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Protective Effects of 4-Hydroxycinnamic Ethyl Ester Derivatives and Related Dehydrodimers against Oxidation of LDL: Radical Scavengers or Metal Chelators?

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4-Hydroxycinnamate derivatives are known to be potent protectors against oxidation of low-density lipoproteins (LDL), via a combination of free radical scavenging and transition metal chelation. Through a series of 4-hydroxycinnamic ethyl ester derivatives and related 8-8 dehydrodimers, we have tried to bring out the structural requirements for radical scavenging and cupric ion chelation. We found that the monomeric compounds, except for highly lipophilic *tert*-butyl derivative **3**, exhibited rather low radical scavenging properties. Furthermore, they did not chelate copper but, in contrast, reduced cupric ion to cuprous ion, affording the related 8-8 dehydrodimers, for which they could be considered as precursors in vitro. In the copper-dependent human LDL oxidation in vitro, the cyclic 8-8 dehydrodimer forms behaved essentially as efficient copper chelators, while related noncyclic 8-8 forms, which were found to be the best protectors, mainly acted as radical scavengers.

KEYWORDS: 4-Hydroxycinnamic acid derivatives; dehydrodimers; LDL oxidation; radical scavenging; lipophilicity; metal chelation

INTRODUCTION

Oxidative modification of low-density lipoproteins (LDL) has been recognized to play a central role in early stage of atherosclerosis (*1*). Consequently, a growing interest is devoted to the intake of antioxidant-rich foods, such as fresh fruits, vegetables, and beverage plants. Catechols and phenolic compounds, which are widely distributed in nature (*2*, *3*), offer a great number of pharmacologically interesting molecules such as flavonoids (quercetin, myricetin, rutin, kaempferol) (*4*), caffeic acid derivatives, and 4-hydroxycinnamic compounds (ferulic and sinapic acids) (*5*, *6*). Among these compounds, 4-hydroxycinnamic derivatives are known to be absorbed across the gastrointestinal barrier, then conjugated and eliminated in human urine (*7*−*10*). In plants, these compounds undergo oxidative cross-coupling leading to the corresponding 8-8 noncyclic, 8-8 cyclic, 8-5, 5-5, and 8-*O*-4 dehydrodimers, natural precursors of lignin (*11*−*14*). The content of dehydrodimers isolated from natural material varied with both hydrolysis conditions and species considered (**Table 1**) (*15*, *16*). Because they can also act as cross-linking agents between polysaccharides and lignin (*17*−*20*), 4-hydroxycinnamic acid derivatives modify the mechanical properties of cell walls, inducing insolubility of cereal dietary fibers (*21*).

Table 1. Distribution of Ferulic Acid Dehydrodimers Released from Insoluble Dietary Fiber of Cereals after 1 M NaOH Treatment[a]

	8-8 cyclic[b]	8-8 noncyclic[b]	8-5[b]	8-*O*-4[b]	5-5[b]
barley	33	17	12	17	31
maize	27	7	17	25	24
oats	37	19	17	6	21
rye	30	8	43	3	16
millet	39	5	19	17	20

[a] Data previously reported in ref 20. [b] Expressed as the percentage of each dimer over the total ferulic acid dehydrodimers.

Previous studies have shown that, in all cereal insoluble dietary fibers, the 8-5 dehydrodimers predominated, whereas in cereal soluble dietary fibers, the 8-8 coupled dimers became the major ones (*21*). Similarly, 8-8 noncyclic and cyclic sinapic acid dehydrodimers were found in wild rice insoluble dietary fibers (*22*), while 8-8 noncyclic dehydroferulate was shown to play a key role in conferring thermal stability of cell−cell

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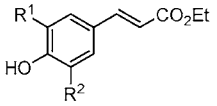
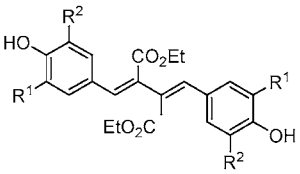
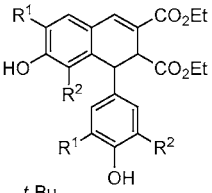
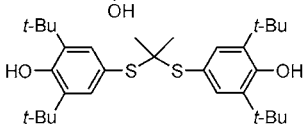
Table 2. Partition Coefficient and Antioxidant Activity toward Cu^{2+} or AAPH-Induced LDL Oxidation of 4-Hydroxycinnamic Ethyl Esters **1**–**3** and Relative Dehydrodimers **4**–**8**

	Compounds	R^1	R^2	Log *P*	IC_{50} (μM) AAPH-induced oxidation[a]	IC_{50} (μM) Copper-induced oxidation[b]
	**1**	OMe	H	2.02	40.0 +/- 3.5	150 +/- 20
	2	OMe	OMe	1.89	30.0 +/- 3.0	80 +/- 6
	3	*tert*-Bu	*tert*-Bu	5.55	5.5 +/- 0.5	70 +/- 5
	**4**	OMe	H	3.40	50.0 +/- 4.0	60 +/- 5
	5	OMe	OMe	3.15	6.0 +/- 0.5	20 +/- 3
	6	*tert*-Bu	*tert*-Bu	10.47	7.0 +/- 0.5	25 +/- 3
	**7**	OMe	H	3.12	40.0 +/- 4.0	75 +/- 5
	8	OMe	OMe	2.87	15.0 +/- 1.0	100 +/- 10
	**Probucol**[c]			11.62	4.0 +/- 0.3	100 +/- 10

[a] Concentration of compounds 1–8 leading to 50% decrease of the amount of TBARS concentration produced after 250 min of incubation. LDL (0.06 g of protein•L^{-1}) was incubated with 4 mM AAPH, at 37 °C, in the presence of 10^{-7} to 10^{-3} M solutions of the compounds in DMSO for 250 min. [b] Data previously reported in ref 40. [c] Probucol, a hypocholesterolemic compound, was evaluated in the same test systems for comparison.

adhesion (*23*). More recently, an 8-8 cyclic/8-*O*-4 dehydroferulic acid was isolated from maize bran (*24*).

At the same time, many studies attempted to determine the antioxidant properties of 4-hydroxycinnamic acid derivatives, especially their capacity to protect LDL from oxidation (*25*−*29*). Many in vitro assays used the copper-catalyzed LDL oxidation model as a mimic of the in vivo oxidation process (*30*−*32*). These assays measure a combination of radical scavenging and transition metal chelation. Radical scavenging by phenols occurs by reducing the reactive oxygen species or lipid peroxyl radicals by means of hydrogen atom donation from free hydroxyl radicals, giving rise to phenoxyl radicals. Metal chelation would be considered as prevention means of the peroxidation, either by sequestering metal ions or by restricting the access of metal ions toward lipid hydroperoxides (*6*, *33*−*35*). As part of our continuing research of efficient antioxidants (*36*−*38*), we showed that electrochemical oxidative coupling of 4-hydroxycinnamic ester derivatives constituted a straightforward one-pot method for the biomimetic synthesis of natural lignin precursors (*39*). Consecutively, we reported the protective effects against copper-induced LDL oxidation, exerted by a series of 4-hydroxycinnamic ethyl ester derivatives **1**−**3** and related dehydrodimers **4**−**8** (**Table 2**) chosen as model of their corresponding saccharidic esters (*40*). Our results showed that, on the whole, dehydrodimers derivatives **4**−**8** acted as better antioxidants than their monomeric counterparts **1**−**3**. In particular, the noncyclized 8-8 dehydrodimers **5** and **6** were characterized as very potent antioxidants, while the corresponding cyclized 8-8 dehydrodimer (dihydronaphthol) derivatives were found less active, in agreement with the final sequence order of activity **5** ≈ **6** > **4** > **7** ≈ **3** > **2** > **8** ≡ probucol > **1**.

As the protection of LDL against oxidation may take place through metal chelation as well as radical scavenging (*31*−*35*, *41*−*44*), the antioxidant activity of the series studied was also tested in the trolox equivalent antioxidant capacity (TEAC) assay, which measured the cation radical 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) $ABTS^{+\bullet}$ scavenging properties. Then, we observed an inversion in the sequence order of activity which became the following: probucol > **8** > **7** > **4** > **1** > **6** ≈ **5** > **3** > **2** (*40*). So, in copper-induced LDL oxidation, 8-8 noncyclic dehydrodimers were found to be the most efficient protectors against copper-induced oxidation of human LDL, while in the TEAC aqueous system, 8-8 cyclic dehydrodimers exhibited the strongest scavenging properties against the $ABTS^{+\bullet}$ cation radical.

As these two assays differed from both the nature of the phase and that of the oxidation initiator, we thought that a more comprehensive investigation of the antioxidant capacity of the studied compounds would be desirable to undoubtedly determine which of the dehydrodimers, cyclized or not, could be considered as the most attractive derivative. Consequently, we sought to evaluate the radical scavenging properties of the title compounds through their respective ability to inhibit 2,2′-azo-bis-(2-amidinopropane)dihydrochloride (AAPH)-induced oxidation of LDL, thus allowing a more rigorous comparison with the copper-induced oxidation assay. Secondarily, we envisaged a second antioxidant mechanism of the title compounds, consecutive to copper chelate formation.

MATERIALS AND METHODS

Chemicals. AAPH, a water-soluble source of peroxyl radicals, was a generous gift from A. Kontush and was obtained from Polysciences (Warrington, PA). BHT was obtained from Carlo-Erba (Rodano, Italy). Tetramethylammonium hydroxide (25 wt % solution in methanol), $Cu(NO_3)_2 \cdot 2.5\ H_2O$ and Probucol were supplied from Sigma-Aldrich (Saint Quentin Fallavier, France). Acetonitrile (anhydrous analytical grade) was purchased from SDS (Peypin, France). Compounds **1**−**8** were prepared according to our published procedures (*39*, *40*).

Isolation of Human Low-Density Lipoproteins (LDLs). LDL was isolated from the pooled plasma of healthy normolipidemic human subjects in the presence of EDTA (1.08 mM) by sequential ultracentrifugation ($1.019 < d < 1.050$), according to the method of Havel et al. (*45*). 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 determination of protein concentration by a pyrogallol technique (Elitech Diagnostics, Sees, France) (*46*), LDL was diluted to a final protein concentration of 0.06 g L^{-1}.

Kinetics of AAPH-Induced LDL Oxidation. The effects of antioxidants on the kinetics of lipid oxidation of human LDL were assessed by spectrophotometric monitoring, at 234 nm, of conjugated diene lipid hydroperoxide formation (*47*), during AAPH-induced oxidation (4 mM AAPH, 10 mM Chelex-treated PBS pH 7.4, 37 °C, in the dark) (*48*). The tested compounds, dissolved in DMSO (10 μL), were added to dialyzed LDL (final volume 1 mL, 0.06 g of protein·L^{-1}), with final concentrations of 5 μM just before the introduction of the AAPH solution. LDL oxidized in the presence of DMSO constituted a reference. Absorbance at 234 nm was recorded every 10 min, during 600 min, on a Uvikon Kontron spectrophotometer (reference, 4 mM AAPH in 10 mM PBS, pH 7.4) and the differential absorbance ($\Delta A = A - At_0$) was calculated. Three phases could be distinguished from the absorbance change pattern, i.e., lag phase of conjugated diene formation, propagation phase, and termination phase. During the lag phase, the lipophilic endogenous antioxidant derivatives protected the polyunsaturated fatty acids of the LDL against oxidation. After their consumption, the lipid peroxidation process underwent the propagating chain reaction phase.

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 AAPH-induced LDL oxidation. Oxidation was performed with dialyzed LDL (0.06 g of protein·L^{-1} final concentration), by the above-described method (4 mM AAPH, 10 mM Chelex-treated PBS pH 7.4, 37 °C, in the dark) (*48*). A nonoxidized LDL sample, incubated in the absence of AAPH and in the presence of 200 μM EDTA, constituted the blank control, while addition of 10 μL of DMSO was used for reference. Oxidation was stopped by adding an EDTA−BHT solution (200 μM EDTA and 20 μM BHT final concentration) and cooling in an ice bath. After a 250 min period of oxidation, the final concentration of TBARS was determined by the spectrofluorometric method of Yagi (λexc = 515 nm, λem = 548 nm), with 1,1′,3,3′-tetraethoxypropane as the standard and the nonoxidized sample as the blank (*49*). 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 formed after oxidation for 250 min (compared to the oxidation in the presence of DMSO) was estimated by linear regression analyses.

Partition Coefficient Determination (Log *P*). Log *P* values, which express the partitioning of the compounds in an *n*-octanol−water system, were calculated using Crippen's fragmentation through the CS Chem Draw Pro (7.0.1 Ultra, Cambridge Soft Company) program (*50*). In this program, the contribution of each atom to molar hydrophobicity was evaluated within a molecular database of experimental partitioning values, using a constrained least-squares technique.

Interactions of Cu^{2+} with Compounds 1−8. *Electronic Absorption Spectroscopy.* A solution of $Cu(NO_3)_2$ (0.1 M) in acetonitrile was added in portions, under stirring, at room temperature, to 0.1 mM solutions of compounds **1**−**8**, in deaerated acetonitrile containing 1 equiv (compounds **1**−**3**) or 2 equiv (compounds **4**−**8**) of tetramethylammonium hydroxide. The absorption spectra of aliquots were recorded in the range of 200−600 nm, after each addition of copper solution.

Isolation of Resulting Compounds. Among the compounds studied, similar behavior was observed (**1** vs **2** and **3**; **4** vs **5** and **6**; **7** vs **8**). Consequently, the reactions of Cu^{2+} with compounds **1**, **4**, **7** were not presented.

Reaction of Cu^{2+} with Compound **2***: Method A.* A solution of $Cu(NO_3)_2$ (0.1 M) in acetonitrile was added stepwise, under stirring at room temperature, to a 2.0 mM solution (100 mL) of compound **2** (50.4 mg, 0.2 mmol) and tetramethylammonium hydroxide (84 μL, 0.2 mmol), in deaerated acetonitrile. After addition of 1.4 equiv of Cu^{2+}, the final solution was poured into a 0.5 M acetic acid buffered aqueous solution of pH ≈ 4.5 (50 mL). The resulting mixture was concentrated to 50 mL under reduced pressure, at 40 °C, and extracted with ethyl acetate (100 mL). The organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure, at 40 °C. Flash chromatography of the residue on silica gel, with a toluene−acetone (85:15) mixture as the eluent, afforded 8-8 noncyclic dehydrodimer **5** (28.0 mg, 55%) and 8-8 cyclic dehydrodimer **8** (6.5 mg, 13%) as pale yellow oils, together with 8% of the starting material **2** (4.0 mg).

Reaction of Cu^{2+} with Compound **3***: Method B.* A solution of $Cu(NO_3)_2$ (0.1 M) in acetonitrile was added stepwise, under stirring, at room temperature, to a 2.0 mM solution (100 mL) of compound **3** (60.8 mg, 0.2 mmol) and tetramethylammonium hydroxide (84 μL, 0.2 mmol), in deaerated acetonitrile. After addition of 1.2 equiv of Cu^{2+}, evaporation of the solvent under reduced pressure, at 40°C, gave a pale yellow oil. Analysis by ^{1}H NMR showed that the bisquinonemethide **9** was the sole product of the reaction. Spectroscopic data for compound **9** have been previously reported (*39*).

Reaction of Cu^{2+} with Compounds **5**, **6**, *and* **8***:* Method A (vide supra) was applied to compounds **5**, **6**, and **8** (0.2 mmol), introducing 2 equiv of tetramethylammonium hydroxide (168 μL, 0.4 mmol). After addition of respectively 1.7, 0.6, and 2.2 equiv of copper, the starting materials **5**, **6**, and **8** were recovered, after chromatography on silica gel, in 77%, 79%, and 78% yields, respectively.

Binding Constants Determinations. The binding constants were determined using the LETAGROP-SPEFO program. This program calculates the global binding constant $\beta = K_1K_2K_3$ ... (and the successive association constants K_i) for a chemical scheme by iterative comparison of calculated data with experimental data, searching for the global minimum of the error function. Equations that do not fit the data are rejected. This program is a multicompartmental approach to spectral fitting, which allows simultaneous fitting at many wavelengths (*51*, *52*).

RESULTS AND DISCUSSION

Radical Scavenging Properties and Partitioning Properties. The radical scavenging properties of compounds **1**−**8** were tested in a nonaqueous system using the 2,2′-azo-bis-(2-amidinopropane) dihydrochloride (AAPH)-induced LDL oxidation. In the AAPH-mediated test, lipid peroxyl radicals are generated indirectly within the lipidic phase, consecutively to spontaneous decomposition of the azo compound producing AAPH-derived peroxyl radicals. This metal-independent oxidation measures the ability of antioxidants to intercept lipid peroxyl radicals and thereby to break the free radical chain reactions.

The effects of the different monomeric and dimeric 4-hydroxycinnamic ethyl esters derivatives **1**−**8** (5 μM) on the kinetics of AAPH-mediated oxidation reaction of LDL were determined by measuring conjugated diene formation at 234 nm. For comparison, Probucol, a well-known hypocholesterolemic and lipophilic antioxidant compound, was used as a reference antioxidant. As shown in **Figure 1a**, monomeric compounds **1**−**3** exhibited moderate antioxidant properties, while noncyclic 8-8 dehydrodimers **4**−**6** inhibited more strongly

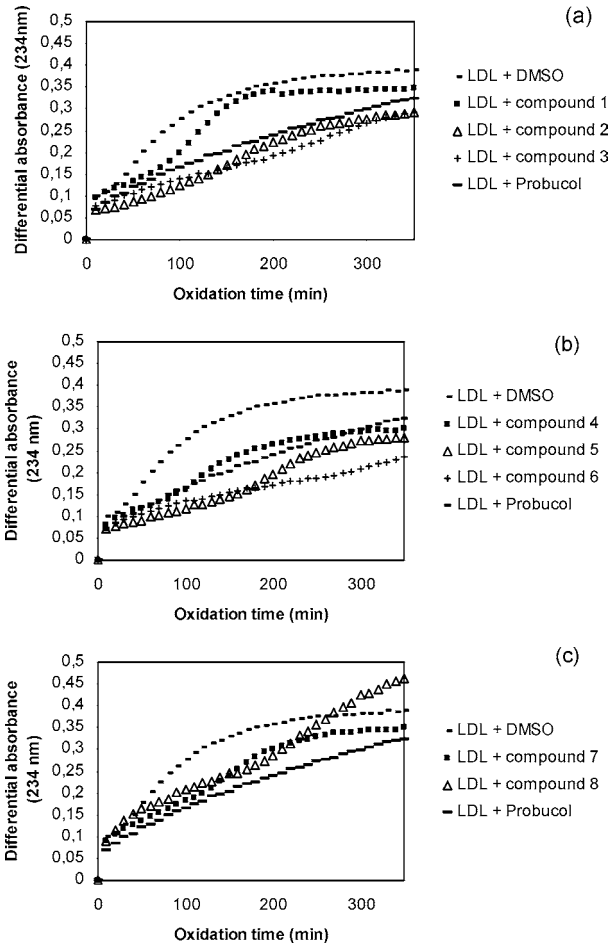


Figure 1. Kinetics of formation of conjugated dienes in the absence or presence of 5 μM phenolic compounds **1**–**8**, measured by the differential absorbance at 234 nm (reference, 4 mM AAPH in 10 mM PBS, pH 7.4; $l = 1$ cm).

the conjugated diene formation (**Figure 1b**) than did the corresponding cyclic 8-8 dehydrodimers **7** and **8** (**Figure 1c**). Note that (a) compounds **3** and **6**, possessing *tert*-butyl substituents, delayed LDL oxidation to a greater extent than did probucol; (b) the dimeric derivatives, except for compound **8**, are more potent antioxidants than the corresponding monomeric 4-hydroxycinnamic derivatives; (c) compound **8** had a biphasic effect. First, it acted as a moderate antioxidant increasing the lag time, and then it behaved as a prooxidant inducing the formation of conjugated diene (**Figure 1c**). Similarly, previous studies have shown that the syringic acid (4-hydroxybenzoic compound bearing the 2,6-dimethoxyphenol framework) also induces LDL oxidation, in the copper-mediated test (*26*).

The protective effects of the different monomeric and dimeric 4-hydroxycinnamic ethyl ester derivatives **1**−**8** were also determined by the TBARS assay. According to the above data, the concentration of oxidative products reached a steady state after 250 min of incubation. The IC_{50} values represent the concentration of phenol derivative which leads to 50% decrease of the amount of TBARS produced at this time-point. As shown in **Table 2**, the order of effectiveness within the series studied was as follows: probucol > **3** ≈ **5** > **6** > **8** > **2** > **1** ≡ **7** > **4**. Although the TBARS method was aspecific (it measures a multistep process and, if malonaldehyde is the purported product (*53*), it would only be formed from fatty acids having three or more double bonds), in our case its use appeared necessary to compare the present results to those previously published (*40*). Then, it appeared that the discrepancy in the order of activities noted between the data obtained through LDL copper-mediated oxidation (giving the noncyclized dehydrodimers **5** and **6** as the most active compounds) and the previous TEAC aqueous assay (showing the cyclic dehydrodimers **7** and **8** as the strongest radical scavengers) was suppressed. Indeed, the radical scavenging properties of the cyclic dehydrodimers **7** and **8** notably decreased in the AAPH lipidic assay, while, in contrast, related noncyclic dehydrodimers **5** and **6** were found to be the best radical scavengers. These would inhibit LDL oxidation through radical scavenging, likely due to the high stability of the corresponding phenoxyl radical, consecutive to delocalization of charge and spin over the whole molecular framework. Accordingly, the lowering of radical scavenging observed with cyclic dehydrodimers **7** and **8** would be consecutive to a decrease of the delocalization of charge and spin over a sole phenyl ring.

Interestingly, all compounds substituted by *tert*-butyl groups on the aromatic rings, probucol together with compounds **3** and **6**, exhibited low IC_{50} values (**Table 2**). At this point, the contribution of partitioning should also be taken into account. The partition coefficient *P* has been the most frequently used physicochemical parameter to rapidly evaluate the permeability of molecules through biological membranes. The log *P* values of the 4-hydroxycinnamic ester derivatives **1**−**8** were calculated in octanol−water mixture, according to the Crippen's fragmentation method. We found that **3** (log $P = 5.55$) and especially **6**, with the highest log *P* value (10.47), also exhibited the highest antioxidant activity in the lipidic AAPH system, while **7** and **8** (log $P = 3.12$ and 2.87, respectively) were notably less active. In contrast, when we compared two derivatives exhibiting very close values of log *P*, **4** and **5** for example (log $P = 3.40$ and 3.15, respectively), we found that the former possessed a markedly lower antioxidant activity than the latter. Therefore, although high lipophilicity improved the antioxidant potential activity of 4-hydroxycinnamic ethyl ester derivatives by increasing their propensity to penetrate into lipidic bilayers, it was not sufficient to explain the variation of the IC_{50}, in the series **4**−**8**. Consequently, we turned our attention toward the chelating properties of the compounds.

Interactions of Cu^{2+} Ions with Compounds 1−8. Transition metals are strongly implicated in the production of highly reactive hydroxyl radicals through the superoxide-driven Fenton reaction, as well as in the direct reductive decomposition of lipid hydroperoxides, to give alkoxyl and lipid peroxyl radicals during the propagation step. Consequently, antioxidative compounds could provide protection against copper-induced LDL oxidation, through reduction of Cu^{2+} ions into Cu^{+} ions or by chelation of Cu^{2+} ion, alternatively. To clarify the ability of 4-hydroxycinnamic ethyl ester derivatives **1**−**3** and related dehydrodimers **4**−**8** to react with cupric ion, their interaction was assessed by electronic absorption spectroscopy, and the resulting products were isolated and characterized by ^{1}H NMR. As the phenolate anion species proved to be a better substrate than the neutral form for oxidation or metal chelation, the study was performed in acetonitrile containing a stoichiometric amount of tetramethylammonium hydroxide.

*Monomeric Compounds **1**−**3**.* Stepwise addition of $Cu(NO_3)_2$ to monomeric compound **3** induced a decrease in the UV−vis absorption band shown by the anionic form at 437 nm, while new bands developed at 293 and 314 nm, respectively (**Figure 2**). After addition of 1.1 equiv of Cu^{2+} ion, the initially yellow solution became colorless and the spectral evolution reached a steady state. Spectral changes showed two isosbestic points at

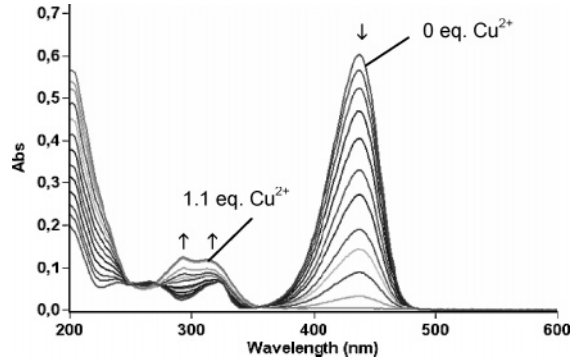
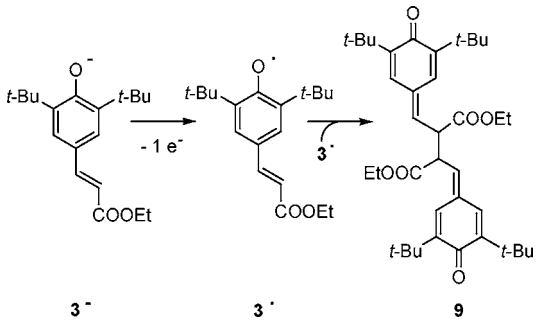


Figure 2. UV–vis absorption spectra of a 10^{-4} M solution of compound **3**, in acetonitrile, in the presence of 1 equiv of tetramethylammonium hydroxide; spectrophotometric titration with $Cu(NO_3)_2$, up to 1.1 equiv. Each spectrum was recorded after addition of 0.1 equiv of Cu^{2+} (cell thickness $l = 0.1$ cm).

Scheme 1. Dimerization Reaction of 3,5-di-*tert*-Butyl-4-hydroxy Cinnamate $\mathbf{3}^-$



252 and 354 nm indicating that a simple equilibrium between two species was operating. The two new bands observed at 293 and 314 nm can be assigned to the bisquinonemethide **9**, by comparison with the UV−vis absorption spectrum of an authentic sample (*39*). Furthermore, the formation of **9** was confirmed after evaporation of the solvent (acetonitrile) and analysis of the isolated solid by ^{1}H NMR, which identified it as the bisquinonemethide **9.** This result indicates that compound **3** did not form a chelate but acted as a reducing agent toward cupric ions, generating cuprous ions together with the bisquinonemethide **9** through the dimerization of the phenoxyl radical **3•** (**Scheme 1**).

Comparatively, the spectral changes observed during the addition of $Cu(NO_3)_2$ to a solution of compound **2** were more complex: up to 0.7 equiv of copper, interaction of Cu^{2+} ions with the phenolate anion $\mathbf{2}^-$ induced hypochromic and hypsochromic shifts of the initial band, from 436 to 393 nm (**Figure 3a**). Further addition of Cu^{2+} up to 1.3 equiv resulted in the observation of four new bands (or shoulders) at 316, 333, 472, and 506 nm, respectively. The lack of well-defined isosbestic points suggested that concomitant reactions take place (**Figure 3b**). Accordingly, a preparative scale experiment allowed the isolation of the 8-8 noncyclic dehydrodimer **5** as the major product (55%), the 8-8 cyclic dehydrodimer **8** (13%) as the minor product, together with 8% of the starting material. Compound **1** behaved similarly, but the isolation of the resulting compounds was not attempted as the oxidation of compound **1** simultaneously generated five coupling products (8-8 noncyclic, 8-8 cyclic, 8-5, 5-5, and 8-*O*-4 dehydrodimers) (*39*).

Finally, as previously reported in the case of ferulic and sinapic acid (*6*), monomeric compounds **1**−**3** did not act as chelators but, in contrast, revealed a reducing activity toward

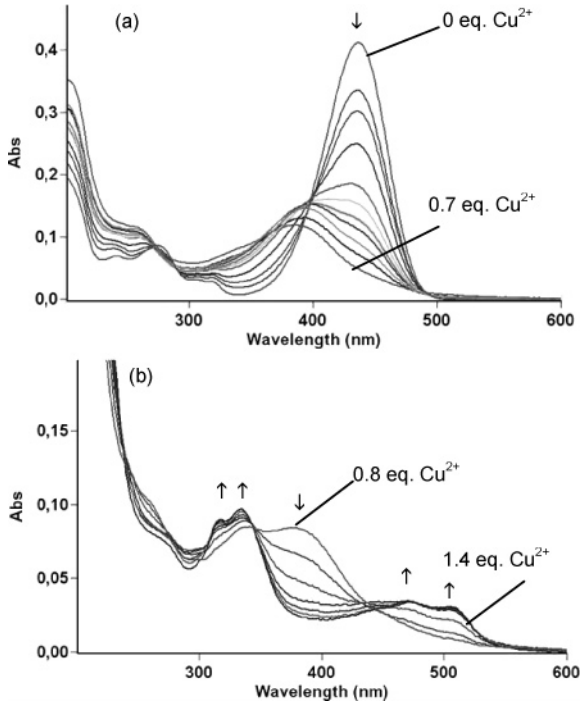


Figure 3. Titration of compound **2** (10^{-4} M), in acetonitrile, in the presence of 1 equiv of tetramethylammonium hydroxide, with Cu^{2+} (cell thickness $l = 0.1$ cm). (**a**) $[Cu^{2+}] = 0$, 0.10, 0.20, 0.30, 0.40, 0.44, 0.48, 0.52, 0.56, 0.6, and 0.70×10^{-4} M; (**b**) $[Cu^{2+}] = 0.8$, 0.9, 1.0, 1.1, 1.2, 1.3, and 1.4×10^{-4} M.

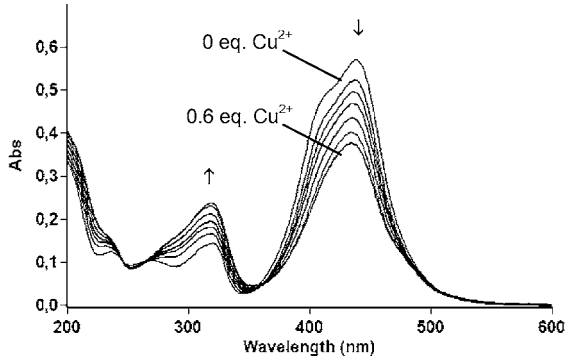


Figure 4. Titration of compound **6** (10^{-4} M), in acetonitrile, in the presence of 2 equiv of tetramethylammonium hydroxide, with Cu^{2+} (cell thickness $l = 0.1$ cm). $[Cu^{2+}] = 0$, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6×10^{-4} M.

cupric ions, generating cuprous ions together with the dehydrodimer oxidized forms.

*Dehydrodimers **4**−**8**.* A distinct behavior was observed with 8-8 linear dehydrodimer **6**, although spectral changes recorded in the course of titration were close to those found for the parent monomer **3** (vide supra) (**Figure 4**). A steady state was reached after addition of only 0.6 equiv of cupric ions, while a new band increased at 322 nm. This spectral evolution, characterized by the presence of three isosbestic points at 245, 263, and 358 nm, indicated that the stoichiometry of the reaction remained unchanged and that no secondary reaction occurred during this time. However, it was not possible to isolate an oxidized product, as extraction only regenerated the starting material in 79% yield. These results indicated that the Cu^{2+} ion was not reduced but likely bridged by the 8-8 dehydrodimer **6** ligand. The resulting chelate would be expected to decompose in water during the workup. At this point, to substantiate the formation of the copper complex we attempted to model the spectral changes with the

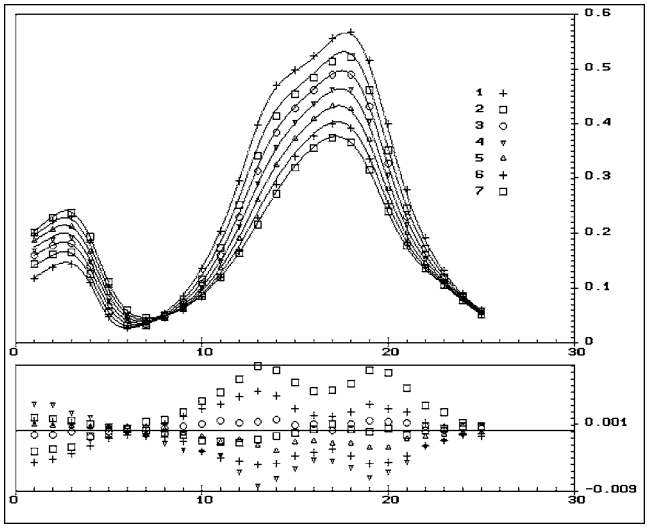


Figure 5. LETAGROP plot for the determination, in acetonitrile, of the stoichiometry and association constants of compound **6** with Cu^{2+} from UV titration between 304 and 488 nm (24 wavelengths and 7 different salt concentrations: geometrical symbols, experimental data; solid lines, calculated spectra; R_i, weighted residuals distribution).

Table 3. Binding Constant Values for Dehydrodimers **4**–**8** and Cu^{2+} Ions

compounds	log $K_{1:1}$	log $K_{1:2}$	log $K_{2:1}$	log β_2
4	5.65		4.65	10.30
5	5.81		5.04	10.85
6			8.63	8.63
7	6.72	5.04		11.76
8	5.81	4.74		10.55

help of the known LETAGROP-SPEFO method (*51*, *52*). This program calculates, for a given chemical scheme (see eqs hereafter), the global binding constant $\beta_2 = K_1K_2$ by iterative comparison of calculated data with experimental data, searching for the global minimum of the error function. Equations that do not fit the data are rejected. As shown in **Figure 5**, a very good agreement between experimental and theoretical spectra was obtained for the formation of a 2:1 dehydrodimer−copper chelate, with a global binding constant log $\beta_2 = 8.63$ (**Table 3**).

$$L + Cu^{2+} \leftrightarrows LCu^{2+} \qquad (L \equiv \text{compound } \mathbf{6}) \qquad K_{1:1}$$

$$LCu^{2+} + L \leftrightarrows L_2Cu^{2+} \qquad K_{2:1} \qquad K_{1:1}K_{2:1} = \beta_2$$

The interaction of the Cu^{2+} ion with compounds **4** and **5** was studied under the same experimental conditions. Spectral changes obtained in the case of compound **5** markedly differed from those recorded with compound **6**. Two steps could be distinguished in the course of the titration. From 0 to 0.6 equiv, the initial band at 435 nm, characteristic of the anionic form $\mathbf{5}^-$, decreased. Simultaneously, a new band around 365 nm increased (**Figure 6a**). Further addition of Cu^{2+} ion up to 1.7 equiv induced the disappearance of the latter in favor of two new bands at 473 and 506 nm (**Figure 6b**), and a steady state was obtained up to 1.7 equiv of Cu^{2+} ion. After extraction, we recovered the starting material in 77% yield as the sole compound. LETAGROP-SPEFO calculations confirmed the simultaneous formation of two types of chelates, a 2:1 and a 1:1 ligand-to-copper chelate. The values of their binding constant are reported in **Table 3**. Compound **4** behaved similarly. Thus,

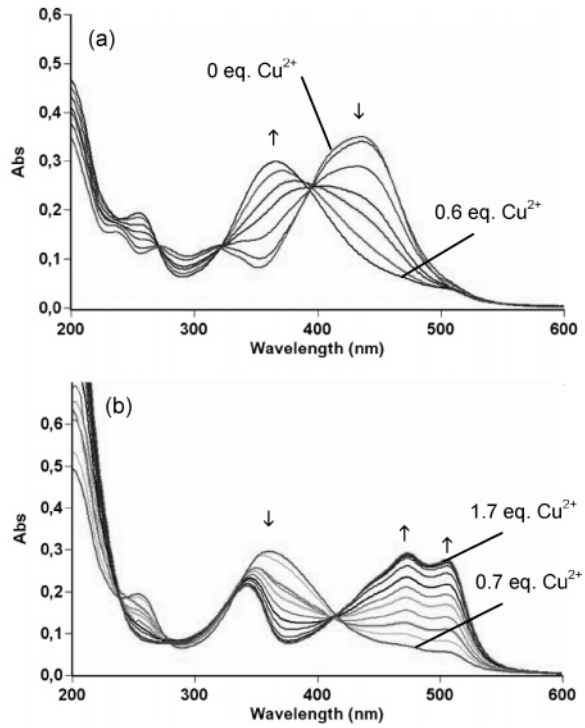


Figure 6. Titration of compound **5** (10^{-4} M), in acetonitrile, in the presence of 2 equiv of tetramethylammonium hydroxide, with Cu^{2+} (cell thickness $l = 0.1$ cm). (**a**) $[Cu^{2+}] = 0.0$, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6×10^{-4} M; (**b**) $[Cu^{2+}] = 0.7$, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 1.7×10^{-4} M.

whatever the compound considered within the 8-8 noncyclic dehydrodimer series, the chelate formed was a 2:1 ligand-to-copper complex stoichiometry.

In the case of 8-8 cyclic dehydrodimers **7** and **8**, spectral shifts were very close to those described above. The spectral evolution involved two steps, and more than 75% of the starting material was recovered at the end of the reaction. However, the stoichiometry of the chelates formed was different: the curves were correctly fitted by considering the generation of 1:1 and 1:2 ligand-to-copper complexes simultaneously.

$$L + Cu^{2+} \leftrightarrows LCu^{2+} \qquad (L \equiv \text{compound } \mathbf{7} \text{ or } \mathbf{8}) \qquad K_{1:1}$$

$$LCu^{2+} + Cu^{2+} \leftrightarrows L(Cu^{2+})_2 \qquad K_{1:2} \qquad K_{1:1}K_{1:2} = \beta_2$$

Concluding Remarks. In this work, we have provided new insight into the antioxidant capacity of a series of 4-hydroxycinnamic ethyl ester and related dehydrodimers. Taking into account both the ability of the compounds to inhibit the metal-dependent and metal-independent oxidation of LDL, the partitioning properties together with the chelating properties, several conclusions could be drawn:

In the AAPH-induced LDL oxidation, the *monomeric compounds*, except for highly lipophilic derivative **3**, exhibited the lowest radical scavenging activities of the series studied. In agreement with previous studies concerning dietary compounds or eugenol compounds (*6*, *44*), they were not endowed with copper chelating properties, but, in contrast, they reduced cupric ions to cuprous ions, affording the corresponding dehydrodimers compounds **4**−**8**, for which they could be considered as precursors.

In the subseries of *dehydrodimers*, two cases had to be considered: the cyclic dihydronaphthol dehydrodimers **7** and **8** were found to be low to moderate inhibitors of both metal-

independent and metal-dependent oxidation of LDL. These results solved the problem of the deviating effects observed earlier (*40*) between the TEAC assay and the copper-mediated oxidation of LDL assay, thus demonstrating that the observed difference was essentially due to the change in the phase (strictly aqueous or not), rather than in the oxidation mediator (cupric ion or stable radical). Conversely, although the cyclic dehydrodimers **7** and **8** behaved as low and moderate radical scavengers respectively, they were found to be effective metal chelators toward cupric ions, affording up to 1:2 ligand-to-copper complexes. The comparison between the powerful chelating properties with the moderate activity found in the copper-mediated LDL oxidation test, which evaluated the exhaustive antioxidant activity including both radical scavenging and metal chelation, indicated that the contribution of metal chelation would not be the major process in the antioxidant activity measured in this assay.

This assumption was substantiated when considering the related noncyclic diphenol dehydrodimers **5** and **6.** As suggested by preliminary results concerning the 8-8 noncyclic diferulic acid (*27*), these compounds proved to be the most potent antioxidants, both in the metal-independent and metal-dependent oxidation of LDL assays. Furthermore, they chelated cupric ion with a lower efficiency than that of the cyclic dehydrodimer counterparts **7** and **8**, since they only gave complexes with a 2:1 ligand-to-copper stoichiometry. It is important to consider that these noncyclized diphenol dehydrodimers were mainly present in the diet as ester-linked compounds in the insoluble fraction of the cell walls. Consequently, at the opposite of their parent monomers, they cannot be released into the intestine after consumption of high-bran cereal, and then are not detected in substantial amount in human plasma (*7*, *9*, *28*). So, they are unable to directly contribute to some of the beneficial effects of a phenolic-rich diet, though they could be spontaneously produced in lipidic phase from their corresponding parent monomers.

Finally, in the series studied, it could be concluded that the noncyclized diphenol dehydrodimers behaved as the most attractive antioxidants through the main antioxidant mechanism involving radical scavenging.

ABBREVIATIONS USED

AAPH, 2,2′-azo-bis-(2-amidinopropane) dihydrochloride; $ABTS^+$, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BHT, butylhydroxytoluene; LDL, low-density lipoprotein; TEAC, trolox equivalent antioxidant capacity; TBARS, thiobarbituric acid-reactive substances.

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