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Article

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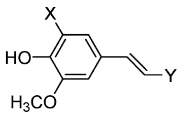
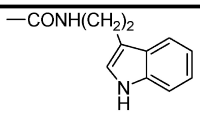
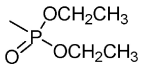
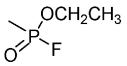


Compd	X	Y	TBARS %	MTT %
1	H		9.6 ± 1.5	93.6 ± 1.1
7c	OCH_3		13 ± 0.7	81 ± 2.8
12c	OCH_3		12 ± 1.4	93 ± 2.8

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Articles

Design, Synthesis, and Evaluation of Pharmacological Properties of Cinnamic Derivatives as Antiatherogenic Agents

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A series of cinnamic and phosphonocinnamic derivatives have been synthesized and their ability to inhibit cell-mediated LDL oxidation and oxidized LDL-induced cytotoxicity was investigated. Electron-donating substituents surrounding the necessary 4-OH group of the aromatic ring showed the best results. Among the different series tested, amide **1**, thioester **5c**, phosphonoester **7c**, and the fluorophosphonocinnamic analogue **12c** exhibited a potent inhibitory effect against LDL oxidation (and subsequent toxicity) mediated by cultured human microvascular endothelial cells (HMEC-1), with an efficacy comparable to that observed with probucol. Beside this indirect protective effect, these compounds exhibited a direct protective effect against the toxicity of previously oxidized LDL in HMEC-1. These data suggest that the newly synthesized cinnamic compounds should protect against early events (cell-mediated LDL oxidation) occurring within the vascular wall in atherosclerosis.

Introduction

Atherosclerosis and related diseases represent the prevalent cause of morbidity and mortality in industrialized countries.[1] There is now an increasing amount of experimental and clinical evidences which shows the involvement of oxidative modifications of low-density lipoproteins (LDL) in the pathogenesis of atherosclerosis.[1] Oxidized LDL (oxLDL) exhibit in vitro a variety of biological properties potentially involved in atherogenesis.[2−5] The role of oxLDL in atherogenesis is assessed by (1) the presence of oxLDL in atheroma plaque,[6] (2) the presence of circulating antioxLDL antibodies,[7] (3) the biological properties of oxLDL on cultured vascular cells[8] and (4) the (globally) protective role of antioxidants, particularly in animal models for atherosclerosis.

One of the strongest lines of evidence of the LDL oxidation hypothesis on atherosclerosis is the ability of a variety of lipid soluble antioxidants to inhibit atherogenesis in animal models of hypercholesterolemia.[9] A large number of antioxidants are phenolic compounds.[10] Several kinds of lipophilic antioxidants such as α-tocopherol, ubiquinol, *β*-carotene are contained in the LDL particles. In addition to the intrinsic antioxidants, other phenolic bioactive substances are widely present in food plants[11] and can be therefore potentially found in the human plasma, depending on diet habits. Synthetic antioxidants have been also studied and evaluation of their protective effects against oxLDL has been undertaken.[12] For instance, probucol, butylated hydroxytoluene, *N*,*N*′-diphenylphenylenediamine inhibit LDL oxidation in vitro and retard the progression of atherosclerosis in hypercholesterolemic rabbits.

Antioxidants may exert their effect through different mechanisms.[13] They have been extensively used to investigate the mechanisms by which cultured cells promote LDL oxidation in vivo. Antioxidants may suppress the formation of active oxygen species by reducing hydroperoxides and hydrogen peroxide, by sequestering metal ions, scavenging active free radicals and/or clearing damage. Nevertheless, even if an increasing amount of evidences supports the role of oxidation in atherogenesis, the protective effect of antioxidants in human is still debated and controversial. The LDLs resistance to oxidation does not systematically correlate with their antioxidant content, the degree of lipid peroxidation in atherosclerotic tissue also failed to correlate with the extent of lesion formation, while considerable amounts of antioxidants can be observed in atherosclerotic lesions.[14] Antioxidants, though highly protective in animal model for atherosclerosis (which investigate mainly the first steps in atherogenesis), fail to protect against atherosclerosis complications in human such as rupture and thrombosis, and subsequent cardiovascular events.

A lot of questions remain to be elucidated so far concerning the mechanisms of LDL oxidation by cells, the respective role of bioreactive oxidation products involved in the biological effects of oxLDL (oxysterols, hydroperoxides, aldehydes, intracellular inductive oxygen radical, etc.).[15] These questions concern also the development of new families of active compounds that may exert effects on the oxidant stress generation but

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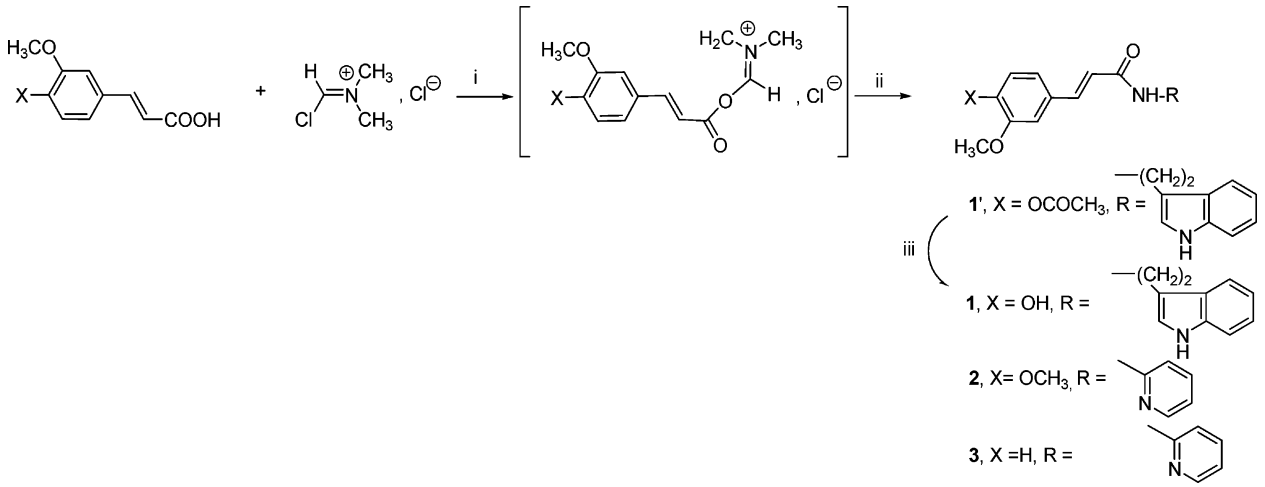
Scheme 1[a]



[a] Reagents and conditions: (i) pyridine, THF, −30 °C; 1 h; (ii) n-BuLi, RNH_2, THF, −30 °C, 10 min, then 2 h at room temperature, 80%, 70%, 76% for compounds **1**−**3** respectively; (iii) K_2CO_3, MeOH, 20 h, quantitative.

also on their ability to interfere with and neutralize the effect of bioreactive oxidation products present in ox-LDL.

Lignins, the second most abundant biopolymer after cellulose, are complex aromatic polymers resulting from the oxidative polymerization of hydroxycinnamyl alcohols, i.e., *p*-coumaryl, coniferyl, sinapyl alcohols.[16] Their biosynthesis begins with the common phenylpropanoid pathway affording para-hydroxylated cinnamic acids and next cinnamoyl CoA esters. They are common precursors of a wide range of products possessing biochemical properties. Polyphenolic secondary plant metabolites such as flavonoids, chalcones, and caffeic acid/esters show activities related to their antioxidant properties.[17] Some cinnamic acid derivatives (amides) have recently been reported to possess inhibitory effects on LDL oxidation. Lignan 2′-hydroxy-dihydroguaiaretic acid and the known neolignan machilin D isolated from root of *S. chinensis* are also reported to exhibit LDL-antioxidant activity.[18]

In the course of our ongoing research program directed toward synthesis of phenolic compounds related to the lignification process,[19] we have described the synthesis of cinnamic compounds (amides)[20] and some phosphonocinnamic analogues.[21]

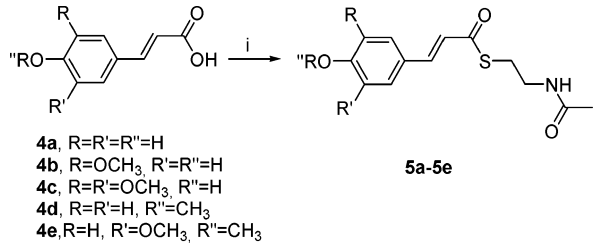
In this report, we wish to describe synthesis of some new analogues, i.e., cinnamic thioesters, phosphonothioesters, and fluorophosphonoesters and the evaluation of their inhibiting effects on LDL oxidation.

Chemistry

The synthesis of the novel compounds is shown in Schemes 1−4. Synthesis of cinnamides **1**−**3** is accomplished in two (or four steps) as we have previously reported, i.e. iminium salt formation of the corresponding cinnamic acid (acetylated on the 4-OH group on the aromatic ring for compound **1′**) followed by reaction with the lithiated amine.[20] Quantitative deacetylation of **1′** afforded feruloylamide **1**. Compounds **1**−**3** were thus obtained in 70−80% yield (Scheme 1).

Synthesis of *N*-acetylcysteamine thioesters of cinnamic analogues was also undertaken. *N*-acetylcysteamine (NAC) might be considered as the simplest mimic of CoA moiety, naturally occurring in many

Scheme 2[a]



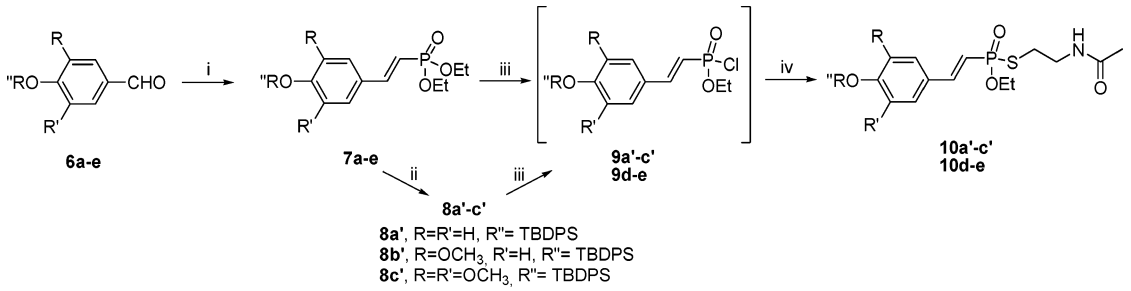
[a] Reagents and conditions: (i) *N*-acetylcysteamine, EDC, DMAP, CH_2Cl_2, room temperature, 1 day, 76%, 90%, 79%, 91%, 85% for compounds **5a**−**e**, respectively.

biological systems. Moreover, thioesters may be viewed as masked forms of the thiol group, a well-known scavenger of oxygen radical playing an important role in growth, differentiation, and detoxification processes in the mammalian organism.[22]

Different strategies have been explored. The thiotransesterification reaction that we had optimized and used for synthesis of the cinnamoyl CoA esters in its biphasic version did not operate. Reaction of 4-chlorothiophenylferuloyl ester with *N*-acetylcysteamine in the presence of triethylamine afforded essentially ferulic acid. Different coupling reagents such as bromo-tris-(dimethylamino)phosphonium (BrOP), dicyclohexylcarbodiimide (DCC), and 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) with 1-hydroxybenzotriazole (HOBt) were then tested. The basic conditions were also varied (diisopropylamine, *N*,*N*-dimethylaminopyridine, triethylamine). The best results were obtained when operating with EDC as coupling agent in the presence of DMAP. The *N*-acetylcysteamine thioesters **5a**−**e** (Scheme 2) were thus synthesized in 76−91% yield after silica gel purification.

The phosphorylated analogues of cinnamic acids were also synthesized. Diethylphosphonates were obtained as previously described by reacting the corresponding aldehydes with lithiated tetraethylmethylenediphosphonate.[21] Thermal treatment of the diphosphorylated intermediate gave exclusively the trans-vinylic phosphonates. Treatment with oxalyl chloride in methylene chloride provided the corresponding phosphonochloridates. Chloration did not occur if 4-OH group on the

Scheme 3[a]

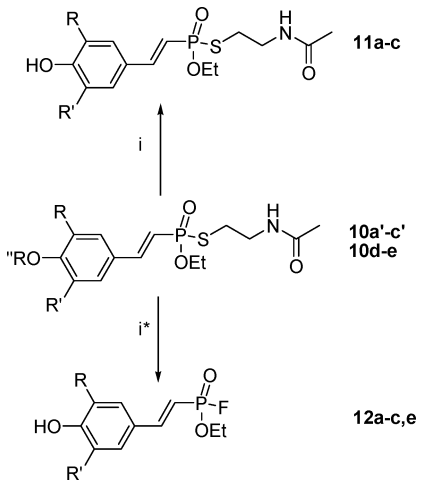


[a] Reagents and conditions: (i) $^{i}Pr_2NH$, n-BuLi, THF, −50 °C, 20 min, tetraethylmethylene diphosphonate, 1 h, then **6a**−**e**, 45 min and reflux 2 h, 60%, 68%, 80%, 80%, 77% for compounds **7a**−**e**, respectively; (ii) imidazole, DMAP, TBDPSCl, DMF, room temperature, 16 h, 67%, 86%, 57%; (iii) $(COCl)_2$, CH_2Cl_2, room temperature, 20 h, quantitative; (iv) *N*-acetylcysteamine, Et_3N, CH_2Cl_2, room temperature, 16 h, 34%, 67%, 59%, 68%, 37% for compounds **10a′**−**c′**, **10d**,**e** respectively.

aromatic was let free, but did effectively operated when the 4-OH group was protected upon silylation (Scheme 3). The reactions were monitored by ^{31}P NMR spectroscopy indicating the complete transformation of compounds **7d**,**e** and **8a′**−**c′** to the corresponding phosphonochloridates. Condensation with *N*-acetylcysteamine in the presence of triethylamine afforded the corresponding phosphonothioesters **10a′**−**c′**, **10d**,**e** in moderate yields (34−68%).[23]

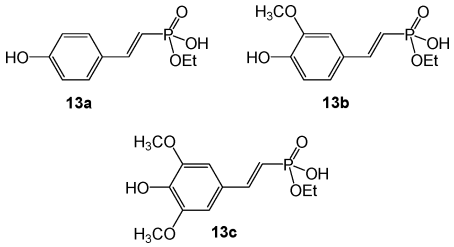
To obtain the desilylated target compounds **11a**−**c**, efforts were undertaken to cleave the silyl protective group selectively. While acidic conditions are deleterious for the phosphonothioester functionality, fluoride anions were used. The in situ prepared complex $Et_3N{\cdot}HF$ was used for that purpose and its reactivity tested on compound **10a′**. When the reaction is carried at room temperature (monitored by ^{31}P NMR), the desilylated thioester **11a** was formed as revealed by ^{31}P NMR spectra (δ = 47.41 ppm in $CDCl_3$), but derivative **11a** was quickly transformed in a new one possessing a P−F coupling (δ = 20.53 ppm J = 1024 Hz in $CDCl_3$). This compound was identified to be the phosphonofluoridate **12a**, obtained after elimination of the protective silyl group and subsequent fluoride substitution of NAC on the phosphorus atom. This method applied to compounds **10a′**−**c′**, **10e** afforded the corresponding phosphonofluoridates **12a**−**c**, **e**, in 64−69% yield after silica gel purification (Scheme 4). The reaction temperature was then checked in order to obtain only the desilylated compounds **11a**−**c**. When operating in THF at −25 °C and quenching the reaction mixture after 20 min, only the desilylated products were observed by ^{31}P NMR along with starting material. Thiophosphonates **11a**−**c** were thus obtained in 43−51% yield after silica gel purification.

Scheme 4[a]



[a] Reagents and conditions: (i) Et_3N−HF, THF, 5−20 min, −25 °C, 51%, 43%, 49% for compounds **11a**−**c**, respectively; (i*) Et_3N−HF, THF, 2−3 h, room temperature, 66%, 64%, 68%, 69% for compounds **12a**−**c**,**e** respectively.

Chart 1



Results and Discussion

Two families of cinnamic derivatives, cinnamic (amides and thioesters) and phosphonocinnamic compounds, were synthesized, and their antioxidant properties were studied on cell-mediated LDL oxidation. Among the phosphonocinnamic derivatives, the monoacids **13a**−**c** (Chart 1), synthesized from the corresponding diesters as previously reported, were also tested.[21]

All compounds were evaluated for their ability to protect native human LDLs from HMEC-1-induced oxidation. HMEC-1 are human microvascular endothelial cells able to oxidize LDL and thus suitable to model the pathophysiological events (LDL oxidation) that occur within the vascular wall, and the protective effect of antioxidants.[24] LDL oxidation was determined by using the thiobarbituric acid reactive substance assay (TBARS) and expressed as a percentage of TBARS formed in LDL in contact with HMEC-1 in the absence of antioxidant. Alternatively, LDL oxidation and protection by antioxidant were evaluated by their relative electrophoretic mobility in agarose gel, (Hydragel, Sebia, France).

Since LDL oxidation by cultured cells renders them cytotoxic[25] and since antioxidants are able to block the LDL oxidation process by cells,[26] we evaluated the residual cytotoxicity of LDL previously oxidized by HMEC-1 in the presence or absence of the newly synthesized cinnamic agents, by the MTT assay. The data were expressed as a percentage of the unstimulated control (cell incubated without LDL).

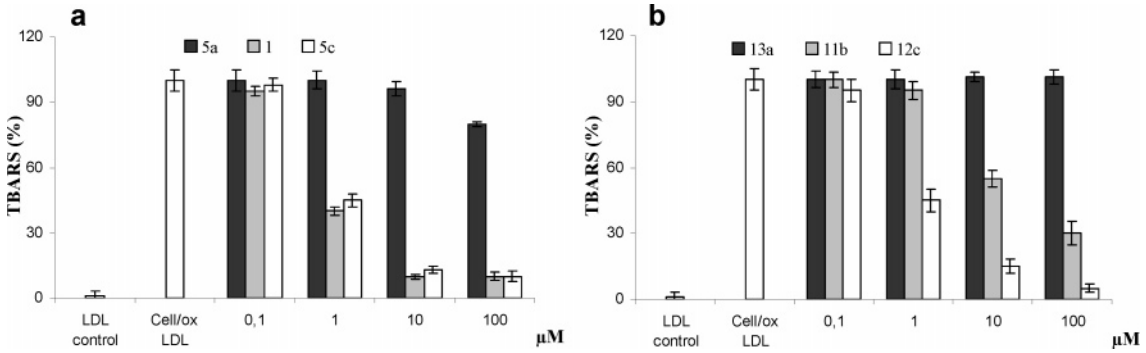


Figure 1. Effect of cinnamic compounds (a) and phosphonocinnamic compounds (b) on HMEC-1-mediated LDL oxidation, evaluated by TBARS formation. Native LDL (100 μg/mL) were incubated with subconfluent HMEC-1 in RPMI 1640, in the presence of 1 μM Cu^{2+}, and in the presence (or absence) of cinnamic compounds. After 6 h contact, the TBARS content was determined in the culture medium as reported in the Experimental section. Results are expressed as % of TBARS formed in LDL oxidized by HMEC-1 in absence of antioxidant (i.e. 20 ± 5 nmoles TBARS/mg apoB protein). The TBARS content of control native LDL (i.e. native LDL in the presence of 1 μM Cu^{2+} without HMEC-1 cells) was evaluated to verify the lack of endogenous oxidation in LDL and was around 0.5 ± 0.15 nmol TBARS/mg apoB protein. Each bar represents the mean for four separate determinations.

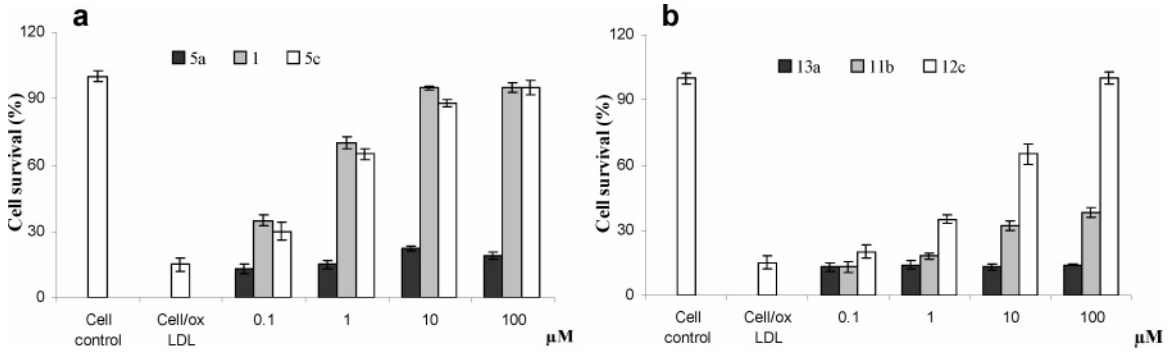


Figure 2. Cytotoxic effect of LDL oxidized by HMEC-1 in the presence or absence of cinnamic (a) and phosphonocinnamic compounds (b). The cytotoxicity of cell-oxidized LDL (in the presence or absence of antioxidants) was evaluated after 24 h of pulse on HMEC-1, by the MTT assay. The results are expressed as % of the unstimulated control (cell incubated in LDL-free medium). Each bar represents the mean for four separate determinations.

Preliminary studies were undertaken on all synthesized compounds in order to determine the dose effect on LDL oxidation as measured by the TBARS formation and the oxLDL cytotoxicity as measured by the MTT assay. Figures 1a and 2a represent selected results for the carbonylated cinnamic derivatives **5a**, **5c**, and **1**. Compounds **1** and **5c** present good antioxidant and cytoprotective properties starting from 1 μM to 100 μM. The same trends were observed concerning the phosphorylated analogues **13a**, **11b**, and **12c** (Figures 1b, 2b), illustrating respectively antioxidant activities and protective effects of the three phosphonocinnamic compounds at concentrations ranging from 0.1 μM to 100 μM. Figure 1 shows that the antioxidant effect is strong for **1**, **5c**, and **12c** derivatives at 10 μM and becomes really stronger for **12c** at 100 μM. The same trends were observed for the cytoprotective effect (Figure 2).

Synthesized antioxidants could either share both radical scavenger and metal chelator activities, or show antioxidant activity only as metal chelators, since LDL oxidation by HMEC-1 was done in the presence of copper (1 μM). Nevertheless, comparatively the same antioxidant effects have been obtained in absence of copper (at 10 μM (100 μM), 18 (8) % and 25 (10) % of TBARS formed in oxidized LDL for compounds **5c** and **12c** respectively), suggesting that a metal chelator effect, though not excluded, is not the only antioxidant activity elicited by these agents.

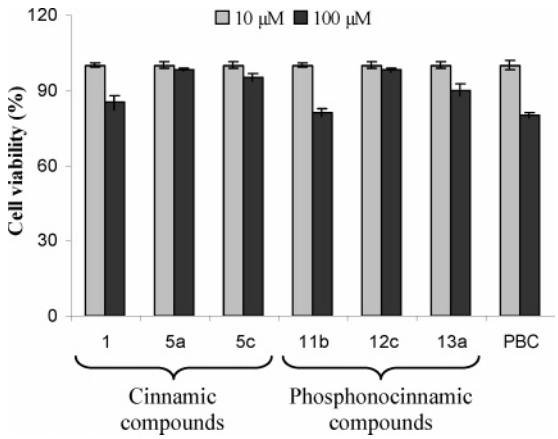


Figure 3. The self-toxicity of cinnamic, phosphonocinnamic compound and probucol (PBC) was tested on HMEC-1 by the MTT assay, after incubation of cells for 24 h in fresh RPMI medium containing or not 10 or 100 μM of antioxidants.

To choose the optimal concentration for testing the different compounds, their self-toxicity was also measured on HMEC-1 by MTT assay. As shown in Figure 3, a self-toxicity of these compounds was not observed until 10 μM, but became apparent and non negligible for some compounds at 100 μM. Therefore, further investigations were done at 10 μM.

LDL oxidation and protection by different antioxidants were evaluated by their relative electrophoretic

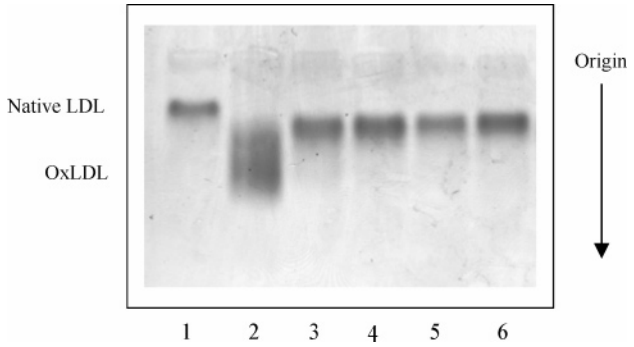


Figure 4. Relative electrophoresis mobility (REM) profiles of the compounds. LDL-containing culture media ± antioxidants were submitted to electrophoresis in agarose (0.7%). Lane 1, control native LDL; lane 2, LDL incubated for 6 h with HMEC-1; lane 3, same as 2 but in the presence of 10 μM probucol; lane 4, same as lane 2 but in the presence of 10 μM **7c**; lane 5, same as lane 2 but in the presence of 10 μM **12c**; lane 6, same as lane 2 but in the presence of 10 μM **1**.

Table 1. Antioxidant Effect of Cinnamic Compounds[a]

	TBARS, %[b]	MTT, %[c]
control	0 ± 5	100 ± 5
native LDL	100 ± 5	15 ± 5
1	9.6 ± 1.5	93.6 ± 1.1
2	100 ± 1.2	20.7 ± 3.5
3	61.3 ± 3.5	48.7 ± 3.1
5a	95.6 ± 4.5	22.6 ± 1.5
5b	40.3 ± 2.5	69.3 ± 5.5
5c	12.7 ± 2.1	88.7 ± 2.1
5d	109 ± 6.6	21.7 ± 3.1
α-tocopherol	55 ± 2.1	29 ± 2.8
probucol	10 ± 0.7	92 ± 11.3

[a] The different compounds were tested at 10 μM on LDL oxidation (TBARS) and subsequent cytotoxicity (MTT) elicited by HMEC-1. [b] The results are expressed as % of TBARS formed in LDL oxidized by cells in the absence of antioxidants. [c] The results are expressed as % of viability in the unstimulated control (MTT done on control cells incubated in fresh RPMI culture medium in the absence of LDL and antioxidant).

mobility in agarose gel (Hydragel, Sebia, France). In Figure 4, the REM of HMEC-1 oxidized LDL was increased indicating that oxLDL move faster than the native one. All the antioxidants tested here were found highly protective on LDL REM, in the same range than probucol, tested at the same concentration of 10 μM. These data confirm the strong protective effect of the compounds synthesized **1**, **7c**, and **12c** on cell-mediated oxidation.

The results (antioxidant and cytoprotective effect) for all compounds tested at 10 μM are reported in Tables 1 and 2.

The antioxidant and cytoprotective effects were first evaluated for the cinnamides **1**−**3**. Compounds **2** and **3** present the same amide functionality and differ on the aromatic ring substitution. Cinnamide **3** possessing only a 3-OMe group was a better antioxidant and had a better cytoprotective effect than the 3-OMe,4-OMe-substituted analogue **2** (61.3%/100% for TBARS and 48.7%/20.7% for MTT assays).

On the contrary, amide **1**, possessing a 4-OH functionality and an adjacent 3-OMe group, presented the best results in this series, i.e., 9.6% of TBARS activity and 93.6% MTT reduction. This result was comparable to recent findings concerning the inhibitory effects on LDL oxidation by cinnamic and caffeic acid derivatives.[17] Note that compound **1** exhibited an antioxidant activity comparable to that observed with probucol, and much higher than that observed with vitamin E, under the same experimental conditions.

Table 2. Antioxidant Effect of Phosphonocinnamic Compounds

	TBARS, %	MTT, %
control	0 ± 5	100 ± 5
native LDL	100 ± 5	15 ± 5
7a[a]	77.6 ± 8.5	65.3 ± 8.1
7b	32.3 ± 7.0	75.7 ± 5.0
7c	13 ± 0.7	81 ± 2.8
11a	52.7 ± 3.2	25.7 ± 3.8
11b	51.0 ± 5.3	32.3 ± 3.1
11c	10.7 ± 0.6	46.7 ± 2.1
10d	75.3 ± 8.1	20.7 ± 2.5
10e	102 ± 3.5	10.3 ± 0.6
12b	50 ± 3.2	25 ± 2.2
12c	12 ± 1.4	93 ± 2.8
13a	101 ± 3.0	13.3 ± 1.5
13b	52.7 ± 3.2	21.7 ± 4.0
13c	16 ± 1.7	45 ± 2.3
α-tocopherol	55 ± 2.1	29 ± 2.8
Probucol	10 ± 0.7	92 ± 11.3

[a] The compounds are tested in the conditions defined in Table 1.

The thioesters **5a-d** effects were then determined. Again, the presence of the electron-donating 4-OMe group on the aromatic ring (compound **5d**) resulted in extremely poor (if no) activity (109% on TBARS and 21.7% on MTT assays). Among compounds **5a**−**c**, all possessing a 4-OH functionality on the aromatic ring, derivative **5c** presented the best results (12.7% and 88.7% on TBARS and MTT activities respectively).

The phosphonated analogues of cinnamic acids were then examined (Table 2). Phosphonocinnamic diesters **7a**−**c** and phosphonothioesters **11a**−**e** presented the same trends as the carbonylated counterparts. Compounds **7c** and **11c**, possessing 3-OMe and 5-OMe groups surrounding the 4-hydroxy function on the aromatic ring, showed the best results. Phosphonothioesters **10d** and **10e** possessing only electron donor groups in the 4 and (or not) 5 position of the aromatic ring showed very poor antioxidant (and thus protective) effects on LDL oxidation by HMEC-1.

Monophosphonic acids **13a**−**c**, follow the same trends as before. Compound **13c** presents the best results. Among the two fluorophosphonocinammic derivatives tested, again, the 4-OH,3-OMe,5-OMe-substituted compound **12c** presented the best results (12% TBARS and 93% MTT assays).

Considering compounds **5**, **7**, **11**, **12**, **13** of the most active **b** and **c** series and also compound **1**, some comments could be done. In all comparative cases, the **c** series compounds (i.e. sinapoyl moiety) presented a much better antioxidant activity as measured by TBARS assay. They also present a variously better cytoprotective effect, the most optimal differentiation being between the fluorophosphonocinnmic derivatives **12b/12c**. Their better inhibitory activities are to be related to the presence of the conjugated α,β-ethylenic moiety[27] and electron-donating substituents on the aromatic ring (3-OMe or 3,5-OMe). The phenolic adjacent methoxy groups may provide stability to the phenoxy radical[10,28] and also weaken the phenolic O−H bond; these additive effects increasing the antioxidant potentiality of the compounds of the **c** versus **b** series.[29,30] Considering

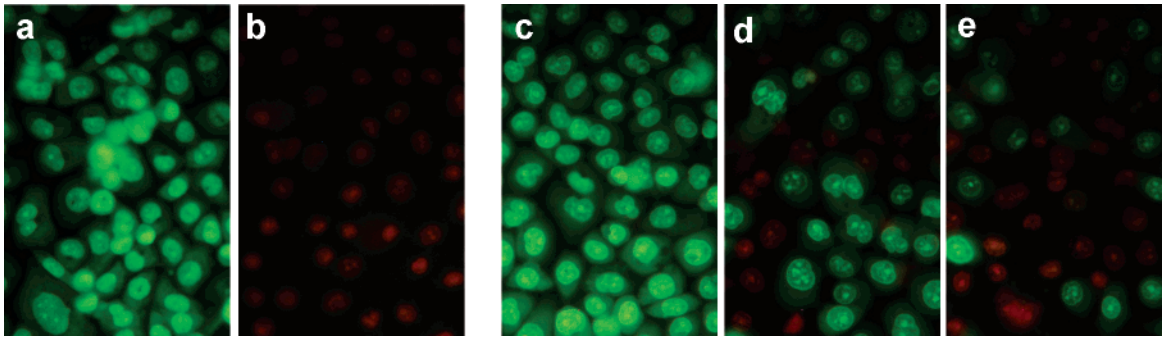


Figure 5. Microscopic pictures of HMEC-1 either control (a) or incubated for 24 h in the presence of LDL oxidized by cells as indicated in the legend to Figure 1, in absence (b), or in the presence of antioxidants **7c** (c), **12c** (d), and **13c** (e). At the end of the pulse period, HMEC-1 were labeled with Syto13/propidium iodide, as indicated in the Experimental Section.

compounds possessing the same aromatic moiety, some comments can be done. For the feruloyl or the sinapoyl series (**b** or **c** series), the antioxidant activities as measured by TBARS assay were comparable for the **c** series (10.7−16%) and more variable for the **b** series (32.3−52.7%) with an important difference concerning the amide **1** (9.6%). So, except this latter compound, the TBARS assay was not influenced in a significant manner by modifying the side chain of the aromatic ring. It should be noted that the phosphonoacids **13b** and **13c** presented the less better results (52.7% and 16%, respectively). Nevertheless, they were in the same range of activities as the other compounds that were more lipophilic, indicating that the degree of lipophilicity, usually influencing the antioxidant properties, was not determinative for the potentiating preexisting antioxidant activity of the tested molecules.[31]

The results obtained for the cytoprotective effect of the compounds of **b** and **c** series were shown to be more "side chain" dependent. For the feruloyl compounds again, the amide functionality presented the best results while the thioester **5b** and the phosphonate **7b** were approaching the value of the amide **1** and were found to be 2−3-fold more cytoprotective than their **11**−**13** counterparts. Finally, among the sinapoyl derivatives tested, we observed a 2-fold side chain dependence between compounds **5c**, **7c**, **12c** and compounds **11c**, **13c**, the former showing a good cytoprotective effect.

The cytoprotective effect of the most active agents (on HMEC-1-mediated LDL oxidation and subsequent toxicity) was also evidenced by a microscopic observation of cells incubated either with LDL alone, or LDL plus antioxidant, and labeled by permeant (Syto 13) or vital (propidium iodide) DNA probes (Figure 5).

The permeant Syto 13 is able to label normal living control cells as well as dying cells (by necrosis or apoptosis), whereas propidium iodide is normally excluded from normal living cells, in which the plasma membrane is intact. In contrast, necrotic cells (resulting from primary necrosis, or after apoptosis) exhibit alterations of the plasma membrane and are labeled by propidium iodide.[29] As shown in Figure 5b, LDL oxidized by HMEC-1 (in the absence of antioxidant) were found highly cytotoxic, by comparison to control unstimulated cells (Figure 5a). This cytotoxic effect resulted from apoptosis evidenced by strong cytoplasm and nucleus condensation, as previously reported in these cells.[32] The nuclei were massively labeled by propidium iodide, indicating the occurrence of necrosis

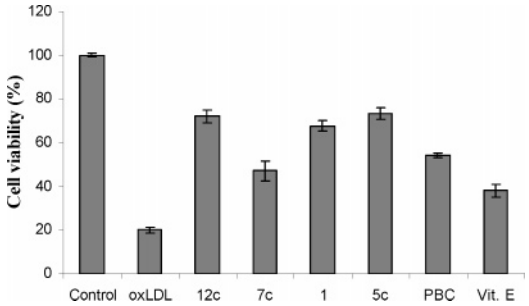


Figure 6. Direct cytoprotective effect of some cinnamic and phosphocinnamic compounds against the cytotoxicity of (previously) oxidized LDL. A fixed concentration of oxidized LDL (200 μg apoB/ml of LDL oxidized by UV irradiation as described in the Experimental Section) were added simultaneously to the culture medium with 10 μM of the different antioxidants. The cell viability was determined after 24 h by the MTT test. The results are a mean (±SEM) of three separate experiments.

that may be post apoptotic necrosis followed by primary apoptosis, as reported.[32] Like that observed with MTT experiments, **7c** and **12c** compounds were highly efficient to protect against the toxicity of LDL, in contrast to **13c** which was much lesser active (Figure 5c−e).

In another set of experiments, we tested the direct cytoprotective effect of the newly synthesized molecules against the cytotoxicity of oxidized LDL. This was studied by incorporating various concentrations of the tested drugs in the culture medium of HMEC-1 simultaneously with previously oxidized LDL (i.e. 200 μg/mL of LDL oxidized by UV irradiation[29] in the absence of any additive). All the tested compounds increased significantly the resistance of cells against the cytotoxic effect of oxidized LDL (at the tested concentration of 10 μM, 65 to 70% living cells vs less than 20% in the presence of oxidized LDL alone) and were found more efficient than probucol and vitamin E (up to 55 and 40% of living cells, respectively) (Figure 6). These data demonstrate that the newly synthesized cinnamic and phosphocinnamic compounds exhibit two types of protective effects: (1) an effective antioxidant activity which inhibits LDL oxidation outside the cell, and (2) a direct cytoprotective effect which prevents the cytotoxicity of oxidized LDL inside the cell.

Conclusion

A series of cinnamic and phosphonocinnamic derivatives were synthesized and evaluated for their antioxidant properties. Some of them possess strong inhibitory

potencies against lipid peroxidation and are very efficient in preventing LDL oxidation by cultured vascular cells (HMEC-1). The protective effect of these compounds evaluated through TBARS content in LDL was shown to be governed mainly by the aromatic ring substitution while viability experiments (MTT) showed also a "side chain" dependence. More electron-donating substituents surrounding the necessary 4-OH group contribute in increasing effectively the antioxidant and cytoprotective effects of these compounds. Concerning the structural modifications α to the ethylenic moiety of the derivatives studied, the amide **1**, the thioester **5c**, the phosphonoester **7c**, and the phosphonofluoridate **12c** presented the best results. Indeed, these compounds were found as efficient as probucol against LDL oxidation and subsequent toxicity, and much more efficient than vitamin E. Moreover, preliminary results indicate that these agents are even more efficient than probucol in protecting cultured cells against the cytotoxicity of oxidized LDL (oxidized in vitro by UV radiations as reported).[29] The relevance of the cell-induced oxidation model fits well with the mechanisms involved in the early events occurring within the vascular wall, namely LDL oxidation by vascular cells, that leads to their uptake by macrophages and the formation of foam cells and renders them cytotoxic.[32] This model is quite suitable to discriminate between the antioxidant and cytoprotective properties of the different newly synthesized agents and allowed selection of several highly efficient agents able to protect (1) against LDL oxidation and their subsequent toxicity, and (2) against the cytotoxicity of LDL oxidized in the absence of any additive. In conclusion, these molecules exhibit two types of (potentially) antiatherogenic properties, (1) an antioxidant effect allowing the prevention of cell-mediated oxidation of LDL (occurring within the plaque and involved in the development of early atherosclerotic lesions), and (2) a direct cytoprotective effect against oxidized LDL-mediated cytotoxicity possibly involved in vascular cell apoptosis and plaque rupture. Further investigations are needed in order to evaluate their complete antioxidant profile and the structure−activity relationship particularly, for the most potent compounds **1**, **5c**, and **12c**. The influence of each factor contributing to the observed global antioxidant effect, particularly metal chelation and radical scavenging, will be studied in order to get a better insight in the mode of action of these compounds. The possibility for these compounds to possess scavenging properties against the oxidized LDL derivatives will be evaluated. In vivo studies will be undertaken for the development of more potent agents that could be of great interest for the treatment and the prevention of atherosclerosis and oxidative injuries. Finally, considering the results obtained, new compounds possessing the optimal "side chain" among the different ones we have investigated and also bulkier groups surrounding the phenolic one will be synthesized and evaluated.

Experimental Section

Materials. All chemicals are of the highest commercially available purity. 2-Thiobarbituric acid and diagnostic kits for total cholesterol, LDL, and triglyceride determination are purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents are purchased from Aldrich-Chemie (Steinheim, Germany). Commercial 2-piperidinemethanol (Aldrich) corresponds to the racemate.

Synthesis. Organic solvents were purified when necessary by methods described by D. D. Perrin, W. L. F. Armarengo, and D. R. Perrin (*Purification of Laboratory Chemicals*; Pergamon: Oxford, 1986) or purchased from Aldrich Chimie.

Melting points (mp) were obtained on a Buchi apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer 1725 infrared spectrophotometer, and the data are reported in inverse centimeters. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a Bruker AC 250 MHz and a Bruker AC 400 MHz spectrometer. Chemical shifts were reported in parts per million (‰) relative to tetramethylsilane (TMS), and signals are given as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were recorded on on an R 10−10 C Nermag (70 eV) quadripolar spectrometer. Elemental analyses were performed with a Perkin-Elmer 2400 CHN analyzer.

(*E*)-3-(3,4-Dimethoxyphenyl)(pyridin-2-yl)acrylamide 2. *N*,*N*-Dimethylchloromethyleniminium chloride was generated in situ from oxalyl chloride and DMF and used immediately after preparation. Oxalyl chloride (15 mmol) was slowly added to anhydrous DMF (5.03 mmol) dissolved in dichloromethane (8 mL) at 0 °C under a nitrogen atmsophere. After stirring for 30 min at 0 °C, solvent and oxalyl chloride were removed in vacuo to give quantitatively the iminium salt. Pyridine (5 mmol) was added to the iminium salt dissolved in THF (20 mL) at −30 °C, and 3, 4-dimethoxycinnamic acid in THF (10 mL) was added.

1-Aminopyridine lithium salt generated from 1-aminopyridine and butyllithium at −30 °C in THF was added to the previous mixture and stirred for 1 h. After being raised to room temperature, the mixture was poured into water, neutralized with aqueous HCl (1 M), and extracted with ethyl acetate. Organic layer was dried over magnesium sulfate and concentrated in vacuo. Silica gel chromatography (CH_2Cl_2:EtOAc 95:5) gave compound **2** in 70% yield. ^{1}H NMR δ_H 3.93 (s, 6H, CH_3O), 6.49 (d, 1H, J = 15.5 Hz, C=CH−CO), 6.88 (d, 1H, J = 8.3 Hz, ortho H), 7.12 (m, 2H, H_3 pyridine, ortho H), 7.15 (dd, 1H, J = 8.25 Hz, J = 1.8 Hz, meta H), 7.73 (d, 1H, J = 15.5 Hz), 7.77 (m, H_4 pyridine), 8.28 (d, 1H, J = 4.87 Hz, H_5 pyridine), 8.40 (d, 1H, J = 8.55 Hz, H_6 pyridine), 8.93 (bm, 1H, NH). ^{13}C NMR δ_C 55.9 (s, 1C, CH_3), 56.0 (s, 1C, CH_3), 109.9 (s, 1C, C_3 pyridine), 111.1 (s, 1C, ortho C), 114.5 (s, 1C, C_5 pyridine), 118.3 (s, 1C, meta C), 119.6 (s, 1C, C=*C*-CO), 127.4 (s, 1C, ortho C), 127.4 (s, 1C, Cq), 138.9 (s, 1C, C_4 pyridine), 143.1 (S, 1C, *C*=C−CO), 147.1 (s, 1C, C_6 pyridine), 151.0 (s, 2C, C−O), 151.7 (s, 1C, C_2 pyridine), 164.5 (s, 1C, CONH). MS (*m*/*z*) 285 (M^+H^+). Anal. ($C_{16}H_{16}N_2O_3$), C, H, N.

General Procedure for the Preparation of Cinnamic Derivatives. The synthesis of the cinnamic thioesters derivatives **5a**−**e** was carried out by stirring a mixture of the corresponding carboxylic acid (4 mmol), *N*-acetylcysteamine (2 mmol), DMAP (2 mmol), and EDC (4.6 mmol) in dichloromethane (10 mL) for 24 h at room temperature. The reaction mixture was washed with diluted aqueous HCl. Aqueous layer was extracted with dichloromethane (3% 10 mL). Organic layers were brought, dried over anhydrous sodium sulfate, and evaporated in vacuo. Silica gel chromatography (EtOAc) gave the title compound.

(*E*)-S-2-Acetamidoethyl-3-(4-hydroxyphenyl)prop-2-enethioate 5a was obtained with EtOAc as eluent in 76% yield. R_f 0.20 (AcOEt). IR (KBr) 1765, 3400 (O−H), 1646 (C=O), 1256 (C−N). ^{1}H NMR δ_H 1.92 (s, 3H, CH_3CO), 3.08 (t, 2H, J = 6.6 Hz, CH_2S), 3.36 (td, 2H, J = 6.6 Hz, 6.6 Hz, CH_2NH), 6.57 (d, 1H, J = 15.7 Hz, C=CH−CO), 6.80 (d, 2H, J = 8.6 Hz, ortho H), 7.41 (d, 2H, J = 8.6 Hz, meta H), 7.53 (d, 1H, J = 15.7 Hz, CH=C−CO). ^{13}C NMR δ_C 23.4 (s, 1C, CH_3CO), 29.4 (s, 1C, CH_2S), 40.7 (s, 1C, CH_2NH), 117.3 (s, 2C, C-3+C-5), 122.6 (s, 1C, C=*C*−CO), 126.8 (s, 1C, C-1), 131.8 (s, 2C, ortho C), 142.9 (s, 1C, *C*=C−CO), 161.5 (s, 1C, para C), 173.5 (s, 1C, CONH), 191.9 (s, 1C, COS). MS (*m*/*z*) 265 (M^+). Anal. ($C_{13}H_{15}NO_3S$), C, H, N.

General Procedure for the Preparation of Silylated Phosphonates Diethyl Esters. The phosphonate diethyl ester **7a**−**c** (3.9 mmol), prepared as described by Kennedy, was dissolved in anhydrous DMF (15 mL). Imidazole (7.9 mmol), DMAP (3.9 mmol), and *tert*-butyldiphenylsilyl chloride (5.9 mmol) were added. The reaction mixture was stirred for 16 h at room temperature, and brine was added. The solution was extracted with ether. Etheral solution was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting oil was purified by flash column chromatography (EtOAc:Et_3N 99:1) to give **8a′**−**c′**.

(*E*)-Diethyl 2-(4-terbutyldiphenylsilyloxyphenyl)vinylphosphonate 8a′ was obtained with EtOAc:Et_3N (99:1) as eluent in 67% yield (oil). R_f 0.50 (AcOEt). IR (KBr) 3071 (C−H), 2959 (C−H), 1617 (C═C), 1270 (P═O), 1170 (P−O−C). ^{1}H NMR δ_H 1.11 (s, 9H, tBu), 1.31 (t, 6H, J = 7.2 Hz, CH_3), 4.08 (qd, 4H, J = 7.2 Hz, CH_2O), 6.01 (dd, 1H, J = 17.7 Hz, CH−P), 6.77 (d, 2H,J = 8.5 Hz, ortho H), 7.25 (d, 2H, J = 8.5 Hz, meta H), 7.38 (m, 7H, CH + Har), 7,71 (d, 4H, Har). ^{13}C NMR δ_C 16.3 (d, 2C, J = 18.5 Hz, CH_3), 19.5 (s, 1C, C−q tBu), 26.5 (s, 3C, CH_3 tBu) 61.8 (d, 2C, J = 5.4 Hz, CH_2O), 111.0 (d, 1C, J = 191.8 Hz, C−P), 120.1 (s, 2C, ortho C), 127.7 (s, 4C, C−o), 128.0 (s, 2C, meta C), 129.3 (s, 2C, C−p), 131.4 (s, 3C, C_{ar}, C_q-ar), 135.4 (s, 4C, C−m), 148.7 (d, 1C, J = 6.6 Hz, C═C−P), 157.7 (s, 1C, para C). ^{31}P NMR δ_P 20.54 (s, P−OEt). MS (EI) *m/z* 494. Anal. ($C_{28}H_{35}O_4PSi$), C, H.

General Procedure for the Preparation of Phosphono Thioester Derivatives. The diethylphosphonate (**8a′**−**c′**, **7d**,**e**) (1.8 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL), and oxalyl chloride (8.6 mmol) was added. The solution was stirred for 20 h at room temperature. The volatile components were evaporated under reduced pressure. The resulting phosphonochloridate (**9a′**−**c′**, **9d**,**e**) was dissolved in CH_2Cl_2 (1 mL) and added dropwise to the mixture of *N*-acetylcysteamine (3.9 mmol) and Et_3N (3.8 mmol) dissolved in CH_2Cl_2 (1 mL). The resulting solution was stirred at room temperature for 16 h, and CH_2Cl_2 was removed. The crude material was filtered and washed successively with $NaHCO_3$ (saturated) and water. The organic layer was dried over Na_2SO_4. After removal of the solvent, the crude mixture was purified by flash column chromatography (EtOAc).

The silylated phosphonothioesters derivatives **10a′**−**c′**, **10d**,**e** and the phosphonic acid derivatives **13a**−**c** have been described previously.[23]

(*E*)-S-2-Acetamidoethyl *O*-ethyl 2-(4-methoxyphenyl)vinylphosphonate 10d was obtained with EtOAc as eluent in 68% yield (oil). R_f 0.35 (AcOEt). IR (KBr) 3286 (N−H), 3074 (C−H), 2982 (C−H), 1661 (C═O), 1603 (C═C), 1212 (P═O),-1174 (P−O−C). ^{1}H NMR δ_H 1.37 (t, 3H, J = 7.0 Hz, CH_3), 1.98 (s, 3H, CH_3CO), 2.95 (m, 2H, CH_2−S), 3.52 (m, 2H, CH_2−N), 3.83 (s, 3H, CH_3O), 4.20 (m, 2H, CH_2O), 6.23 (dd, 1H, J = 17.4 Hz, J = 23.8 Hz, CH−P), 6.89 (d, 2H, J = 7.3 Hz, meta H), 7.19 (s, 1H, NH), 7.42 (m, 1H, C*H*═CH−P), 7.47 (d, 2H, J = 7.3 Hz, ortho H). ^{13}C NMR δ_C 16.3 (d, 1C, J = 6.7 Hz, CH_3), 23.0 (s, 1C, *C*H$_3$CO), 29.2 (s, 1C, CH_2−S), 40.6 (s, 1C, CH_2−N), 55.4 (s, 1C, CH_3O), 62.2 (d, 1C, J = 6.5 Hz, CH_2O), 114.3 (s, 2C, meta C), 114.9 (d, 1C, J = 155.0 Hz, C═*C*-P), 127.0 (d, 1C, J = 23.4 Hz, C_{q-ar}), 129.7 (s, 2C, ortho C), 147.7 (d, 1C, J = 5.3 Hz, *C*═C−P), 161.6 (s, 1C, para C), 170.7 (s, 1C, CONH). ^{31}P NMR δ_P 45.84 (s, P−S). MS (CI) *m/z* 344 (MH^+). Anal. (C15H22NO4PS), C, H, N.

General Procedure for the Preparation of Fluorophosphonocinnamic Derivatives. Phosphonocinnamic thioester (0.64 mmol) was dissolved in anhydrous THF (4 mL) under N_2 flux. Et_3N (2.1 mmol) and Et_3N·3HF complex (1.1 mmol) were added, and the reaction mixture was stirred for 2 h at room temperature. The solvent was removed in vacuo, and the residue was purified via flash column chromatography (EtOAc).

(*E*)-O-Ethyl 2-(4-hydroxyphenyl)vinylphosphonofluoridate 12a was obtained with EtOAc as eluent in 66% yield (oil). R_f 0.64 (AcOEt). IR (KBr) 3233 (O−H), 3020 (CH), 1604 (C═C), 1585, 1515 (C═C), 1230 (P═O), 1170 (P−O−C). ^{1}H NMR δ_H 1.41 (t, 3H, J = 7.0 Hz, CH_3), 4.30 (qd, 2H, J = 7.0 Hz, CH_2O), 6.03 (dd, 1H, J = 17.5 Hz, J = 20.4 Hz, C═C*H*-P), 6.92 (d, 2H, J = 8.7 Hz, ortho H), 7,37 (d, 2H, J = 8.7 Hz, meta H), 7.54 (dd, 1H, J = 17.5 Hz, J = 25.0 Hz, C*H*═CH−P), 8.54 (s, 1H, OH). ^{13}C NMR δ_C 174 (d, 1C, J = 6.0 Hz, *C*H$_3$-CH_2), 65.0 (d, 1C, J = 6.2 Hz, CH_2O), 105.5 (dd, 1C, J = 208.4 Hz, J = 31.5 Hz, C═*C*−P), 117.3 (s, 2C, ortho C), 126.9 (d, 1C, J = 25.7 Hz, C_q-ar), 131.3 (s, 2C, meta C), 153.3 (dd, 1C, J = 7.8 Hz, J = 4.7 Hz, *C*═C−P), 161.1 (s, 1C, para C). ^{31}P NMR δ_P 20.53 (d, J_F = 1024 Hz, P−F). ^{19}F NMR: δ_F 11.17 (d, J = 1024 Hz, F−P). MS (CI NH_3, MNH_4^+) *m/z* 248. Anal. ($C_{10}H_{12}$-FO_3P), C, H.

Pharmacological Methods. Cell Culture. HMEC-1 line was obtained from CDC (Atlanta, US)[24] and was a generous gift from Dr. F. Trottein (Institut Pasteur, Lille). Cells were grown in MCDB-131 supplemented with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and 24 h before LDL incorporation, cells were starved in serum-free RPMI medium.

LDL Isolation and Oxidation. Effect of Antioxidants. LDL were isolated by ultracentrifugation from the pooled plasma of healthy normolipidemic human subjects and dialyzed against PBS containing 100 μmol/L EDTA, as previously indicated.[29]

To evaluate cell-mediated LDL oxidation, HMEC-1 were seeded in 24 multiwell plates. The standard culture medium (on sparse proliferative cells) was removed and replaced by serum-free RPMI-1640 containing native LDL (100 μg apoB/mL) and incubated at 37 °C for 6 h with cells or in cell-free medium, as previously used.[33] The compounds in DMSO were added to the culture medium at variable concentrations, just before beginning the oxidation and in duplicate. At the end of the incubation, LDL-containing medium was immediately used for determining thiobarbituric acid reactive substances (TBARS) formation using the fluorimetric procedures of Yagi.[34] Alternatively, the relative electrophoretic mobility of LDL oxidized by HMEC-1 in the presence or absence of antioxidant was evaluated on Hydragel (Sebia, France).

Alternatively, to test the direct cytoprotective effect of newly synthesized molecules against oxidized LDL-mediated cytotoxicity (i.e. LDL oxidized in the absence of antioxidants), we used LDL oxidized by (UV + copper/EDTA) as previously described:[29] briefly, LDL solution [2 mg apoprotein B (apoB)/mL, containing 2 μmol/L $CuSO_4$] was irradiated for 2 h, as a thin film (5 mm) in an open beaker placed 10 cm under the UV-C source (HNS 30W OFR Osram UV-C tube, λ_{max} 254 nm, 0.5 mW/cm^2 determined using a Scientech thermopile Model 360001). At the end of the irradiation, aliquots were taken up for analyses, and oxidized LDL (200 μg apoB/mL under standard conditions) was immediately incorporated in the culture medium.

Cell Viability. Cells were incubated in the presence of LDL (previously oxidized by cells for 6 h in the presence of variable concentrations of antioxidants) until the cell viability was evaluated at the indicated time (24 h pulse period, under standard conditions). To test the direct cytoprotective effect of antioxidants, cells were incubated for 24 h with UV/copper oxidized LDL (200 μg/mL) in the presence of variable concentrations of the newly synthesized molecules. The cell viability was determined by using the MTT test (based on the reduction of diphenyltetrazolium bromide salt by mitochondrial enzymes, according to Price and McMillan),[35] and the microscopic observation of morphologically apoptotic cells after fluorescent double staining with SYTO-13/propidium iodide, under the previously described conditions.[29] Briefly, cells were incubated with two vital fluorescent dyes, 0.6 μM SYTO-13 (a permeant DNA intercalating green-colored probe) and 15 μM propidium iodide (a nonpermeant intercalating red probe). Normal nuclei exhibited a loose chromatin colored in green by SYTO; apoptotic nuclei exhibited condensed green-colored chromatin and/or fragmentation. Postapoptotic necrosis, usually considered as apoptotic cell death, was characterized by nuclei exhibiting the same apoptotic morphological features but red-colored.

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Supporting Information Available: Spectroscopic and analytical data for compounds **5b**−**e**, **8b′**, **8c′**, **12b**, **12c**, and **12e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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