solutions of the compounds in DMSO for 24 h. [c] Probucol, a hypocholesterolemic compound, was evaluated in the same test system for comparison.

phase of conjugated diene formation, propagation phase, and termination phase. During the lag phase, the lipophilic endogenous antioxidants protected the polyunsaturated fatty acids of the LDL against oxidation. After their consumption, the lipid peroxidation process underwent the propagating chain reaction phase. A tangent to the ΔA versus t curve was drawn during the propagation phase. The time interval between the addition of $CuSO_4$ ($t = 0$) and the intersection point of the tangent on the time axis was defined as the lag phase (expressed in minutes). The propagation rate was calculated from the slope of the tangent and expressed in micromoles per liter per minute (molar extinction coefficient for conjugated dienes at 234 nm = 27 000 M^{-1}·cm^{-1}).

Antioxidant Activity upon LDL Oxidation Evaluated by TBARS. The antioxidant activity of each compound was also estimated through their ability to inhibit the formation of products of lipid peroxidation during copper-induced LDL oxidation. Oxidation was performed with dialyzed LDL (0.4 g of protein·L^{-1} final concentration), by the above-described method (5 μM $CuSO_4$, 10 mM PBS, pH 7.4, 37 °C). A nonoxidized LDL sample, incubated in the absence of $CuSO_4$ and in the presence of EDTA, constituted the blank control, while addition of 10 μL of DMSO was used for solvent control. Oxidation was stopped by adding an EDTA solution (200 μM final concentration) and cooling in an ice bath. After completion of the oxidation (24 h), the final concentration of thiobarbituric acid-reactive substances (TBARS) was determined by the spectrofluorometric method of Yagi ($\lambda_{exc} = 515$ nm, $\lambda_{em} = 548$ nm), with 1,1′,3,3′-tetraethoxypropane as a standard and a nonoxidized sample as a blank (*37*). For each compound, 10 concentrations, ranging from 10^{-7} to 10^{-3} M, were tested in duplicate, the variability between the results being less than 5%. The presence of DMSO, or of the studied compounds at 10^{-3} M, did not significantly interfere on the calibration curve used for the assay. The concentration (IC_{50}) leading to 50% decrease of the amount of TBARS was estimated by linear regression analyses.

Free Radical Scavenging Capacity Evaluated by the TEAC Assay. The radical scavenging activity of tested compounds was measured by the Trolox equivalent antioxidant capacity (TEAC) assay, previously described by Re et al. (*38*). This method is based on the ability of hydrogen-donating antioxidants to decolorize the preformed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), generated by oxidation of ABTS with potassium persulfate. The extent of quenching of the $ABTS^{\bullet+}$ radical is measured by spectrophotometry at 734 nm and compared to standard amounts of Trolox C. For each compound tested, concentrations ranged from 2.5 to 10 μM.

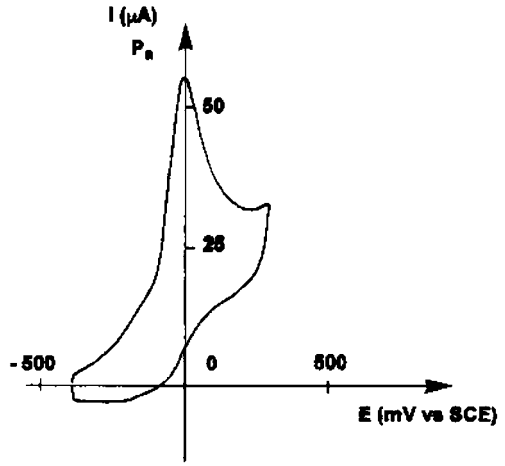


Figure 1. Cyclic voltammogram at a glassy carbon electrode of 0.5 mM ferulic ethyl ester **2**, in deaerated anhydrous acetonitrile containing 10 mM TEAP and 0.5 mM TMAOH. Scan rate $v = 0.2$ V s^{-1}.

RESULTS AND DISCUSSION

Electrochemical Measurements. This study was performed at a glassy carbon electrode, in acetonitrile containing tetraethylammonium perchlorate (TEAP) as the supporting electrolyte, and a stoichiometric amount of tetramethylammonium hydroxyde (TMAOH). Under these experimental conditions, the voltammogram of the ferulic ethyl ester anion $\mathbf{2}^-$ exhibited an oxidation peak P_a due to a diffusion-controlled one-electron process at 0 mV vs SCE, the sweep rate being 0.2 V s^{-1} (**Figure 1**). This peak could be assigned to the formation of the phenoxyl radical $\mathbf{2}^{\bullet}$. No cathodic peak was recorded in the reverse sweep, indicating that the dimerization reaction rapidly occurred after the electron transfer (*28*). The cyclic voltammograms of 4-hydroxycinnamic ethyl ester derivatives $\mathbf{1}^-$–$\mathbf{4}^-$ and dehydrodimer derivatives $\mathbf{8}^-$–$\mathbf{10}^-$ very closely resembled that described for $\mathbf{2}^-$. The respective values found for the one-electron oxidation potential E_{P_a} are listed in **Table 2**; they are related to the overall reaction reported in **Scheme 1**.

The noncyclic 8–8 dehydrodimer derivative $\mathbf{5}^-$ exhibited the same irreversible behavior as its monomer counterpart $\mathbf{2}^-$, except that two electrons were involved in the oxidation reaction (one electron for each monomeric moiety). In the specific case of 8–8 dehydrodimers $\mathbf{6}^-$ and $\mathbf{7}^-$, a cathodic peak P_c appeared on the reverse sweep, around − 350 mV vs SCE, illustrating the partial reversibility of the two-electron transfer that could be assigned to the $\mathbf{6}^-/\mathbf{6}^{\bullet}$ and $\mathbf{7}^-/\mathbf{7}^{\bullet}$ redox couples (**Figure 2**, **Scheme 2**). However, the redox potential E'° could not be accurately evaluated under our experimental conditions, as the system P_a/P_c did not fulfill all the diagnostic criteria required for a reversible process, at least when v was ≤500 V s^{-1} (*39*): the ratio of the height of P_a over that of P_c never reached unity ($i_{P_a}/i_{P_c} = 0.8$), and the value of $E_{P_c} - E_{P_a}$ was found to be higher than 30 mV.

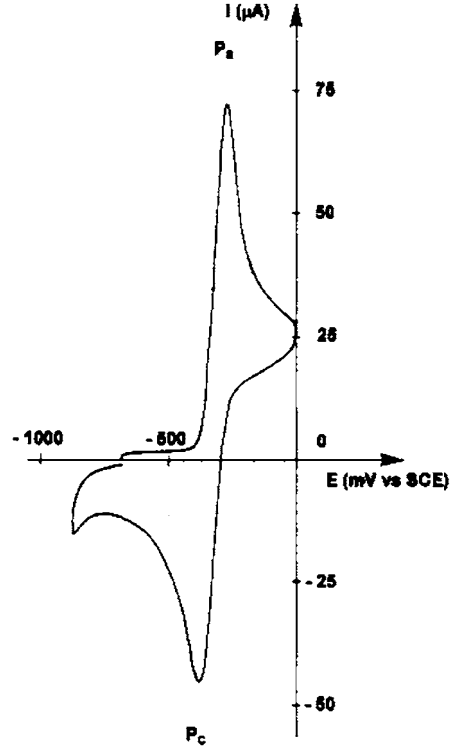
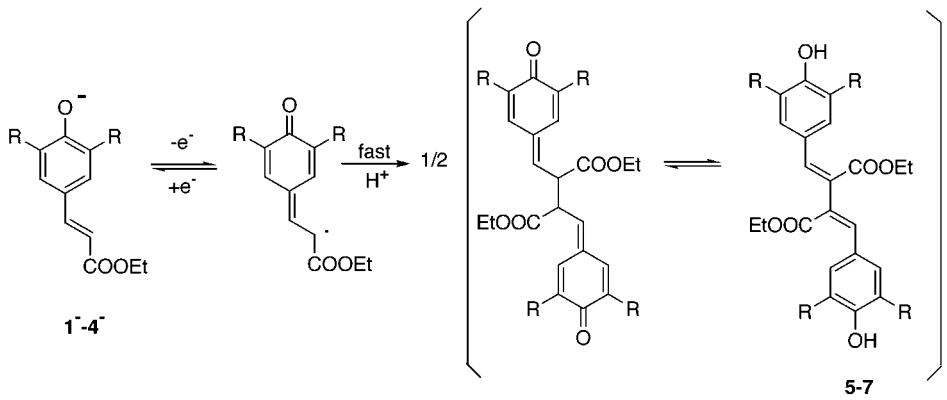


Figure 2. Cyclic voltammogram at a glassy carbon electrode of 0.5 mM noncyclized 8–8 dehydrodimer **7**, in deaerated anhydrous acetonitrile containing 10 mM TEAP and 0.5 mM TMAOH. Scan rate $v = 0.2$ V s^{-1}.

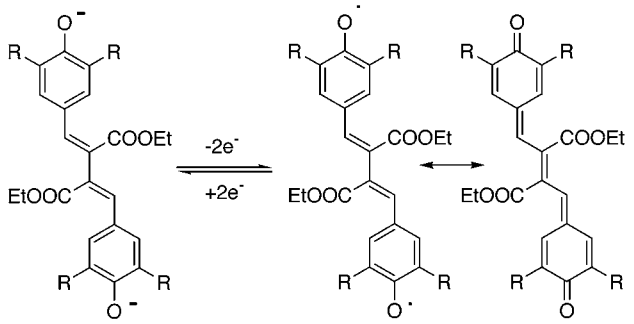
Because the 8–8 noncyclized dehydrodimers **6** and **7** could no longer undergo the dimerization reaction (**Scheme 1**), quasi-reversible behavior was observed. However, with 8–8 ferulic ethyl ester dehydrodimer **5**, no cathodic peak was detected on the reverse scan up to 500 V s^{-1}, indicating that a chemical reaction rapidly occurred after the electron transfer. Among the possible reactions, an intramolecular ring closure of the bisquinone methide leading to a naphthol derivative could be suggested (**Scheme 3**).

Very likely, when the 8–8 dehydrodimer $\mathbf{6}^-$ (or $\mathbf{7}^-$) was the starting material, the steric crowding exerted by the methoxy substituents (or *tert*-butyl groups) in ortho positions would prevent the ring-closure step (1), so that a quasi-reversible behavior was observed in cyclic voltammetry.

Scheme 1. One-Electron Oxidation of 4-Hydroxycinnamic Ethyl Ester Derivatives $\mathbf{1}^-$–$\mathbf{4}^-$



Scheme 2. Two-Electron Oxidation of the 4-Hydroxycinnamate 8–8 Dehydrodimers **6**$^-$–**7**$^-$



6$^-$: R= OMe
7$^-$: R= *t*-Bu

Kinetics of Copper-Induced LDL Oxidation in the Presence of Compounds 1−10. The effects of the different monomeric and dimeric 4-hydroxycinnamic ethyl ester derivatives **1**−**10** (50 and 5 μM) on the kinetics of conjugated diene formation, during 5 μM copper-mediated oxidation modification of LDL, are shown in **Figure 3**. For comparison, Probucol, a well-known hypocholesterolemic compound, was evaluated by the same procedure. The results are summarized in **Table 3**. It could be noted that addition of DMSO in LDL (solvent control) resulted by itself in a slight antioxidant activity, although it did not have any effect on the lag phase.

Except for the noncyclized 8−8 diphenol dehydrodimers **5**−**7**, all tested compounds were found to be less efficient than Probucol. Within the series of 4-hydroxycinnamic ethyl esters **1**−**3**, kinetic profiles are very close to those of the corresponding 4-hydroxycinnamic acid derivatives previously reported (**Figure 3a**) (*22*): (i) *p*-Coumarate **1** did not prolong the induction time of LDL oxidation. (ii) Although ferulate **2** seemed to act as prooxidant by diminishing the lag phase, it reduced the rate of LDL oxidation and decreased the maximal concentration of conjugated dienes formed after 300 min of oxidation. (iii) Sinapate **3** enhanced the induction time and reduced the rate of LDL oxidation at both concentrations tested. So, it behaved as a better antioxidant than ferulate **2**. Note that replacement of the methoxy substituent by the bulky *tert*-butyl group (compare monomers **3** and **4**) did not increase the antioxidant activity. Within the series of dehydrodimer derivatives **5**−**10**, the 8−5 benzofuran **8** roughly showed the same activity than the parent monomer **2**, whereas cyclized 8−8 dihydronaphthol dehydrodimers **9** and **10**, characterized by long induction times, acted as better protectors of LDL (**Figure 3b**). Interestingly, the noncyclized 8−8 diphenol dehydrodimers **5**−**7** were found to be very potent antioxidants. Especially, compound **7** extended the lag phase from 56 to 137 min and lowered the propagation rate from 0.76 to 0.49 μmol·L^{-1}·min^{-1}.

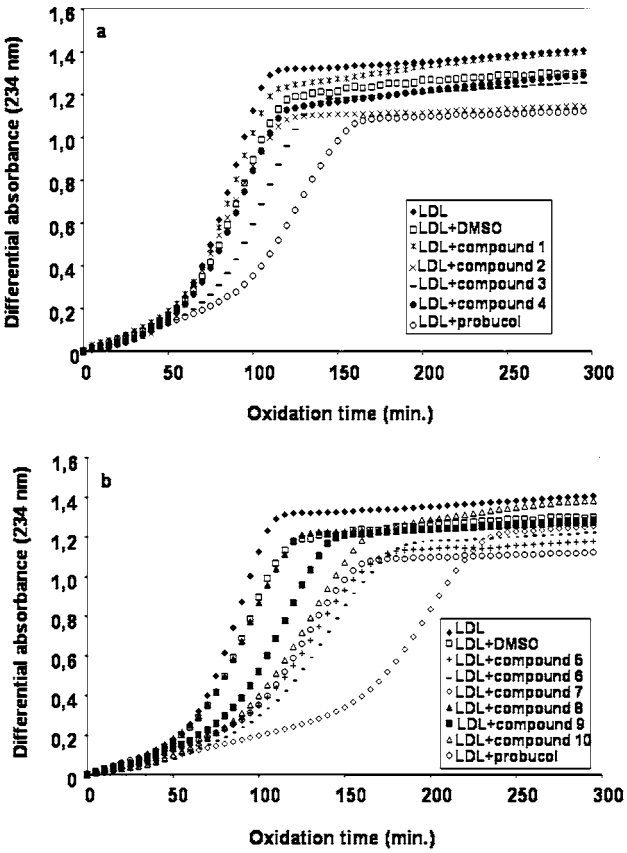


Figure 3. Formation of conjugated dienes in the absence or presence of 50 μM phenolic compounds measured by the differential absorbance at 234 nm (reference, 5 μM $CuSO_4$ in 10 mM PBS, pH 7.4; l = 1 cm). (**a**) Monomeric 4-hydroxycinnamic ethyl ester derivatives **1**–**4**; (**b**) dimeric 4-hydroxycinnamic ethyl ester derivatives **5**–**10**.

Antioxidant Activity upon LDL Oxidation Evaluated by TBARS. The protective effects of phenol derivatives **1**−**10** on copper-catalyzed LDL oxidation were also evaluated by monitoring thiobarbituric acid-reactive substances (TBARS) production. According to previous kinetic data, the concentration of oxidative products reached a steady state after 24 h of incubation. IC_{50} values represented the concentration of phenol derivative leading to 50% decrease of the amount of TBARS produced at this time point.

Scheme 3. Plausible Mechanistic Origin of the Irreversible Oxidation of 8–8 Dehydrodimer **5**

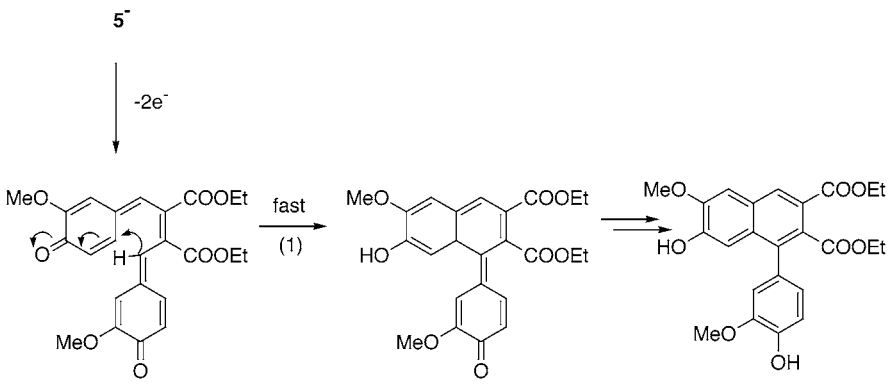


Table 3. Influence of the Presence of Compounds **1**–**10** on LDL Oxidation Evaluated by Continuous Monitoring at 234 nm[a]

compd	compd concn (μM)	lag phase (min)	propagation rate (μM·min^{-1})
1	50	53 ± 3	0.83 ± 0.08
	5	45 ± 4	0.83 ± 0.08
2	50	45 ± 4	0.59 ± 0.06
	5	45 ± 4	0.60 ± 0.06
3	50	65 ± 3	0.66 ± 0.07
	5	50 ± 2	0.68 ± 0.07
4	50	54 ± 3	0.71 ± 0.07
	5	51 ± 3	0.75 ± 0.07
5	50	78 ± 4	0.48 ± 0.05
	5	67 ± 4	0.48 ± 0.05
6	50	85 ± 4	0.48 ± 0.05
	5	73 ± 4	0.66 ± 0.07
7	50	137 ± 7	0.49 ± 0.05
	5	73 ± 4	0.60 ± 0.06
8	50	54 ± 3	0.71 ± 0.07
	5	49 ± 3	0.80 ± 0.08
9	50	69 ± 3	0.64 ± 0.06
	5	52 ± 3	0.68 ± 0.07
10	50	75 ± 4	0.52 ± 0.05
	5	57 ± 3	0.80 ± 0.08
Probucol	50	78 ± 4	0.52 ± 0.05
	5	57 ± 3	0.59 ± 0.05

[a] Results are given as mean ± SD of triplicate experiments. In the same test system, the solvent control (LDL + DMSO) and the blank sample (LDL alone) gave (a) lag phase 56 ± 3 μM and (b) propagation rate 0.76 ± 0.08 and 0.97 ± 0.10 μM·min^{-1}, respectively.

In agreement with the sequence order previously established with 4-hydroxycinnamic acid derivatives (*15*, *17*, *22*), the antioxidant activity of the monomer ethyl esters **1**−**4** decreased in the following order: sinapate **3** ≈ *t*-Bu analog **4** > ferulate **2** > 4-hydroxycinnamate **1**. With an IC_{50} value of 7×10^{-5} M (**Table 2**), the *tert*-butyl derivative **4** was the most efficient monomer derivative. This result highlighted the well-known antioxidant character of the 3,5-di-*tert*-butyl-(4-hydroxyphenyl) moiety, which is present in the widespread food antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT).

The noncyclized 8−8 dehydrodimers **5**−**7** showed significant antioxidant activity, higher than that exhibited by their monomer counterparts **2**−**4**, with IC_{50} values lower than 7×10^{-5} M. Interestingly, **6** and **7**, with a 2×10^{-5} M IC_{50} value (**Table 2**), behaved as very potent antioxidants. In contrast, cyclized 8−8 dihydronaphthol dehydrodimers **9** and **10** and 8−5 dihydrobenzofuran dehydrodimer **8** showed lower activity with IC_{50} values ranging from 7.5×10^{-5} to 10^{-4} M.

Correlation of E_{P_a} with Log 10^5 IC_{50}. Apart from 8−8 dihydronaphthol dehydrodimers **9** and **10**, the compounds examined here showed a suitable qualitative relationship between one-electron oxidation potential E_{P_a} and antioxidant activity in the lipid peroxidation assay (*40*, *41*): the log 10^5 IC_{50} vs E_{P_a} was a straight line whose equation was log 10^5 $IC_{50} = (3.40 \times 10^{-3})E_{P_a} + 1.25$ (correlation coefficient value $r = 0.973$) (**Figure 4**). From these results, several structure−activity relationships could be deduced.

Within the series of monomeric species, 4-hydroxycinnamic ethyl ester **1** was found to be inactive, with high values of E_{P_a} and IC_{50}. Introduction of a methoxy group, in the ortho position, induced a decrease in the respective values of E_{P_a} and IC_{50}, so that ferulic ethyl ester **2** behaved as a moderately active compound. Sinapic ethyl ester **3,** bearing two methoxy groups in ortho positions, proved to be the most efficient antioxidant of the monomer series, with the lowest values of IC_{50} and E_{P_a}. Replacement of the methoxy substituent by the bulky *tert*-butyl group (compare monomers **3** and **4**) induced no noticeable

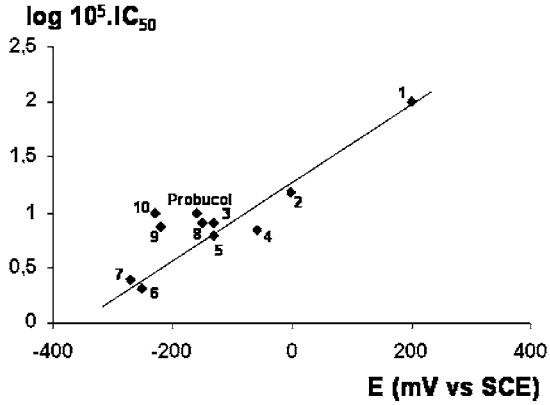


Figure 4. Variation of the logarithm of inhibitory concentration 50% (log 10^5 IC_{50}) as a function of the oxidation peak potential (E_{P_a}).

change in these parameters. All these results were in agreement with those previously reported in the literature for antioxidative phenolic compounds (*15*, *17*, *22*).

Within the series of related 8−8 dehydrodimers, similar observations could be made from the noncyclized diphenol dehydrodimer derivatives **5**−**7**, the most attractive antioxidants being the most substituted dehydrodimers **6** and **7**. In contrast, in the case of cyclized dihydronaphthol dehydrodimers, introduction of a second methoxy group in the ortho position had no significant effect on the E_{P_a} and IC_{50} values (compare compounds **9** and **10**). Hence, although cyclized 8−8 dihydronaphthol dehydrodimers **9** and **10** exhibited markedly low E_{P_a} values, their antioxidant activity did not reach those of the noncyclized 8−8 diphenol dehydrodimers **6** and **7**. This result was apparently not surprising, since it is known that the phenolic antioxidants may act as inhibitors of LDL oxidation by several different mechanisms, including metal chelation and protein binding. So, in an attempt to explain the differences observed between the noncyclized 8−8 diphenol dehydrodimer derivatives and the related cyclized 8−8 dihydronaphthol derivatives, the antioxidant capacity of compounds **1**−**10** was measured by the TEAC assay, which strictly measured the free-radical scavenging activity.

Free Radical Scavenging Capacity Evaluated by the TEAC Assay. The monomeric and dimeric 4-hydroxycinnamic ethyl ester derivatives **1**−**10**, together with Probucol, were examined for their radical scavenging activity toward the stable cation radical 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$). The activity of antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation was measured by the decrease in its absorbance at 734 nm and compared with that of Trolox, the water-soluble vitamin E analogue (*38*). The TEAC values of compounds **1**−**10** and Probucol, calculated from the $ABTS^{\bullet+}$ decolorization assay at specific times, are given in **Table 4**. From these results, it appeared that Probucol exhibited the highest TEAC value, with a scavenging activity of 2.24-fold higher than that of Trolox at 4 min, that is, at the most appropriate time to screen the relative antioxidant activities (*38*). Interestingly, the phenolic dehydrodimer derivatives **5**−**10** were found to be more efficient radical scavengers than the parent monomers **1**−**4**. The sequence order of scavenging capacity was

$$\text{Probucol} > \mathbf{10} > \mathbf{9} > \mathbf{8} > \mathbf{5} > \mathbf{7} \approx \mathbf{6}$$

According to the TEAC assay, derivatives **9** and **10** could exert potent antioxidant activity when compared to the Trolox, the water-soluble analogue of vitamin E. According to this aqueous assay system, the ability of an antioxidant to scavenge

Table 4. TEAC Values of the Compounds Calculated From the ABTS•+ Decolorization Assay at Specific Time Points[a]

compd	TEAC (1 min)	TEAC (4 min)	TEAC (6 min)
1	0.81	0.81	0.86
2	0.64	1.11	1.16
3	0.43	0.51	0.77
4	0.65	0.68	0.68
5	0.96	1.22	1.35
6	0.97	0.97	1.01
7	0.78	1.00	1.01
8	1.30	1.43	1.51
9	1.34	1.53	1.95
10	1.74	1.83	1.88
Probucol	1.97	2.24	2.42

[a] TEAC, Trolox equivalent antioxidant capacity assay system; ABTS•+, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).

the artificial long-lived radical monocation ABTS•+ would depend on the resonance stabilization energy of the phenoxyl radical species. In the case of dehydrodimers, the odd electrons were spread over both the aromatic rings, affording the bis-quinone methide species (*42*), resulting in an increase of the resonance energy. So, the dissociation energy of the OH bonds in the 8−5 and 8−8 dimers would be lower than that of the OH bond in the parent monomer, because more resonance stabilization forms can be formed in the dimer radicals. However, the scavenging capacity may not reflect the antioxidant activity due to other mechanisms in physiologically relevant substrates, including metal chelation and effects of antioxidant partitioning among phases of different polarities.

Finally, the noncyclized dehydrodimers **6** and **7** could be regarded as the most attractive compounds in the lipid phase system, with the greatest antioxidant ability to inhibit LDL oxidation, while in the aqueous assay phase, cyclized dehydrodimers **8**−**10** were found to be the best radical scavengers. These results once again highlighted the difficulty in evaluating the true protective effects of antioxidants. As previously emphasized (*43*), a reliable antioxidant protocol requires the measurement of more than one property relevant to biological systems. In this respect, the measurement of the oxidation potential could be used to obtain simple chemical information that can be related directly to the antioxidative properties. Thus, in the series of dehydrodimers studied, it could be concluded that (a) all dehydrodimers derivatives could contribute to the overall intake of antioxidants in the diet and (b) a low oxidation potential value is in favor of a satisfactory antioxidant activity in the in vitro tests used.

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