Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 411–418

BIOORGANIC & MEDICINAL CHEMISTRY

Medium Sized Lactones with Hypolipidaemic and Antioxidant Activity: Synthesis and Biological Evaluation of Promising Dual-action Anti-atherosclerosis Drugs

Claudia Baldazzi,[b] Francesca Calderoni, Emanuela Marotta,[a] Silvano Piani,[b] Paolo Righi,[a] Goffredo Rosini,[a,*] Stefano Saguatti,[b] Roberta Tiozzo,[c] Sebastiano Calandra[c] and Francesca Venturelli

[a]*Dipartimento di Chimica Organica, "A. Mangini," Università di Bologna, Viale Risorgimento 4, 40136 Bologna, Italy*
[b]*Dipartimento Chimico e Biologico, Alfa-Wassermann S.p.A., Via Ragazzi del '99, 5, 40133 Bologna, Italy*
[c]*Dipartimento di Scienze Biomediche, Università di Modena, Via Campi 287, 41100 Modena, Italy*

Received 15 July 1998; accepted 20 October 1998

Abstract—Macrocyclic lactones **1a–b** have been synthesized and their potential therapeutic value evaluated. The key structural feature of these active 'chimera' compounds is the 12-membered lactone ring that brings together the well-known polysubstituted hydroquinone moiety of antioxidants and the α,α-dimethyl substituted acyl residue of gemfibrozil. Lactones **1a–b** showed better activity than probucol, a classical phenolic antioxidant, in preventing the Cu^{++}-induced oxidative modification of human LDL. The hypolipidaemic activity of the new lactones, evaluated as the inhibition of lipids biosynthesis in Hep-G_2 cells, was comparable to that of gemfibrozil. These features, added to the lack of cytotoxicity, make this new class of medium sized lactones promising dual-action drugs useful as anti-atherosclerosis agents.

Introduction

Atherosclerosis is a process of degeneration of the arterial wall and is associated with many vascular pathologies like ischaemic cardiopathies (angina pectoris and myocardial infarction) and cerebral thrombosis, main causes of death in the industrial countries. Many efforts have been carried out to understand the aetiology of this pathology of wide diffusion and importance and to find out the possible therapeutic treatments.

Atherosclerosis is recognized to be a process with composite aetiology that involves in varied measure different factors and cell types. Endothelial damages represent the main event in the genesis of the atheroma[1] and are promoters of the progress and the complication of the atheromatous plaque in the high serum rates of lipids and cholesterol that characterize the hyperlipidaemias and that often aggravate the diabetic pathologies.[2] The pharmacological interventions aimed at lowering the plasma levels of cholesterol and low-density lipoproteins (LDL) have proved to be effective in the prevention of the vascular coronary pathologies and in the treatment of atheromatous plaques.[3,4] The atherogenic risk associated with the LDL appears to be related not only to their plasma concentration but also to their qualitative characteristics. The possible modifications of the structure and composition of the LDL in the plasma or in the arterial wall can in fact make these macromolecules more atherogenic, namely more apt to trigger off and to stimulate the formation of the atheromatous plaque.[5] The oxidative processes that occur by action of oxidizing agents present in the plasma or in the endothelial cells of the arterial wall are of main importance among the modifications that the LDL can undergo in vivo. Many in vitro and in vivo experiments, together with the results of epidemiological investigations, support the hypothesis that this mechanism represents an important event in the development of atherosclerosis.[6–9] The LDL are particles not only enriched in cholesterol but also polyunsaturated fatty acids (PUFA) highly susceptible to undergo the peroxidation from which they are protected by the presence of many endogenous antioxidizers like tocopherols, carotenes, lycopene, and ubiquinol-10.

The oxidation of the LDL is a chain reaction of lipidic peroxidation lead by free radicals able to quickly

Key words: Antioxidants; radical scavengers; medium sized lactones; hypolipidaemics.
*Corresponding author. Fax: +39 051 644 3654; e-mail: rosini@ms.fci.unibo.it

0968-0896/99/$ - see front matter
PII: S0968-0896(98)00252-1

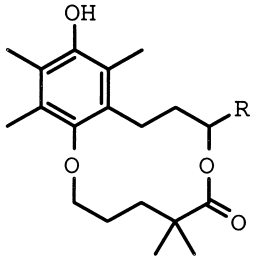
transform the PUFA into lipidic hydroperoxides (propagation period). The endogenous antioxidizers act as *chain-breaking* agents and interrupt the propagation by means of an effective scavenging of the peroxylic radicals and the concentration of hydroperoxides increases only when the endogenous antioxidizers are depleted (latency period).[10–12]

Chain-breaking antioxidants play a key role in the control of LDL and can be depicted as *anti*-atherosclerotic drugs,[13–15] as well as the lipid-soluble antioxidant vitamin E (tocopherol) is an effective inhibitor of LDL oxidation and have great importance in the prevention of atherosclerosis. Perspective studies on a large scale show a relation between the taking of considerable amounts of vitamin E and the lowering of the risk of vascular pathologies, both in men and in women.[16,17]

At present, therapy of disorders of the lipidic metabolism (the familial heterozygotic hypercholesterolaemia, the combined familial hyperlipidaemia, the dis-β-lipoproteinaemia, the familial hypertriglyceridaemia and the polygenic hypercholesterolaemia, all important causes of the atherosclerosis), is based on the use of drugs having hypolipidaemic activity like the fibrates (for instance gemfibrozil (Fig. 1), nicotinic acid, the resins sequestering the bile acids and the inhibitors of the enzyme HMGCoA reductase. The metabolic effect of these drugs is that of lowering the plasma concentration of cholesterol (LDL) and partly of the triglycerides (VLDL-LDL) through two mechanisms: an increase of the mediated receptor-removal of the LDL, and/or a lowering of the synthesis of the VLDL-LDL.[18]

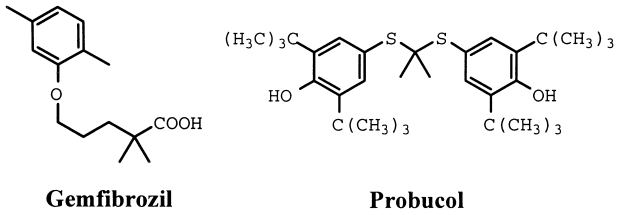
The above mentioned considerations point out that molecules having both hypolipidemic and antioxidant activity can represent a therapeutic development important in the treatment of the disorders of the lipidic metabolism and in the prevention of the atherosclerosis. Many hindered phenol-derived antioxidant with both of these features have been screened since the initial work on probucol (Fig. 1).

Recently, we have envisioned the possibility to build up new dual-action drugs by the combination of two important pharmacophores, the polysubstituted hydroquinone structure of classical antioxidants and the 2,2-gem-dimethyl substituted acyl structure typical of gemfibrozil, in a novel class of 'chimera' compounds with a 12-membered lactone ring as structural feature.



1a : R = CH_3
1b : R = C_2H_5

In this paper, we report the synthesis of macrocyclic lactones **1a**–**b** and some evidences that indicate they are endowed with antioxidant and hypolipidaemic properties.



Gemfibrozil **Probucol**

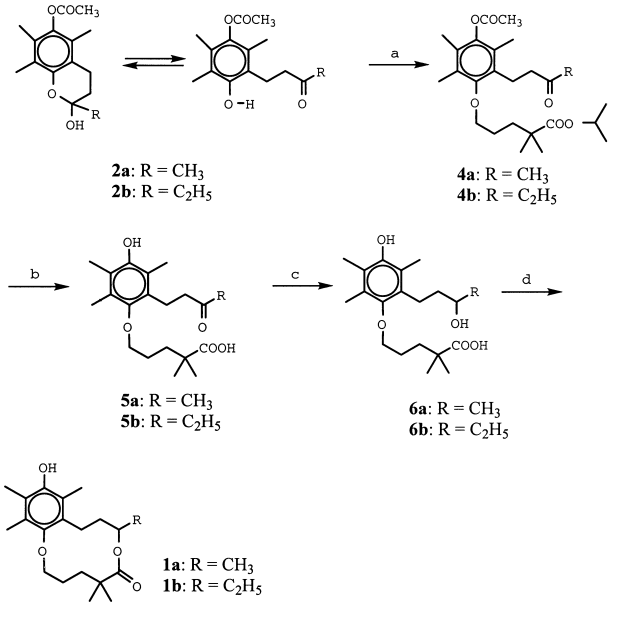
Figure 1.

In particular they seems to prevent and/or to delay the oxidative modification of the human LDL, i.e. they compete with the chain of propagation of the lipidic peroxidation through an effective scavenging of the peroxylic radicals.

The oxidative modifications of LDL represent, as it has already been seen, a key event in the pathogenesis of atherosclerosis and therefore the lactones **1a**–**b** can find useful therapeutic application in the treatment of the atherosclerosis and in the prevention of many vascular pathologies like ischaemic cardiopathies (angina pectoris and myocardial infarction), cerebral thrombosis, and periferal arteriopathies.

Results

The synthesis of lactones **1a**–**b** with a 12-membered ring including the hydroquinone moiety, joined by an ether bridge, was performed according to Scheme 1. Compounds **2a**–**b** were prepared following the well-established procedure of Scott,[19] which uses trimethylhydroquinone and alkyl vinyl ketones as inexpensive starting materials. The reaction of the sodium salts of **2a**–**b** with isopropyl 5-bromo-2,2-dimethylpentanoate (**3**) afforded compounds **4a**–**b**, which upon saponification



2a: R = CH_3
2b: R = C_2H_5

4a: R = CH_3
4b: R = C_2H_5

5a: R = CH_3
5b: R = C_2H_5

6a: R = CH_3
6b: R = C_2H_5

1a: R = CH_3
1b: R = C_2H_5

Scheme 1. Synthesis of 14-hydroxy-2,8-dioxabicyclo[10.4.0]hexadec-12,14,16-trien-7-ones **1a**–**b**. (a) 1. NaH/DMSO, benzene; 2. 2,2-dimethyl-5-bromopentanoic acid, isopropyl ester (3). (b) KOH/EtOH. (c) $NaBH_4$/MeOH. (c) 2,2′-dipyridyl disulfide, PPh_3, CH_3CN, $AgClO_4$.

Table 1. Latency period evaluation by TBARS method (As reported in Figure 2(a), the latency period for ethanol as negative control is 38 min)

Compd	Latency periods (min)		
	1 μM	5 μM	10 μM
Probucol	–	–	82
1a	73	120	> 180
1b	96	> 180	> 180

Table 2. Relative electrophoretic mobility (REM) on agarose gel of copper-oxidized LDL

Compd (μM)	REM (%) at 180 min	REM (%) at 360 min
Ethanol (–)	60	100
Probucol (10)	40	100
1a (10)	15	30
1b (10)	15	30

gave the corresponding acids **5a**–**b**. The carbonyl group on the other chain was converted into a hydroxy group by reduction and the resultant hydroxy acids (**6a**–**b**) were finally macrolactonized using the Gerlach modification[20] of Corey's 'double activation' methodology.[21] The target molecules **1a**–**b** were obtained in fair yields and were used for the biological evaluation.

They were first evaluated by in vitro tests considered predictive of the antioxidant and hypolipidaemic properties. In particular, these lactones showed their ability to prevent and/or to delay the oxidative modification of LDL, chemically induced by incubation of native LDL with cupric ions.[22] The oxidation of the LDL was evaluated by monitoring at different time points the concentration of the aldehydes derived from the degradation of lipid peroxides as well as the modifications of the apolipoprotein B-100 (ApoB). The antioxidant activity of lactones **1a–b** has been expressed in terms of duration of the latency period which is a parameter of the resistance to the oxidation of the human LDL. The results obtained from lactones **1a**–**b** are reported in Table 1 along with probucol as standard (Fig. 2).

The *inhibition of the oxidative process* was also qualitatively studied through the evaluation of the modification of the surface-negative charge (relationship) of the ApoB by electrophoretic analysis on agarose gel.[23] The relative electrophoretic mobility (REM) of lactones **1a** and **1b** and probucol at 10 μM expressed as percent variation at 180 and 360 min is reported in Table 2.

Table 4. Cytotoxicity effect evaluated in vitro on Hep-G_2 cells. The data are expressed as ID_{50} (mol)

Compd	DMSO	EtOH
Probucol	–	$> 10^{-3}$
Gemfibrozil	$> 10^{-4}$	–
1a	$(3.2 \pm 1.5) \times 10^{-5}$	$(3.2 \pm 1.3) \times 10^{-5}$
1b	$(6.3 \pm 1.0) \times 10^{-5}$	$> 10^{-4}$

The electrophoretic mobility of native LDL is taken as a reference equal to 0, while the migration of the band of the copper-oxidized LDL at the time of 360 min, only in presence of ethanol, is taken as reference equal to 100.

The inhibition of the oxidative process was evaluated also by means of the study of the fragmentation of the ApoB through the electrophoretic analysis on polyacrylamide gel in presence of sodium dodecylsulfate (SDS-PAGE).[24,25] The densitometric values related to the areas of the peaks of the ApoB, expressed as absorbance units per millimeter, for the lactones **1a** and **1b** and for the probucol, as reference, all used at the concentration of 10 μM, are reported in Table 3.

Preliminarily the cytotoxicity was evaluated to establish the dose to use in lipid synthesis model. The toxicity of the lactones **1a**–**b** was evaluated in vitro by means of the cytotoxic effect on human hepatocarcinoma (Hep-G2 American Type Culture Collection) cells.[26] The cells growth was evaluated by spectrophotometric determination of nucleic acids and proteins. The value of the absorbance is a direct function of the amount of the macromolecules present and therefore indirectly of the number of the cells. In Table 4 are reported the values of ID_{50}, expression of the concentration of compounds **1a**–**b**, in solution both of ethanol and of dimethylsulfoxide, able to cause a 50% lowering of the total macromolecules.

Lactones **1a**–**b** were evaluated in vitro by testing their effect on the synthesis of lipid cells like cholesterol, fatty acids, triglycerides, phospholipids, and esters of the cholesterol.[27]

Lipid synthesis was studied by incubating cells coming from a human hepatocarcinoma in presence of ^{14}C-acetate (Amersham) and by measuring[28] the incorporation of the radioactive precursor in the main lipid classes after chromatographic separation on silica gel.[29] The data reported in Table 5 refer to the incorporation of

Table 3. Oxidative modifications on SDS-PAGE of ApoB-100

Compd (μM)	Area[a] (AU/mm)						
	0 min	60 min	90 min	120 min	150 min	180 min	360 min
Ethanol (-)	2.3	1.7	1.5	0.5	0.4	0.3	0.2
Probucol (10)	2.7	1.8	1.6	0.9	0.9	0.6	0.3
1a (10)	2.2	2.0	1.9	1.6	1.5	1.5	1.5
1b (10)	2.5	2.3	2.1	2.0	1.7	1.6	1.6

[a]Areas of the peaks of the ApoB-100 expressed as absorbance units per millimeter.

^{14}C-acetate in the various lipid classes, expressed as radioactivity in count per minute (cpm) for culture plate, for lactones **1a**–**b**, in comparison with the probucol and the gemfibrozil, as standards, and ethanol as negative control.

Discussion

It should be pointed out that the *antioxidant activity* of lactones **1a**–**b** reported in Table 1 is far higher than that showed by probucol. As a matter of fact, a comparable

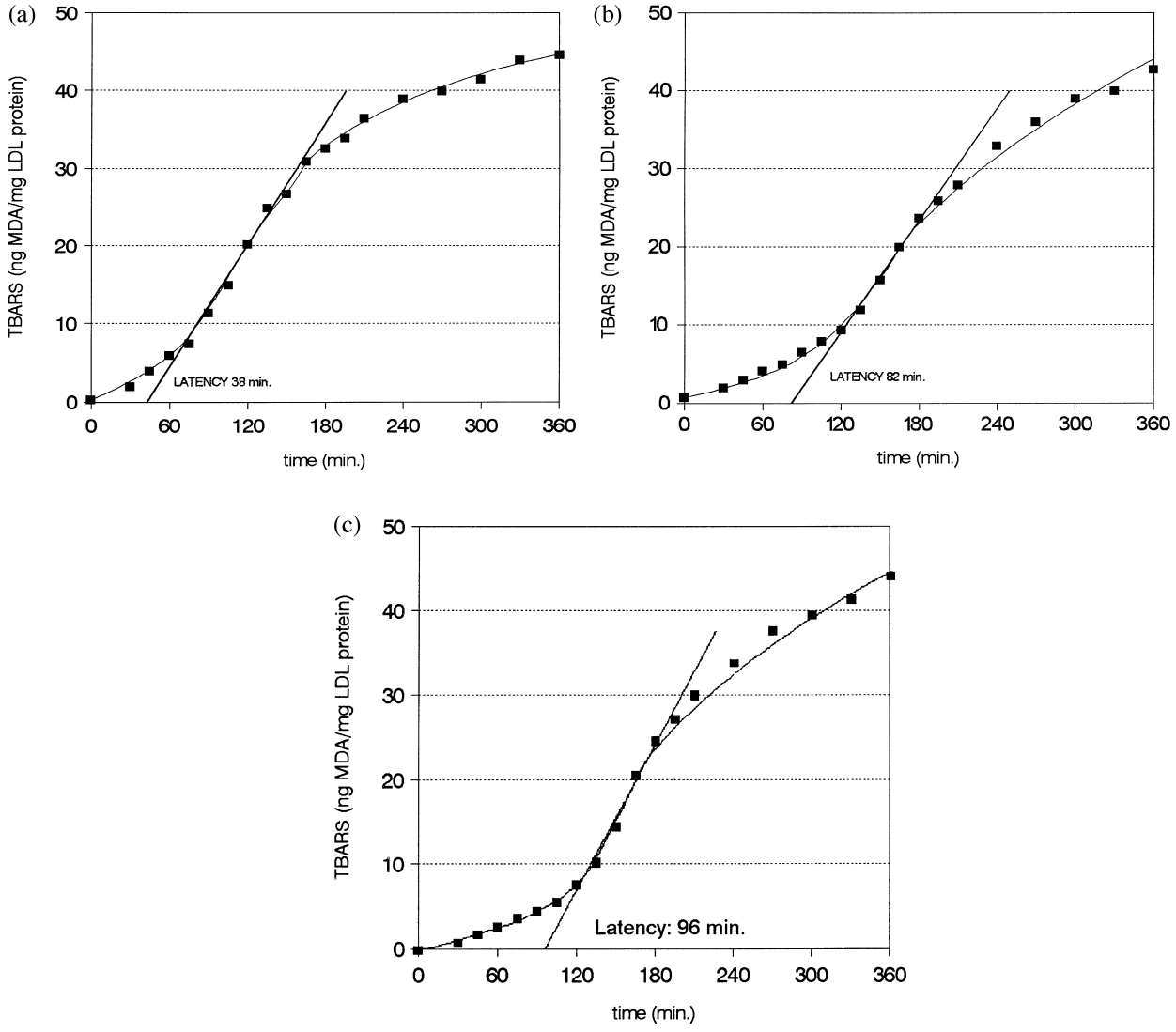


Figure 2. Antioxidant activity of (a) ethanol (negative control); (b) probucol (reference compound); and (c) lactone **1b** on Cu^{2+} modified LDL. LDL (0.5 mg protein/mL) containing ethanol (1.7 μL EtOH/mL LDL); probucol (10 μM) or lactone **1b** (1 μM) were incubated at 37 °C in the presence of 5 μM $CuSO_4$. At the indicated times aliquots were removed, EDTA was added (0.24 mM) after cooling at 4 °C and TBARS were determined as described in Experimental. The intercept on the axis of abscissas of the tangent to the curve at the flex point represents the latency period.

Table 5. In vitro activity on lipid synthesis by incorporation in human hepatocarcinoma cells (Hep-G_2) of ^{14}C-acetate[a]

Compd		Cholesterol (%)	Cholesterol esters (%)	Triglycerides (%)	Fatty acids (%)	Phospholipids (%)
Ethanol		−16	−64	+7.0	−30	−24
Probucol	1 μM	−55	−54	−8.0	−36	−31
	10 μM	−64	−35	−3.0	−42	−31
Gemfibrozil	1 μM	−34	−76	−22	−55	−47
	10 μM	−20	−71	−11	−44	−34
1a	1 μM	−26	−67	−1.1	−29	−27
	10 μM	−40	−73	−4.2	−45	−22
1b	1 μM	−25	−73	−0.9	−42	−35
	10 μM	−45	−72	+1.1	−50	−29

[a]Data are expressed as the variation of ^{14}C-acetate radioactivity (determined as counts per min for culture plate) referred to that of a physiological solution.

prolongation of the latency period was observed at a concentration 10 times lower than that of probucol. In addition, the data reported in Table 2 show that the lactones **1a** and **1b** are more effective than probucol in lowering the electrophoretic mobility of LDL, by preventing their oxidative modifications. Furthermore, the data regarding the fragmentation of ApoB, collected in Table 3, show how lactones **1a**–**b** prevent the disappearance of the ApoB in a more efficient manner than probucol. This consideration is obvious from the almost constant maintenance of the area of the peaks of the ApoB.

The *activity on lipid synthesis* reported in Table 5 show that lactones **1a**–**b** interfere with the synthesis of cholesterol in a comparable extent to that of gemfibrozil and lower than probucol. Concerning the synthesis of the esters of the cholesterol, the compounds show an activity greater than that of the probucol and comparable to that of gemfibrozil. The data referred to the other lipid classes emphasize how the two lactones practically show the same effect on the synthesis of fatty acids and of phospholipids in comparison to both probucol and gemfibrozil whereas the influence on the synthesis of the triglycerides, on which only the gemfibrozil shows a remarkable activity, is practically absent. The preliminary cytotoxicity data, reported in Table 4, show how the active concentrations of lactones **1a**–**b** used in the previous experimental model are far away from ID_{50} as well as probucol and gemfibrozil.

Conclusions

New 12-membered lactones **1a**–**b**, structurally related to phenol-induced antioxidant and gemfibrozil, have been prepared by a practical and efficient synthetic sequence. Compounds **1a** and **1b** showed good antioxidant properties in vitro along with a favorable activity on lipid biosynthesis. These features make this new class of medium-sized lactones promising dual-action drugs for anti-atherosclerosis treatments.[30]

Experimental

Melting points were measured on a Büchi apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer or a Varian Gemini 300 NMR spectrometer. Data were reported as follows: chemical shifts in ppm using tetramethylsilane (TMS) as internal standard on the δ scale, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) in hertz, and integration (for ^{1}H NMR). Mass spectra were measured on a VG 7070E mass spectrometer, at a 70 eV ionization voltage and with a 6 kV acceleration voltage and the data are reported with the base peak underlined. The IR spectra were obtained on a Perkin–Elmer model 983/G spectrophotometer and generally, when not otherwise specified, preparing the specimen in potassium bromide (KBr) and recording the spectrum between 4000 and 600 nm.

Sodium hydride, 5-bromo-2,2-dimethylpentanoic acid isopropyl ester, 2,2′-dipyridyldisulfide, triphenyl phosphine, and silver perchlorate hydrate are commercially available materials and did not require further purification. All solvents were dried and purified by standard techniques before use. All reactions were run under nitrogen. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck). Compounds were visualized by illumination with ultraviolet light (254 nm) or with iodine or with 7% phosphomolybdic acid in ethanol. Column chromatography (flash) was performed using silica gel (0.040–0.063 mm, Merck), with the eluents cited in each case.

Chemistry

5-[4-Acetoxy-2-(3-oxobutyl)-3,5,6-trimethylphenoxy]-2,2-dimethylpentanoic acid, isopropyl ester (4a). A solution of **2a** (14.0 g, 0.053 mol) in benzene (240 mL) was slowly added under stirring to a suspension of 97% sodium hydride (1.82 g, 0.074 mol) in dimethylsulfoxide (100 mL). The temperature of the mixture was maintained at 30–40 °C with an oil bath. After 20–40 min the hydrogen evolution ceased and the solution turned brown-red. A second flask was charged with 5-bromo-2,2-dimethylpentanoic acid isopropyl ester (13.3 g, 0.052 mol) in benzene (60 mL) and the solution of the first flask was transferred into the second while stirring by using a 100 mL syringe under a continuous flushing of nitrogen through both flasks. The mixture was stirred and heated at 65°C for 19 h. The reaction mixture was cooled to room temperature, diluted with ethyl ether, and the water layer acidified to pH 5 by means of a 10% aqueous solution of hydrochloric acid. The layers were then separated, the aqueous phase was extracted again with ether, and the extracts were washed with water and dried over Na_2SO_4. The evaporation of the solvent under reduced pressure furnished a residue that was purified by flash-chromatography on silica gel column (9/1 methylene chloride/ethyl ether). The evaporation of the solvent gave compound **4a** (13.35 g, 58% yield) pure as an oil: IR (film) ν 1757, 1719 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.20 (6H, s, geminal C**H**$_3$), 1.23 (6H, d, J = 6.2 Hz, 2 isopropyl C**H**$_3$), 1.71 (4H, m, -C**H**$_2$C**H**$_2$C), 2.02 (3H, s, arom. C**H**$_3$), 2.04 (3H, s, arom. C**H**$_3$), 2.15 (3H, s, C**H**$_3$-CO-), 2.16 (3H, s, arom. C**H**$_3$), 2.34 (3H, s, C**H**$_3$COO), 2.54–2.68 (2H, m, -C**H**$_2$-), 2.78–2.90 (2H, m, -C**H**$_2$-), 3.60–3.70 (2H, m, C**H**$_2$O), 5.00 (1H, m, J = 6.2 Hz, isopropyl C**H**); ^{13}C NMR ($CDCl_3$) δ 12.6 (arom. **C**H$_3$), 13.1 (arom. **C**H$_3$), 13.2 (arom. **C**H$_3$), 20.6 (**C**H$_3$COO-), 21.7 (-CO**C**H$_3$), 21.9 (OCH(**C**H$_3$)$_2$), 25.2 ((**C**H$_3$)$_2$CCO), 26.1 (**C**H$_2$), 29.9 (**C**H$_2$), 37.1 (**C**H$_2$), 42.1 (CH$_2$**C**CO-), 44.0 (CH$_2$**C**H$_2$CO), 67.6 (isopropyl **C**H), 73.8 (O**C**H$_2$), 126.8, 128.2, 128.7, 131.3, 144.9, 153.9 (arom. 6 **C**), 169.6 (CH$_3$**C**OO-), 177.7 (**C**COO-), 208.6 (-**C**O-); MS m/z 434 (M^+), 222, 171, <u>129</u>, 43.

5-[4-Acetoxy-2-(3-oxopentyl)-3,5,6-trimethylphenoxy]-2,2-dimethyl pentanoic acid, isopropyl ester (4b). This compound was obtained in the same way as described for **4a**, starting from **2b** (20.0 g, 0.072 mol in 500 mL of benzene), 97% sodium hydride (2.07 g, 0.086 mol in 150 mL of DMSO) and 5-bromo-2,2-dimethyl-pentanoic

acid isopropyl ester (21.6 g, 0.086 mol in 100 mL of benzene). Column flash-chromatography (6/4 petroleum ether/ethyl ether) gave compound **4b** (16.5 g, 51% yield) as an oil: IR (film) ν 1758 (C=O), 1716 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.95 (3H, t, $J=7.3$ Hz, $COCH_2CH_3$), 1.12 (6H, s, $C(CH_3)_2$), 1.18 (6H, d, $J=6.2$ Hz, $OCH(CH_3)_2$), 1.63 (4H, m, CH_2CH_2), 1.91 (3H, s, arom. CH_3), 1.98 (3H, s, arom. CH_3), 2.10 (3H, s, arom. CH_3), 2.22 (3H, s, $COCH_3$), 2.35 (2H, q, $J=7.4$ Hz, $COCH_2CH_3$), 2.51 (2H, m, CH_2), 2.80 (2H, m, CH_2), 3.58 (2H, m, CH_2O), 4.90 (1H, m, $J=6.5$ Hz, $OCH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ 8.5 ($COCH_2CH_3$), 13.1 (arom. CH_3), 13.6 (arom. CH_3), 13.7 (arom. CH_3), 21.1 ($COCH_3$), 22.35 ($OCH(CH_3)_2$), 25.7 ($(CH_3)_2CCO$), 26.6 (CH_2CH_2O), 36.5 (CH_2), 37.6 (CH_2), 42.5 ($C(CH_3)_2$), 43.0 (CH_2), 67.9 ($CH(CH_3)_2$), 74.1 (OCH_2), 126.9, 128.3, 128.8, 131.65, 144.9, 154.0 (6 arom. **C**), 169.7 (CH_3COO), 177.7 (**C**OO), 211.3 (**C**O). MS *m/z* 448 (M^+), 279, 236, 171, 129, 43.

2,2-Dimethyl-5-[4-hydroxy-2-(3-oxobutyl)-3,5,6-trimethylphenoxy]pentanoic acid (5a). Compound **4a** (20.0 g, 0.046 mol) dissolved in 95% ethanol (100 mL) was added to potassium hydroxide (22.6 g, 0.40 mol) in 95% ethanol (300 mL). The reaction mixture was refluxed for 18 h. After cooling, it was diluted by addition of water and acidified to pH 5 with a 10% aqueous solution of hydrochloric acid. This solution was concentrated under reduced pressure and extracted with diethyl ether. The crude product obtained by evaporation of the solvent at reduced pressure was purified by filtration on a short column of silica gel eluting with diethyl ether. Solvent removal under reduced pressure gave **5a** (10.6 g, 65% yield) as pure product: mp 108–110 °C (from *n*-hexane); IR (KBr) ν 3499 (OH), 1715 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.05 (3H, t, $J=7.3$ Hz, $COCH_2CH_3$), 1.25 (6H, s, $C(CH_3)_2$), 1.75 (4H, s, 2 CH_2), 2.15 (3H, s, arom. CH_3), 2.2 (6H, s, 2 arom. CH_3), 2.45 (2H, q, $J=7.0$ Hz, $COCH_2CH_3$), 2.6 (2H, m, CH_2), 2.85 (2H, m, CH_2), 3.7 (2H, s, OCH_2), 4.9 (2H, broad s, 2 O**H**); ^{13}C NMR ($CDCl_3$) δ 8.2 ($COCH_2CH_3$), 12.1 (arom. CH_3), 12.6 (arom. CH_3), 13.3 (arom. CH_3), 22.0 ($COCH_2CH_3$), 25.2 ($C(CH_3)_2$), 26.2 (CH_2), 36.2 (CH_2), 37.2 (CH_2), 42.2 ($C(CH_3)_2$), 43.0 (CH_2), 73.9 (OCH_2), 120.2, 121.6, 128.0, 130.7, 148.6, 149.6 (6 arom. **C**), 184.3 (**C**OOH), 211.9 (**C**O); MS *m/z* 364 (M^+), 236, 164, 57.

2,2-Dimethyl-5-[4-hydroxy-2-(3-oxopentyl)-3,5,6-trimethylphenoxy]pentanoic acid (5b). This compound was obtained in the same way as described for **5a**, starting from **4b** (20.0 g, 0.045 mol in 100 mL of 95% of ethanol) when treated with potassium hydroxide (22.6 g, 0.40 mol in 300 mL of 95% ethanol) in refluxing conditions for 18 h. Solvent removal at reduced pressure gave pure **5b** (11.1 g, 67% yield): mp 108–110 °C (from *n*-hexane); IR (KBr) ν 3499 (OH), 1715 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.05 (3H, t, $J=7.3$ Hz, $COCH_2CH_3$), 1.25 (6H, s, $C(CH_3)_2$), 1.75 (4H, s, 2 CH_2), 2.15 (3H, s, arom. CH_3), 2.2 (6H, s, 2 arom. CH_3), 2.45 (2H, q, $J=7.0$ Hz, $COCH_2CH_3$), 2.6 (2H, m, CH_2), 2.85 (2H, m, CH_2), 3.7 (2H, s, OCH_2), 4.9 (2H, broad s, 2 O**H**); ^{13}C NMR ($CDCl_3$) δ 8.2 ($COCH_2CH_3$), 12.1 (arom. CH_3), 12.6 (arom. CH_3), 13.3 (arom. CH_3), 22.0 ($COCH_2CH_3$), 25.2 ($C(CH_3)_2$), 26.2 (CH_2), 36.2 (CH_2), 37.2 (CH_2), 42.2 ($C(CH_3)_2$), 43.0 (CH_2), 73.9 (OCH_2), 120.2, 121.6, 128.0, 130.7, 148.6, 149.6 (6 arom. **C**), 184.3 (**C**OOH), 211.9 (**C**O); MS *m/z* 364 (M^+), 236, 164, 57.

2,2-Dimethyl-5-[4-hydroxy-2-(3-hydroxybutyl)-3,5,6-trimethylphenoxy]pentanoic acid (6a). Sodium borohydride (4.9 g, 0.129 mol) was added portionwise to a solution of compound **5a** (4.2 g, 0.012 mol) in methanol (125 mL). After stirring 4 h at room temperature, the reaction mixture was concentrated under reduced pressure, diluted with ethyl ether (250 mL) and a 6 M aqueous solution of hydrochloric acid was slowly added to destroy the unreacted hydride. The ethereal layer was separated, washed with water, and dried over Na_2SO_4. The evaporation of the solvent at reduced pressure gave compound **6a** (3.93 g, 93% yield) as pure product: mp 44–46 °C; IR (KBr) ν 3213 (OH), 1702 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.15 (3H, d, $J=6.8$ Hz, CH_3CH), 1.30 (6H, s, geminal CH_3), 1.50–1.90 (6H, m, 3 CH_2), 2.10 (3H, s, arom. CH_3), 2.18 (6H, s, 2 arom. CH_3), 2.65–2.88 (2H, m, CH_2), 3.52–3.72 (3H, m, CH_2 and O**H**), 5.48 (2H, broad s, 2 O**H**); MS *m/z* 352 (M^+), 224, 206, 164.

2,2-Dimethyl-5-[4-hydroxy-2-(3-hydroxypentyl)-3,5,6-trimethylphenoxy]pentanoic acid (6b). This compound was obtained in the same way as described for **6a**, starting from **5b** (8.0 g, 0.022 mol) dissolved in methanol (240 mL) by treatment with sodium borohydride (9.04 g, 0.24 mol). Work up of the reaction mixture was performed as depicted before after stirring at room temperature for 1 h to give pure **5b** (7.49 g, 93% yield): mp 38–42 °C; IR (KBr) ν 3499 (OH), 1710 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.9 (3H, t, $J=8$ Hz, CH_2CH_3), 1.25 (6H, s, $C(CH_3)_2$), 1.4–1.65 (m, 4H, CH_2CH_2), 1.7–1.9 (m, 4H, $CHCH_2CH_2$), 2.15 (s, 9H, 3 arom. CH_3), 2.7–2.85 (2H, m, CH_2CH_3), 3.3–3.45 (m, 1H, C**H**), 3.6–3.75 (m, 2H, OCH_2), 6.1–7.0 (bs, 3H, 3 O**H**); ^{13}C NMR ($CDCl_3$) δ 10.4 (CH_3), 12.25 (arom. CH_3), 12.7 (arom. CH_3), 13.5 (arom. CH_3), 23.5 (CH_2), 25.45 (CH_3), 26.3 (CH_2), 30.2 (CH_2), 37.2 (CH_2), 42.35 (q, **C**), 72.8 (**C**H), 74.5 (CH_2), 120.5, 121.6, 127.75, 131.3, 149.0, 149.7 (6 arom. **C**), 183.3 (**C**O); MS *m/z* 366 (M^+), 348, 322, 238, 220, 165.

6,6,9,13,15,16-Hexamethyl-14-hydroxy-2,8-dioxabicyclo-[10.4.0]hexadec-12,14,16-trien-7-one (1a). A solution of compound **6a** (2.0 g, 0.005 mol), 2,2′-dipyridyldisulfide (2.5 g, 0.011 mol) and triphenyl phosphine (3.0 g, 0.011 mol) in anhydrous acetonitrile (60 mL) was prepared. The resulting mixture was kept at 25 °C for 4 h after which it was diluted with *p*-xylene (70 mL) and transferred into a pressure-equalizing dropping funnel equipped on a three-necked flask in which silver perchlorate hydrate (2.74 g, 0.012 mol) in *p*-xylene (150 mL) was heated to reflux. The solution containing compound **6a** was slowly added (1 h) to the refluxing perchlorate solution. The mixture was refluxed for an additional 18 h, the reaction mixture was then cooled and the reflux condenser was changed with a Claisen head for distillation. The solution was concentrated by distilling the solvents at atmospheric pressure. The residue

was added to silica gel for chromatography (20 g), collected into a round-bottom flask and the residual solvent of this mixture was evaporated at reduced pressure. The powder so obtained was added to the head of a silica gel column previously prepared with 1/1 diethyl ether/petroleum ether. This mixture was used as eluent and the chromatographic fractions were monitored by TLC. The evaporation at reduced pressure of the solvent of the collected fractions gave pure lactone **1a** (0.77 g, 46% yield): mp 127–130 °C; IR (KBr) ν 3452 (OH), 1709 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.18 (3H, s, CH_3C), 1.22 (3H, d, $J = 6.4$ Hz, CH_3CH), 1.27 (3H, s, CH_3C), 1.50–1.90 (5H, m, 2 CH_2 and CH_2), 2.12 (3H, s, arom. CH_3), 2.14 (3H, s, arom. CH_3), 2.16 (3H, s, arom. CH_3), 2.35 (1H, m, CH_2), 2.55 (1H, m, CH_2), 3.05 (1H, m, CH_2), 3.65 (2H, m, CH_2O), 4.50 (1H, s, OH), 4.85 (1H, m, CHO); ^{13}C NMR ($CDCl_3$) δ 12.3 (arom. CH_3), 12.6 (arom. CH_3), 13.6 (arom. CH_3), 19.2 (CH_3CH), 21.8 (CH_2), 23.5 (CH_3C), 26.1 (CH_2), 28.6 (CH_2), 36.0 (CH_2), 38.1 (CH_2), 42.7 (C), 69.6 (CH), 73.1 (CH_2), 120.0, 121.2, 128.1, 131.5, 148.5, 150.2 (6 arom. C), 177.8 (COO); MS m/z 334 (M^+) 206, 164, 119, 91.

9-Ethyl-14-hydroxy-6,6,13,15,16-pentamethyl-2,8-dioxabicyclo[10.4.0]hexadec-12,14,16-trien-7-one (1b). A solution of compound **6b** (4.00 g, 11.4 mmol), 2,2′-dipyridyldisulfide (4.76 g, 21.6 mmol), and triphenylphosphine (5.68 g, 21.6 mmol) in anhydrous acetonitrile (110 mL) was diluted with *p*-xylene (120 mL) and added over 1 h to a boiling solution of silver perchlorate hydrate (5.2 g, 23.0 mmol) of *p*-xylene (300 mL). After 12 h of refluxing, the crude product was recovered by evaporation of the solvent as previously described and purified by column chromatography (1/1 diethyl ether/petroleum ether). Evaporation at reduced pressure of the solvent of the collected fractions gave pure lactone **1b** (1.80 g, 46% yield): mp 126–128 °C (from ethyl ether and *n*-hexane); IR (KBr) ν 3448 (OH), 1699 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.9 (t, $J = 8$ Hz, 3H, CH_2CH_3), 1.2 (s, 3H, CH_3), 1.3 (s, 3H, CH_3), 1.4–1.9 (m, 7H, CH_2CH_3, CH_2CH_2 1H CH_2CH_2CH), 2.15 (s, 9H, 3 arom. CH_3), 2.35–2.6 (m, 2H, CH_2CH), 2.9–3.1 (m, 1H, 1H CH_2CH_2CH), 3.5–3.8 (m, 2H, CH_2), 4.55 (s, 1H, OH), 4.75–4.9 (m, 1H, CH); ^{13}C NMR ($CDCl_3$) δ 10.4 (CH_3), 12.3 (arom. CH_3), 12.7 (arom. CH_3), 13.7 (arom. CH_3), 21.5 (CH_2), 23.5 (CH_3), 26.6 (2 CH_2), 28.6 (CH_3), 33.7 (CH_2), 37.3 (CH_2), 42.7 (q, C), 72.8 (CH_2), 74.9 (CH), 120.0, 121.2, 128.1, 131.9, 148.4, 150.3 (6 arom. C), 178.2 (CO); MS m/z 348 (M^+), 220, 165.

Biological assays

Native LDL isolation from human plasma. Human plasma was obtained from blood collected, in presence of EDTA, according to the Havey's method,[31] from healthy volunteers fasting for 8 h. LDL were isolated from the plasma by sequential ultracentrifugation at densities between 1.006 and 1.063 g/mL in a Sorval OTD 65 B ultracentrifuge for 20 h at 55,000 rpm and at 4 °C. LDL were dialysed at 4 °C against 0.01 M phosphate buffer (pH 7.4) and 0.15 M NaCl containing EDTA (0.24 mM) (PBS-EDTA solution), filtered through a 0.45 μm membrane (Millex HV filters) and the protein concentration determined, according to Lowry's method.[32] The solution of LDL was diluted with PBS-EDTA to a concentration of 500 μg protein/mL.

Antioxidant activity determination: LDL oxidation. The LDL solution so obtained (1 mL) was incubated at 42 °C for 45 min in presence either of 1.7 μL of a solution of lactones at various concentration in ethanol to a final concentration of lactones as reported in Table 1 and ethanol and probucol as standards. At the end of the incubation the solutions were placed on ice for 45 min and dialysed at 4 °C for 36 h in PBS EDTA-free. The oxidation of LDL was carried out by the addition of a cupric sulfate solution at a final concentration of 5 μM, at 37 °C either in the presence of the testing compounds or of ethanol alone. At defined time intervals the reaction was stopped by placing the test tubes at 4 °C after the addition of 0.24 mM EDTA.

TBARS method. The degree of oxidation of LDL was checked by spectrophotometric evaluation (λ = 535 nm) of the condensation products between thiobarbituric acid and aldehydes formed from oxidative breakdown of LDL, using malondialdehyde (MDA) as standard, according to the TBARS method.[33] The results of the spectrophotometric reading, expressed as ng of MDA/mg LDL protein, were reported as a function of the oxidation time. The latency interval was determined as the intercept on abscissas of the tangent to the curve at the flex point. Whenever the oxidation of LDL was inhibited over the experimental time we conventionally indicated a latency period more than 180 min (see Table 1).

Agarose gel electrophoresis. The electrophoretic mobility of ApoB was measured applying LDL to a 0.6% agarose gel in barbital/sodium barbital buffer pH 8.4–8.8 (Helena Laboratories kit 'Titan Gel Multi-slot lipo-17 electrophoresis system') and using the Bio-Phoresis Horizontal Electrophoresis Cell (BIO-RAD). The native LDL solutions (2 μL) and samples of copper-oxidated LDL at incubation times reported were applied on the agarose gel plate and 7 min were waited before running. The electrophoretic conditions were: the application point was on the cathodic side (—), voltage 60 V, electrophoresis time 40 min, temperature 15 °C. At the end of the electrophoresis period, the plate was dried with air at 60–70 °C for 10 min and placed in the lipoprotein staining solution (0.1% (w/v) Fat Red 7B in 95% methanol) for 2 min. The plate was destained in two consecutive washes of destaining solution (75% (v/v) methanol in water) for 15 s and dried at 60–70 °C for 5 min. The migration of native LDL was taken as equal to 0 while the migration of copper-oxidized LDL at 360 min of oxidation in the presence of ethanol was taken as 100. The electrophoretic mobility of copper-oxidized LDL, in the presence of testing compounds, was expressed as relative mobility (REM) at 180 and 360 min.

SDS-polyacrylamide gel electrophoresis. LDL were dissolved in sample buffer containing 48% 0.7 M SDS,

20% 2-mercaptoethanol, 12% 1 M TRIS pH 6.8, 20% glycerol by incubating in boiling water for 4 min. Vertical gel electrophoresis was performed according to the method of Laemmli[24] using a 5% acryilamide gel at a constant voltage of 30 V for 4.5 h in a BioRad Protean II cell with tap water cooling. Gels were fixed in 10% acetic acid in methanol/water 1/1, stained with Coomassie Brilliant Blue G250 and subjected to densitometric scanning by LKB Mod. 2222-020 Ultrascan XL with integrators LKB 2220 and LKB 2190 GelScan software package.

In vitro cytotoxic activity

The confluent Hep-G2 cells were exposed to the products tested or reference compounds, dissolved in DMSO or ethanol (0.01% v/v versus incubation medium volume) as reported in Table 4, for 24 h in Dulbecco's Modified Eagle Medium (DMEM), containing 5% of fetal calf serum. At the end of incubation, the medium was removed and the monolayer was washed twice with PBS-phosphate buffered saline and fixed twice with 60% methanol at 4 °C for 30 min. The dried monolayer was solubilized with NaOH 0.3 M for 12 h at 25 °C and the optical density of the lysate at 260 nm was measured. Calibration curve was obtained with suspended Hep-G2 cells culture containing different amount of cells. DMSO and ethanol at experimental concentration did not express any toxicity.

Effect on lipid synthesis in Hep-G2 cells

Hep-G2 cells monolayers were incubated for 24 h according to the method of Barnhard[27] in the presence of tested products and reference compounds at the concentrations reported in Table 5. Hep-G2 cells were incubated for 3 h in the presence of ^{14}C-acetate. After removing medium and washing, the lipids were extracted twice with heptane–isopropyl alcohol mixture and the solutions evaporated. The radioactivity incorporated in the main lipid classes was measured after chromatographic separation[29] on silica gel (Merck F254) and the quantifying radioactivity by liquid scintillation counting.[28]

Acknowledgements

This research was supported by two Alfa Wassermann S.p.A. Postdoctoral Fellowships to F.C. and F.V. and by research grants from the Ministero dell'Università e della Ricerca Scientifica (MURST), Italy, the Consiglio Nazionale delle Ricerche (CNR), Italy, and by the University of Bologna (Progetto d'Ateneo: Biomodulatori Organici: Sintesi, Proprietà ed Applicazioni). We wish to thank Cristina Carrara and Dr. Eleonora Romagnoli for technical assistance and Dr. Miriam Barbanti for useful discussions.

References and Notes

1. Ross, R. *N. Engl. J. Med.* **1986**, *314*, 488.
2. Ross, R. *Am. J. Pathol.* **1993**, *143*, 987.
3. Steinberg, D. *JAMA* **1985**, *253*, 2080.
4. Steinberg, D. *Arteriosclerosis* **1983**, *3*, 283.
5. Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. *N. Engl. J. Med.* **1989**, *320*, 915.
6. Esterbauer, H.; Waeg, G.; Puhl. H. *Br. Med. Bull.* **1993**, *49*, 566.
7. D. Steinberg showed the presence of antibodies anti-OX-LDL and of OX-LDL, oxidized low density lipoproteins, in the atheromatous lesions of patients suffering from advanced atherosclerosis.[5] Moreover, OX-LDL appeared toxic towards the endothelial cells and a pro-inflammatory activity towards monocytes and lymphocytes and an increased susceptibility to the taking from the macrophages and the smooth muscle cells with formation of foam cells was showed.[8] Such properties can have an important part in the processes of formation and progression of the atheromatous lesion.[9]
8. Witztum, J. L.; Steinberg. D. *J. Clin. Invest.* **1991**, *88*, 1785.
9. Ross, R. *Nature* **1993**, *362*, 801.
10. Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1989**, *19*, 194.
11. Hall, E. D.; Braughler, J. M. *Free Radical Biol. Med.* **1989**, *6*, 303.
12. Van Acker, S. A. B. E.; Koymans, L. M. H.; Bast, A. *Free Radical Biol. Med.* **1993**, *15*, 311.
13. Carew, T. E.; Schwenke, D. C.; Steinberg, D. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 7725.
14. Esterbauer, H.; Dieber-Rotheneder, M.; Striegl, G.; Waeg, G. *Am. J. Clin. Nutr.* **1991**, *53*, 314S.
15. Kita, T.; Nagano, Y.; Yokode, M.; Ishii, K.; Kume, N.; Ooshima, A.; Yoshida, H.; Kawai, C. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 5928.
16. Rimm, E. B.; Stampfer, M. J.; Ascherio, A.; Giovannucci, E.; Colditz, G. A.; Willet, W. C. *New Engl. J. Med.* **1993**, *328*, 1450.
17. Stampfer, M. J.; Hennekens, C. H.; Manson, J. E.; Colditz, G. A.; Rosner, B.; Willet, W. C. *New Engl. J. Med.* **1993**, *328*, 1444.
18. Generally, the best reductions of the plasma LDL have been obtained by means of the resins sequestering the bile acids and with the statins, inhibitors of the HMGCoA reductase, while the plasma levels of the triglycerides are mostly affected by the treatment with nicotinic acid and fibrates. Betteridge, D. *J. Curr. Opin. Lipidol.* **1993**, *4*, 49.
19. Scott, J. W.; Bizzarro, F. T.; Parrish, D. R.; Saucy, G. *Helv. Chim. Acta* **1976**, *59*, 290.
20. Gerlach, H.; Thalmann, A. *Helv. Chim. Acta* **1974**, 57, 2661.
21. Corey, E. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1974**, *96*, 5614.
22. Steinbrecher, U. P. *J. Biol. Chem.* **1987**, *262*, 3603.
23. Vanderyse, L.; Dvreese, A. M.; Baert, J.; Vanloo, B.; Lins, L.; Ruysschaert, J. M.; Rosseneu, M. *Atherosclerosis* **1992**, *97*, 187.
24. Laemmli, U. K. *Nature* **1970**, *227*, 680.
25. Fong, L. G.; Parthasarathy, S.; Witsum, J. L.; Steinberg, D. *J. Lipid Res.* **1987**, *28*, 1466.
26. Cingi, R.; De Angelis, I.; Fortunati, E.; Reggiani, D.; Bianchi, V.; Tiozzo, R.; Zucco, F. *Toxicol. in vitro* **1991**, *5*, 119.
27. Barnhard, J. W.; Li, D. L. *Am. J. Cardiol.* **1988**, *62*, 52B.
28. Gherardi, E.; Calandra, S. *Lipids* **1980**, *15*, 108.
29. Skipski, V. P.; Smolowe, A. F.; Sullivan, M.; Barklay, J. *Biochem. Biophys. Acta* **1965**, *106*, 386.
30. Rosini, G.; Baldazzi, C.; Romagnoli, E.; Saguatti, S.; Piani, S. Eur. Patent Appl. 1996, EP 718295 A1; US Patent 5,608,084, 1997; *Chem Abs.* **1996**, *125*, 114726.
31. Havel, R. J.; Eder, H. A.; Bragdon, J. H. *J. Clin. Invest.* **1955**, *34*, 1345.
32. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
33. Fleisher, S. In *Methods in Enzymology*; Packer, L., Ed.; New York Press: New York, 1978; Vol. 52.