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Novel Antioxidant Agents Deriving From Molecular Combinations of Vitamins C and E Analogues: 3,4-Dihydroxy-5(*R*)-[2(*R*,*S*)-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2(*R*,*S*)-yl-methyl)-[1,3]dioxolan-4(*S*)-yl]-5*H*-furan-2-one and 3-*O*-Octadecyl Derivatives

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Abstract—Molecular combinations of two antioxidants (i.e., ascorbic acid and the pharmacophore of α-tocopherol), namely the 2,3-dihydroxy-2,3-enono-1,4-lactone and the chromane residues, have been designed and tested for their radical scavenging activities. When evaluated for their capability to inhibit malondialdehyde (MDA) production in rat liver microsomal membranes, the 3,4-dihydroxy-5*R*-2(*R,S*)-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (**11a–d**), exhibited an interesting activity. In particular the 5*R*,2*R*,2*R*,4*S* and 5*R*,2*R*,2*S*,4*S* isomers (**11c,d**) displayed a potent antioxidant effect compared to the respective synthetic α-tocopherol analogue (**5**) and natural α-tocopherol or ascorbic acid, used alone or in combination. Moreover, the mixture of stereoisomers **11a–d** also proved to be effective in preventing damage induced by reperfusion on isolated rabbit heart, in particular at the higher concentration of 300 μM. In view of these results our study represents a new approach to potential therapeutic agents for applications in pathological events in which a free radical damage is involved. Design, synthesis and preliminary biological activity are discussed.

Introduction

The area of free radical biology and medicine is developing faster since the discovery, in recent years, of the involvement of free radicals in oxidative tissue injury and a growing number of diseases. The production of free radicals and various reactive oxygen species (ROS), derives from oxidative injuries related to pollution, radiation, food constituents and is also a function of the body's normal defence system and metabolic reactions.[1]

The involvement of free radicals in the pathology of human diseases (i.e., intestinal diseases, atherosclerosis, reperfusion injuries, cardiac diseases, neurodegeneration, respiratory disorders, inflammation, diabetes, cancer and aging) has been recognized by a number of authors.[2] Potential therapeutic interventions might include natural antioxidants or synthetic pharmacological agents with antioxidant activity.[2,3]

The importance of vitamins E and C in disease prevention has become widely recognized and is supported by several clinical and epidemiological studies.[4] α-tocopherol (vitamin E) provides the most important line of defence against lipid peroxidation of cell membranes, and some of its effects are mediated by interaction with specific and saturable binding sites on cell membranes. However, this activity is strongly dependent on the regeneration of vitamin E by ascorbic acid (vitamin C) and glutathione present in the cytosol. Electron spin resonance studies (ESR) have demonstrated the interaction between vitamin C and E during the free radical mediated oxidative stress, thus indicating the role of ascorbic acid as the terminal small-molecule antioxidant in biological systems.[5] Therefore, ascorbic acid itself is not able to prevent lipid peroxidation.[6] It is well known that dietary supplementations of these vitamins are scarcely active, due to their low stability and bioavailability: oxygen-derived free radicals have short half-life and are involved in fast rate reactions (i.e., free radical chain reactions). Thus, the principal requisite that a radical scavenger must possess is to be available, in the

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Table 1. Antioxidant efficiency of the studied compounds **3**, **5**, **11**, **12** and L-ascorbic acid in rat liver microsomal membranes initiated with AAPH[a]

Compound	Concentration (μM)	Malondialdehyde inhibition (%)
5	10	15
	25	20
	100	22
3	10	8
	25	20
	100	24
11a,b (*cis*)	10	20
	25	48
	100	93
11c,d (*trans*)	2.5	54
	10	77
	25	80
	100	100
12	10	8
	25	18
	100	27
L-ascorbic acid	10	47
	25	91
	100	93

[a]Membranes were incubated with 25 mM AAPH for 15 min. Values are the means of three different experiments. Each mean has a standard error less than 10%.

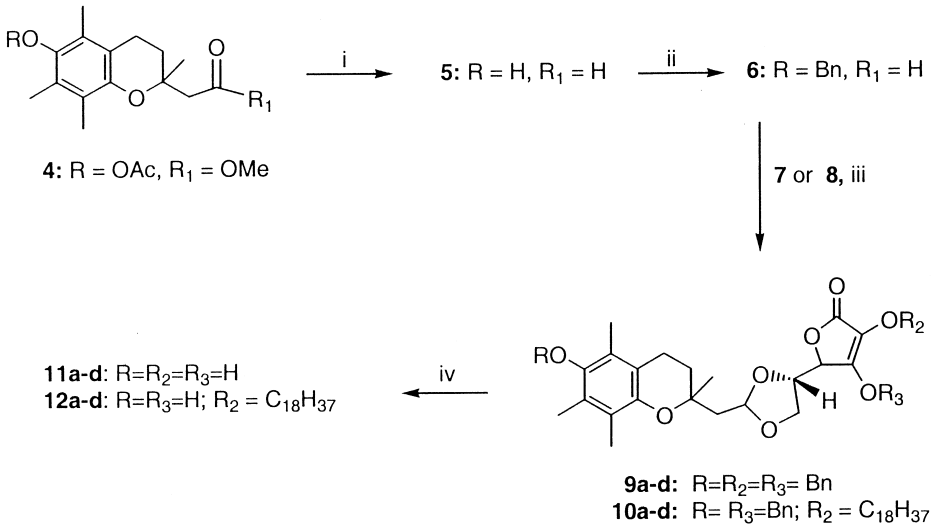
appropriate concentration, at the place where the radical-promoted degradation of the cellular components initiate (i.e., membrane or nucleus and cytoplasm). Up to date, attempts have been made to develop analogues of these vitamins with improved pharmacokinetics (i.e., esters),[2,7] and a few conjugates between vitamin C and E have been reported.[8,9] However, the strategy generally employed for the design of these compounds makes use of the more accessible acid hydroxy residues for the conjugation, namely at the 2 and 3 position. Because these functional groups are responsible for the scavenging activity, these compounds act as merely pro-drugs that require activation in the body of the pro-vitamin and do not warrant a correct delivery of the active moieties to the oxidation site. Taking this into account and continuing our efforts toward the study of radical scavenging agents,[10,11] we have now designed new analogues of these vitamins in the attempt to overcome the above cited drawbacks. In particular, we have studied molecular combinations of the pharmacophores of the two vitamins (**1**), namely the chromane and the 2,3-dihydroxy-2,3-enono-1,4-lactone rings. The title compounds were synthesized and investigated for their antioxidant activity by evaluation of their capability to inhibit malondialdehyde (MDA) production in rat liver microsomal membranes (Table 1). Moreover, in view of the high relevance of myocardial infarct among cause of death, the compounds (**11a–d**), emerging as promising from the primary antioxidant test were further evaluated for their capability to reduce ischemia-reperfusion damage.

Our study, initiated by a careful examination of the literature, led us to select, as suitable candidates for antioxidant and conjugable moieties, the following molecules: (i) the(*R,S*)-6-hydroxy-(2,5,7,8-tetramethylchroman-2-yl) acetaldehyde (**5**),[12,13] (ii) the ascorbic acid itself (**2**) and (iii) the 2-*O*-octadecyl-ascorbic acid (**3**), which has shown, together with important antioxidant properties, an increased stability by comparison to ascorbic acid.[6,14]

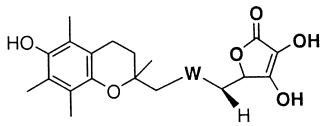
Chemistry

Synthesis

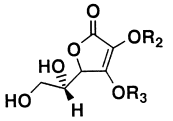
After having evaluated several functionalities, the retrosynthetic analysis suggested us to select an aldehyde on the chromanyl fragment, in order to employ acetalization of the 5- and 6-hydroxy groups on ascorbic acid, for the conjugation of the two fragments. This strategy allowed us to use, for the conjugation process, functional groups of the molecules not involved in the radical scavenging process.



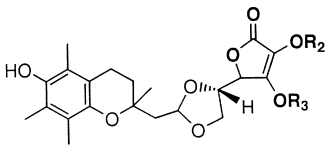
Scheme 1. (i) DIBAL-H, hexane, −70 °C; (ii) benzylbromide, K_2CO_3, DMF, 25 °C; (iii) TsOH, toluene, reflux; (iv) H_2, Pd/C, EtOAc, 25 °C.



1: W = linker



2: $R_2 = R_3 = H$
3: $R_2 = C_{18}H_{37}$; $R_3 = H$
7: R_2, R_3 = Bn
8: $R_2 = C_{18}H_{37}$; R_3 = Bn



11: $R_2 = R_3 = H$
12: $R_2 = C_{18}H_{37}$; $R_3 = H$

Title compounds were synthesized (Scheme 1) in two simple steps from compound **6**, prepared by reduction of (*R,S*)-6-ethyl-(2,5,7,8-tetramethylchroman-2-yl)acetate (**4**)[12] with diisobutylaluminum hydride (DIBAH) to give the corresponding aldeyde **5,** which was protected at the hydroxy function in position 6 by benzylation to afford **6**. Compound **6** was next acetalized, in the presence of *p*-toluenesulfonic acid (TsOH), as catalyst, with 2,3-di-*O*-benzyl-ascorbic acid (**7**)[14] or with 2-*O*-octadecyl-3-*O*-benzyl-ascorbic acid (**8**).[15] Acetalisation proceeded smoothly to give in a 1:1:1:1 mixture of the four expected diastereoisomers, namely 3,4-dibenzyloxy-5*R*-2(*R,S*)-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (**9a–d**) and 4-*O*-benzyloxy-5*R*-2(*R,S*)-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (**10a–d**). Compounds **10a–d** could be isolated only as inseparable mixtures of the two couple of diastereoisomers: **a,b** and **c,d**. The composition of the mixture was estimated, by ^{1}H NMR analysis, as the relative intensity of the H-C4 proton, on the furanone ring, in the **a,b**/**c,d** isomers. These data were also corroborated by NOESY and NOE-difference experiments, indicating a shielding effect, of the methyl at C2 of the chromanyl moiety on the H-C5 at the furanone ring, in the case of the **c,d** isomers. In particular the 5*R*,2*S*,2*S*,4*S* and 5*R*,2*S*,2*R*,4*S* (**a,b**) and the 5*R*,2*R*,2*R*,4*S* and 5*R*,2*R*, 2*S*,4*S* (**c,d**) isomers may be considered as *cis* and *trans* isomers respectively, with regards to the relative stereochemistry of the protons at the C-2 and C-4 of the dioxolane ring.

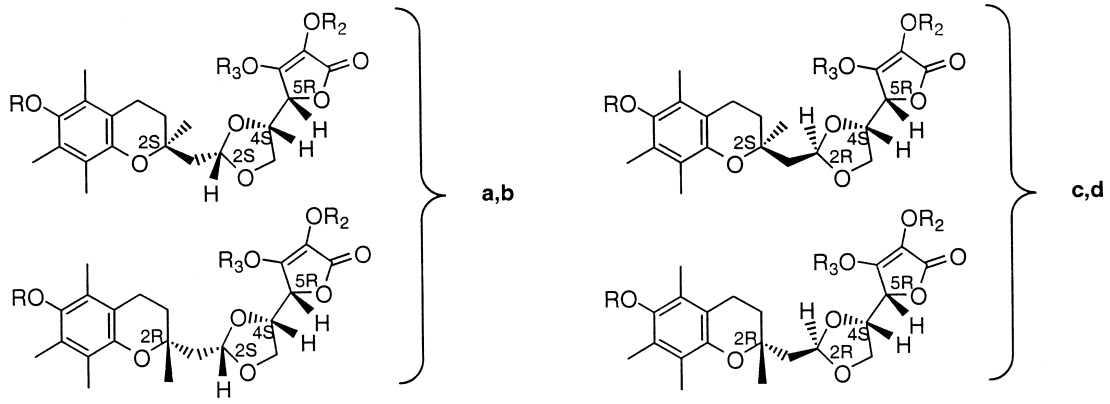
Protective benzyl groups, at positions 3 and/or 2 on the ascorbic acid moiety and at position 6 on the chromanyl residue, were concomitantly removed by hydrogenation in the presence of 5% Pd/C to give compounds **11a–d** and **12a–d** (Fig. 1).

Structure assignments were also conducted on the base of our previous findings[16] and of quantum-mechanics calculations. Briefly, the structure of **a,b** and **c,d** isomers of compound **11** were optimized and subjected to a systematic conformational search. The structures having a maximum energy difference of 20 kcal/mol were further optimized until convergence. The geometry was optimized both with a molecular mechanics force field (MAXIMIN2) and with a semi-empirical molecular orbital method (AM1). The conformations having closer distances between C5 proton at the furanone ring and C3 proton and C2 methyl at the chromanyl residue were studied with ab initio Hartree–Fock methods. Molecular modeling confirmed the upfield shift of about 0.06 ppm observed in the **11c,d** (*trans*) isomers for the C-5 proton at the furanone ring, that might be explained on the base of a preferred conformation in which the C-5 proton was in the shielding region of the chromanyl ring. In particular, the **11c,d** isomers show a closer contact of the C5 proton at the furanone ring with the methyl at the C2 of the cromanyl residue, about 4.19 Å, versus 6.43 Å, as compared to the corresponding **11a,b** (*cis*) isomers. Although molecular modeling calculations have permitted the structure assignments for the **a,b** and **c,d** couple of isomers, because no crystalline structure suitable for X-ray spectroscopy could be obtained, it was impossible to assign the absolute configuration of the single diastereoisomers.

Biology

Prevention of lipid peroxidation

The above prepared compounds were evaluated for their ability to inhibit peroxyl radical dependent lipo-



9a-d: R=R_2=R_3= Bn; **10a-d:** R= R_3=Bn; $R_2 = C_{18}H_3$; **11a-d**: R=R_2=R_3=H; **12a-d**: R=R_3=H; $R_2 = C_{18}H_{37}$

Figure 1. Absolute configuration of stereogenic centers of compounds **9–12**.

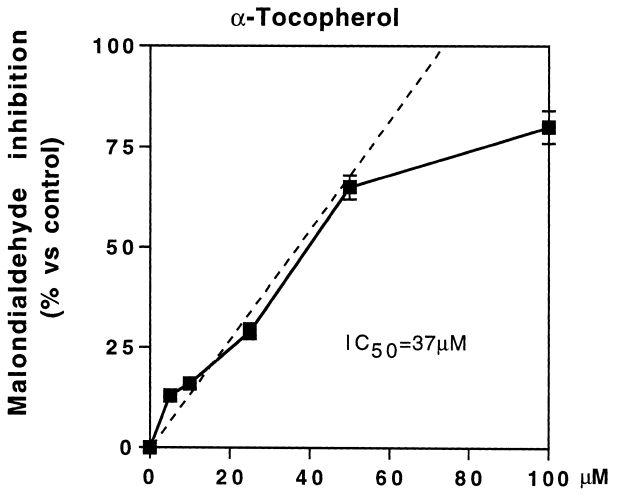


Figure 2. Inhibition of AAPH-induced malondialdehyde production by different concentrations of α-tocopherol in rat liver microsomal membranes. Values are the means±SEM of 10 different experiments. Each mean has a standard error less than 10%.

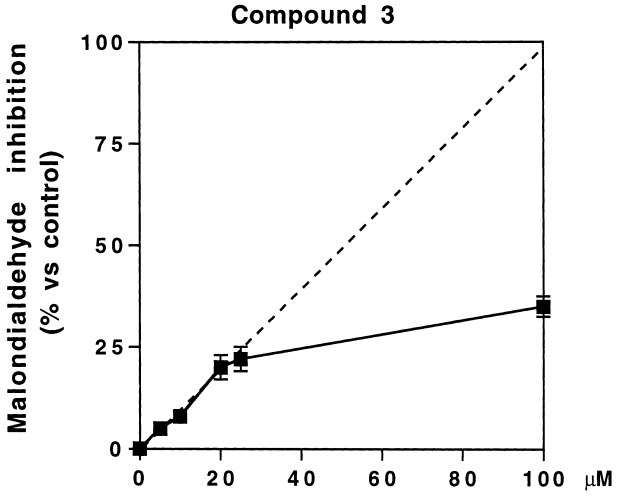


Figure 4. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compound **3**, in rat liver microsomal membranes. Values are the means±SEM of 10 different experiments. Each mean has a standard error less than 10%.

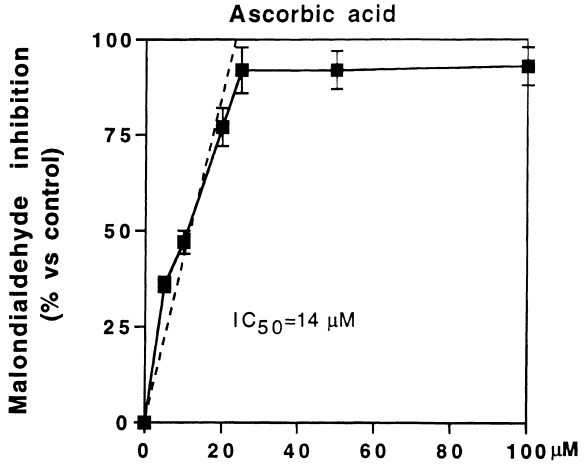


Figure 3. Inhibition of AAPH-induced malondialdehyde production by different concentrations of L-ascorbic acid in rat liver microsomal membranes. Values are the means±SEM of 10 different experiments. Each mean has a standard error less than 10%.

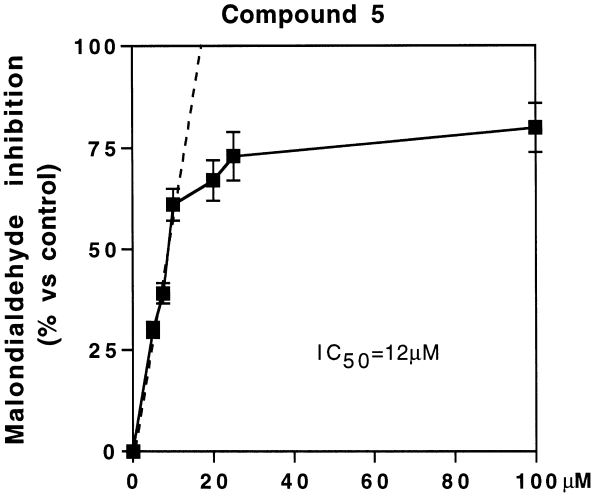


Figure 5. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compound **5**, in rat liver microsomal membranes. Values are the means±SEM of 10 different experiments. Each mean has a standard error less than 10%.

peroxidation in rat liver microsomal membranes. Microsomes were prepared from Winstar rats as previously reported by some of us[17] and microsomal proteins were determined by the Bio-Rad method. Lipid peroxidation was initiated by 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH), which produces peroxyl radicals by thermal decomposition. Reactions were carried out at 37 °C with 25 mM AAPH for 15 min and lipid peroxidation was measured by the appearance of malondialdehyde (MDA) formation.[18] All compounds were used at the concentration of 5 μM. Similar results were also obtained using antioxidant concentrations ranging from 2.5 to 100 μM (data not shown).

In the absence of antioxidants, the formation of MDA in microsomal membranes treated with 25 mM AAPH for 15 min, was 5.40±0.5 nmols/mg protein. Under these conditions, we have compared the antioxidant ability of the natural antioxidants, α-tocopherol (Fig. 2) and L-ascorbic acid (Fig. 3) to that of the above described synthetic compounds, including **3** (Fig. 4), **5** (Fig. 5), **11a–d** (Fig. 6) and **12a–d** (Fig. 7). It is evident that all synthetic compounds, were able to act as antioxidants, inhibiting AAPH-induced MDA production. In particular, compounds **11a,b** and **11c,d,** which are the molecular combination between the α-tocopherol analogue **5** and ascorbic acid, exhibited greater antioxidant efficiency (IC_{50} 12 and 7 μM) than the natural antioxidants, α-tocopherol (IC_{50} 37 μM) and ascorbic acid (IC_{50} of 14 μM). Moreover, this compound was a more powerful antioxidant than the synthetic analogue of ascorbic acid, **3**, and comparable to the other synthetic analogue of α-tocopherol, **5**.

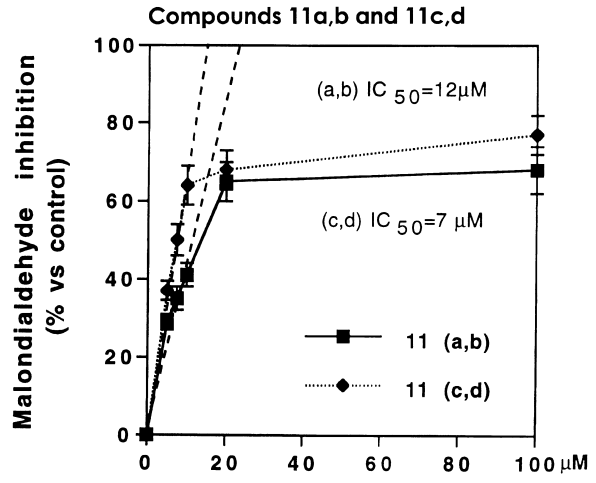


Figure 6. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compounds, **11a,b** and **11c,d** in rat liver microsomal membranes. Values are the means±SEM of 10 different experiments. Each mean has a standard error less than 10%.

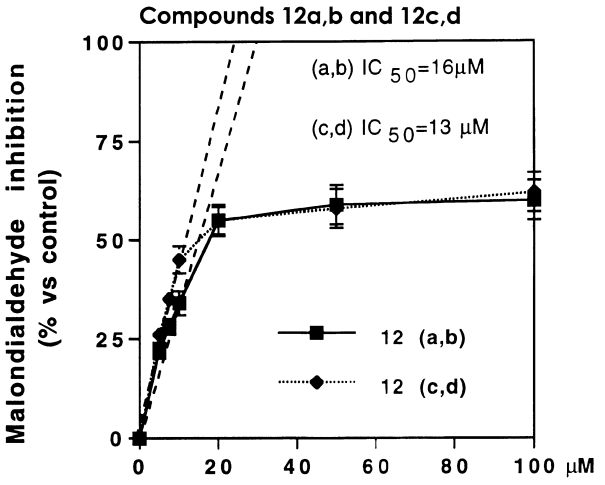


Figure 7. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compounds **12a,b** and **12c,d** in rat liver microsomal membranes. Values are the means±SEM of 10 different experiments. Each mean has a standard error less than 10%.

The configuration at the dioxolane ring and chromane ring also influenced the antioxidants properties of this compound, as shown by the different IC_{50} values of compounds **11a,b** and **11c,d**. Finally, **11c,d** exhibited a greater antioxidant efficiency than the respective **12**, confirming that the substition at position 2 of the ascorbyl residue is important for the antioxidant properties of these compounds, indeed specific enzymes, not present in our preliminary assay, may be responsible of the reported activity of **3**.[14]

The mechanism at the base of the improved antioxidant efficiency of compounds **11c,d**, as compared to other natural and synthetic antioxidants here tested, is currently under investigation. We may advance the hypothesis that the concomitant presence of the ascorbic acid moiety and α-tocopheryl residue may facilitate a radical trapping activity in both hydrophilic and lipophilic compartments by improving the antioxidant potential through concomitant scavenging of lipoperoxyl radicals (preferred by tocopherol) and reactive oxygen species (ROS, preferred by ascorbic acid). Moreover, it may induce increased stability of the compound by limiting its oxidation:[19] the ascorbic acid moiety on α-tocopheryl residue may reduce the production of tocopheroxyl radicals, which are usually formed during the radical scavenging activity and, consequently, may inhibit oxidation induced by these radicals.

In order to verify the influence of possible metabolic transformation, and to compare its activity with that of simple physical mixtures of the corresponding antioxidant moieties, stereoisomers **11c,d** were also compared to associations of the natural α-tocopherol and ascorbic acid and of the respective synthetic analogues **5** and **3**. As shown in Fig. 7, although **3** was the less active among the test compounds (compare Fig. 4 with Figs 2, 3 and 5–7) no significant differences in AAPH-induced MDA inhibition were found among the compounds **11c,d**, and the association of both **3** and **5**. This occurrence may be explained on the basis of the high activity displayed by **5** itself (Fig. 5) which represent an unexpected result of this study. The lack of significative

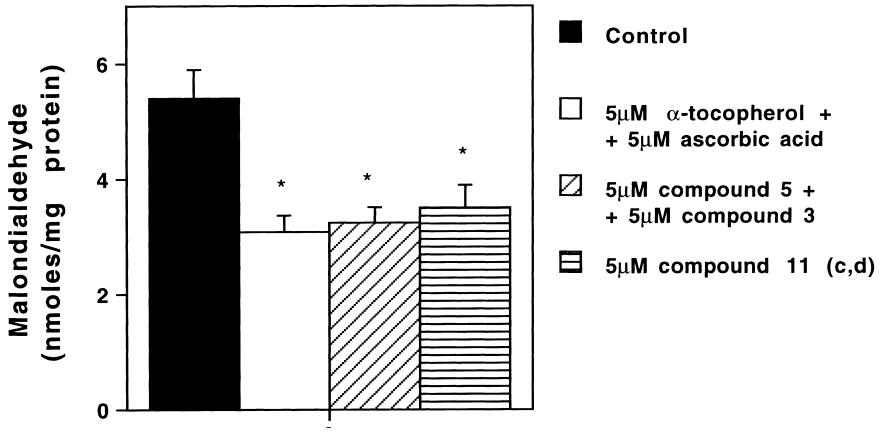


Figure 8. AAPH-induced MDA production in the absence (control) or in the presence of α-tocopherol + ascorbic acid, compound **5** + compound **3** or compounds **11c,d**, in rat liver microsomal membranes. Values are the means SEM of three different experiments. The value with asterisk are significantly different from control values ($p < 0.05$).

increasing in potencies may also be explained by a reduced mobility of compounds **11c,d** in aqueous phase or within the membrane bilayer with respect to α-tocopherol and ascorbic acid.

Pharmacology

Ischemia-reperfusion in isolated rabbit heart

Ischemic tissues, i.e., heart after myocardial infarct, are vulnerable to damage when rapidly reperfused with oxygen-rich blood in order to reduce the extent of the injury; this occurrence is known as reperfusion damage. The further damage is due to the presence of oxygen-derived free radicals and reactive oxygen species present in the reperfusion medium. Many classes of drugs and antioxidants such as α-tocopherol offer protection against reperfusion injury and, in view of the relevance of ischemic diseases, there is an increasing interest in the research of new agents. In view of this evidence, we thus decided to investigate the potential of our compounds in preventing damages induced by reperfusion on isolated rabbit heart.

When the perfusion flow rate of electrically paced isovolumic left rabbit heart preparations was reduced from 20 to 1 mL/min for a period of 40 min, a progressive increase in LVEDP values (from 4.1±0.5 to 72.2±6.8 mmHg; $p < 0.001$) occurred suggesting that an ischemic phenomenon was taking place. In fact, ventricular function was impaired since the recovery of post-ischemic LVDP was low and after 20 min of reperfusion, only 38% of the preischemic strength of heart contractility was restored (38.9±4.2 versus 102.5±8.5 mmHg; $p < 0.001$). At this time, CPP considerably augmented over the basal values (from 52.5±5.1 to 96.6±6.3 mmHg; $p < 0.001$) suggesting an increase in coronary vascular resistance owing to the stiffness of the heart (Figs 8 and 9). Furthermore, the partial functional recovery of the hearts during reperfusion was accompanied by a consistent release of CK into the cardiac perfusates. In fact, peak concentration of CK was increased 9.1-fold compared to preischemic values (from 49±6 to 448±40 mU/min/g wet tissue; $p < 0.001$) (Fig. 10).

When the hearts were perfused for 30 min in the preischemic period with graded concentrations of **11a–d** (100–300 μM), a myocardial protection against mechanical changes due to ischemia-reperfusion was significantly evident at the higher concentration of 300 μM. In fact, the increase in LVDP values observed in untreated preparations at the end of the ischemic period (from 5.8±0.5 to 34.1±2.5 mmHg; $p < 0.05$) was sig-

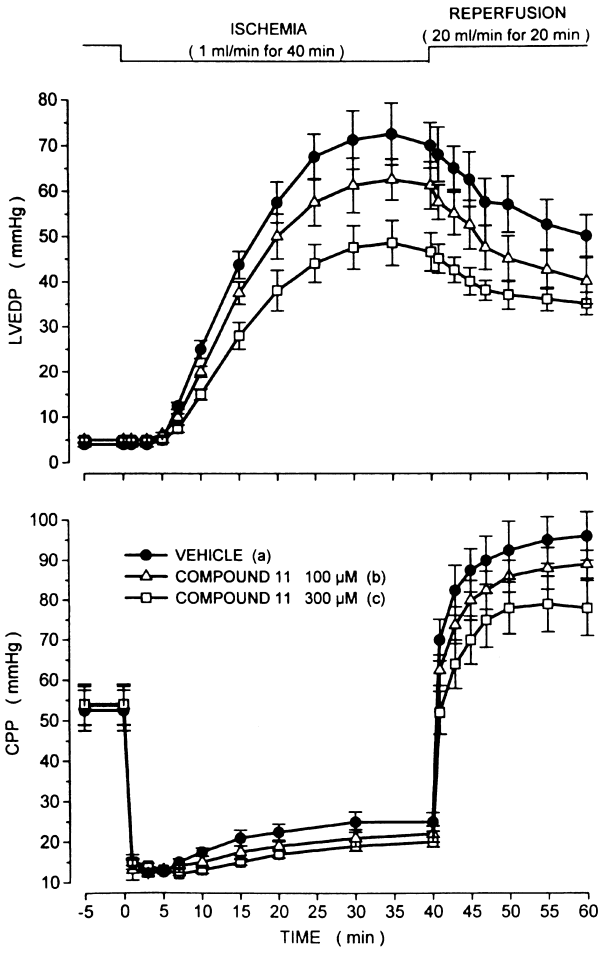


Figure 9. Effects of compounds **11a–d** in perfused rabbit hearts subjected to ischemia-reperfusion: trend of left ventricular end diastolic pressure (LVEDP) and coronary perfusion pressure (CPP). Data are mean±SEM of five different heart preparations per group. Compound **11** (100–300 μM) was perfused through the hearts for a period of 30 min before reduction of coronary flow. The areas under the curve (AUCs) related to LVEDP are: *a*, 3046±284; *b*, 2577±191; *c*, 2021±177. The AUCs related to CPP (increase in mmHg over the preischemic values) are: *a*, 716±83; *b*, 593±49; *c*, 424±23; with statistical differences for both LVEDP and CPP: *a* versus *b*, $p > 0.05$; *a* versus *c*, $p < 0.05$. The AUCs was evaluated according to the trapezoid method: in ordinate, LVEDP or CPP in mmHg; in abscissa, time from 0 to 60 min (for LVEDP) or from 40 to 60 min (for CPP).

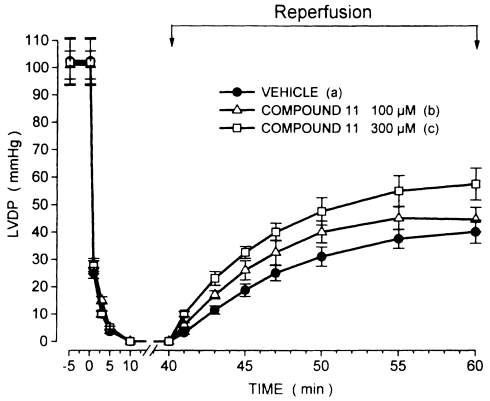


Figure 10. Effects of compounds **11a–d** on left ventricular developed pressure (LVDP) in perfused rabbit heart subjected to ischemia-reperfusion. Data are mean±SEM of 5 different heart preparations per group. Compounds **11a–d** (100–300 μM) were perfused through the hearts for a period of 30 min before reduction of coronary flow. The areas under the curve (AUCs) related to LVDP are: *a*, 593±42; *b*, 672±57, *c*, 835±52; with statistical differences for LVDP: *a* versus *b*, $p > 0.05$; *a* versus *c*, $p < 0.05$. The AUCs was evaluated according to the trapezoid method: in ordinate, LVDP in mmHg; in abscissa, time from 40 to 60 min.

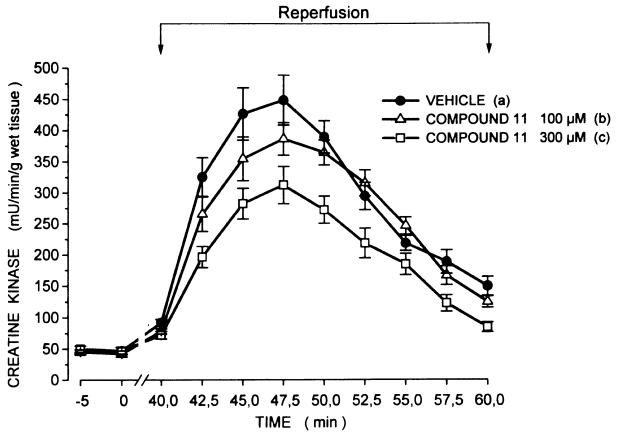


Figure 11. Kinetic profile of creatine kinase (CK) activity released in perfusates of rabbit hearts subjected to ischemia-reperfusion. Data are mean±SEM of five different heart preparations per group. Compound **11a–d** (100–300 μM) was perfused through the hearts for a period of 30 min before reduction of coronary flow. The areas under the curve (AUCs) related to CK are: *a*, 5081±408; *b*, 4553±254, *c*, 3220±272; with statistical differences for CK: *a* versus *b*, $p > 0.05$; *a* versus *c*, $p < 0.01$. The AUCs was evaluated according to the trapezoid method: in ordinate, CK in mU/min/g wet tissue; in abscissa, time from 40 to 60 min.

nificantly less in **11a–d** in infused hearts and at the end of reperfusion period LVDP reached 57% of the pre-ischemic values (57.5±5.8 versus 101.4±9.3 mmHg; $p < 0.01$). At the same time, the increase in CPP in untreated hearts was diminished (from 96.6±6.3 to 77.5±6.9 mmHg; $p < 0.05$) indicating a reduction of coronary vascular resistance (Figs 9 and 10). In keeping with these results, the kinetic profile of CK released in the cardiac effluent was significantly different from that observed in untreated preparations. At the peak of the concentration, CK was increased 6.2-fold (from 50±5 to 312±30 mU/min/g wet tissue; $p < 0.001$) (Fig. 11).

Stability

In order to investigate if the observed activity may be related to a possible hydrolysis of our molecules, stability of the mixture of stereosiomers **11a–d** was evaluated in water at 37 °C, at pH ranging from 3 to 8. Compounds **11a–d** proved stable, with half-lives ranging from 1 to 3 days (Table 2). The obtained half-lives confirm that they act per se and not after transformation into aldehyde **5** and L-ascorbic acid.

Table 2. In vitro hydrolysis of stereoisomers **11a–d** at different pH; $t_{1/2}$ 37 °C

Compound	Product	In vitro hydrolysis	
		pH	$t_{1/2}$ 37 °C, h[a]
11a–d	**5**, ascorbate	3	24
		5	38
		7	72
		8	60

[a]HPLC analysis; $t_{1/2}$ is the time required for 50% hydrolysis to the corresponding **5** and ascorbic acid.

In conclusion, some new molecules, based on the concept of the molecular combination of cooperative antioxidants, have been prepared. In a peroxyl radical-dependent lipoperoxidation assay, these compounds were all inhibitors of MDA formation. However, the synthetic compounds **11c,d**, which are the 5*R*,2*R*,2*R*,4*S* and 5*R*,2*R*,2*S*,4*S* isomers of the molecular combination of an α-tocopherol-analogue and L-ascorbic acid, resulted in interesting antioxidants with a potency comparable, if not slightly superior, to the respective synthetic α-tocopherol analogue (**5**) and natural α-tocopherol or L-ascorbic acid, used alone or in combination. Moreover, the major advantages of this compound derives from its capability to trap free radicals simultaneously in both hydrophilic and lipophilic environments. In view of these data **11a–d** were also investigated for their ability to prevent damage induced by reperfusion on isolated rabbit heart resulting effective at the higher concentration of 300 μM. Taken together, these results are of significance for possible therapeutic applications in pathological events in which free radical damage is involved and for the possible extension of our approach to other synergistic antioxidants.

Experimental

Materials

Compounds **4**,[13] **7**[15] and **8**[14] were prepared following and adapting reported procedures.

General

Melting points were determined by a Kofler apparatus and are uncorrected. Reaction courses were routinely monitored by thin layer chromatography (TLC) on silica gel precoated Durasil-25 UV_{254} Machery–Nagel plates with detection under 254 nm UV lamp and/or by spraying the plates with 10% H_2SO_4/MeOH and/or with 5% $KMnO_4/H_2O$ solutions and heating. Nuclear magnetic resonance (^{1}H NMR) spectra were determined in DMSO-d_6, $CDCl_3$ solutions with a Bruker AC-200 spectrometer and chemical shift are presented in ppm from internal tetramethylsilane as a standard. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) were obtained using the HPG2025A mass spectrometer. HPLC analysis was performed on Beckman System Gold with a Beckman ultrasphere ODS column (5 mm; 4.6×250 mm). Analytical determinations were carried out by two solvent systems: (method 1) A = water, B = acetonitrile, a linear gradient was initiated after injection of a sample and run from 100% A to 100% B in a 25 min. All compounds showed a purity higher than 99% following analytical HPLC monitored at 220 and 254 nm. Column chromatography was performed with Macherey–Nagel 70–230 mesh silica gel. Purity of final compounds was also assessed by high resolution mass and combustion analyses. Where not differently stated, microanalyses were in agreement with calculated values (sd±0.4%).

(*R,S*)-6-Hydroxy-(2,5,7,8-tetramethylchroman-2-yl)-acetaldehyde (5). A solution of 6-ethyl-(2,5,7,8-tetramethylchroman-2-yl)acetate (**4**) (2.5 g, 7.8 mmol) in 130 mL of anhydrous hexane, was cooled under argon atmosphere, in a dry ice/ethanol bath to −70 °C. To the resulting suspension was added, dropwise, a solution of diisobutyl–aluminum hydride in hexane (25%, 13 mL, 23.4 mmol). The reaction was monitored by TLC (hexane:Et_2O 7:3). After 15 min, the reaction was quenched at −70 °C with methanol and water, and worked-up with ether, washed with 2M HCl, dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography (hexane:Et_2O 7:3) to give 1.55 g of white solid; yield 82%. Mp 82–85 °C (Et_2O/hexane). ^1H NMR ($CDCl_3$): δ 1.32 (s, 3H, CH_3-C2); 1.79–1.87 (m, 4H, $ArCH_2\underline{CH_2}$); 2.03, 2.05, 2.09 (3s, 9H, 3 CH_3 Ar); 2.4–2.7 (m, 4H, $Ar\underline{CH_2}CH_2$, $\underline{CH_2}COH$); 9.85 (dd, 1H, $J=6$ and 2.4 Hz, CHO). MALDI-TOF MS: m/z 248.7 $(M)^+$, $C_{15}H_{20}O_3$ requires 248.03. Anal. C, H.

(*R,S*)-6-Benzyloxy-(2,5,7,8-tetramethylchroman-2-yl)-acetaldehyde (6). A mixture of **5** (2.2 g, 8.8 mmol), anhydrous K_2CO_3 (1.28 g, 9.2 mmol), benzyl bromide (1.2 mL, 10.8 mmol), and 20 mL of anhydrous DMF was stirred for 22 h at room temperature. The obtained suspension was poured into H_2O, worked-up with ether, dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography (hexane:Et_2O 9:1) to give 1.3 g of yellow oil; yield 43%. ^1H NMR ($CDCl_3$): δ 1.32 (s, 3H, CH_3-C2); 1.79–1.87 (m, 4H, $ArCH_2\underline{CH_2}$); 2.03, 2.05, 2.09 (3s, 9H, 3 CH_3 Ar); 2.48–2.61 (m, 4H, $Ar\underline{CH_2}CH_2$, CH_2COH); 4.73 (s, 2H, CH_2Ph); 7.40–7.56 (m, 5H, Ph); 9.85 (dd, 1H, CHO, $J=6$ and 2.4 Hz). MALDI-TOF MS: m/z 338.7 $(M)^+$, $C_{22}H_{26}O_3$ requires 338.44.

3,4-Dibenzyloxy-5*R*-2*S*-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R* and *S*)yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (9a,b) and 3,4-dibenzyloxy-5*R*-2*R*-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R* and *S*)yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (9c,d). A mixture of 2,3-*O*-benzyl-ascorbic acid (**7**) (1.2 g, 3.3 mmol), **6** (2.3 g, 6.8 mmol), *p*-toluenesulfonic acid (150 mg, 0.78 mmol) in 90 mL of benzene, was refluxed in a Dean–Stark apparatus for 8 h. The reaction was then poured into H_2O, worked-up with EtOAc, dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure, to give a 1:1:1:1 mixture of the four diastereoisomers (HPLC analysis). The crude product was purified by silica gel chromatography (hexane: Et_2O 6:4) to give compounds **9a–d**, overall yield 55%.

9a oil; 230 mg, yield 10%; TLC (hexane:Et_2O 6:4, 0.35); ^1H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $ArCH_2\underline{CH_2}$ benzopyran); 1.9 (dd, 2H, CH_2, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH_3 Ar); 2.5 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.49–4.50 (d, 1H, H-C5 furanone, $J=2.4$ Hz); 4.47 (d, 2H, CH_2-Ph, benzopyran); 5.01(d, 2H, CH_2-Ph at C3 furanone); 5.09 (m, 1H, H-C2 dioxolane); 5.14 (s, 2H, CH_2-Ph at C4 furanone); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 676.9 $(M)^+$, $C_{42}H_{44}O_8$ requires 676.79.

9b oil; 320 mg, yield 14%; TLC (hexane:Et_2O 6:4, 0.34); ^1H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $ArCH_2\underline{CH_2}$); 1.9 (dd, 2H, CH_2, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH_3 Ar); 2.5 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.47–4.49 (d, 1H, H-C5 furanone, $J=4$ Hz); 4.46 (d, 2H, CH_2-Ph, benzopyran); 5.01 (d, 2H, CH_2-Ph at C3 furanone); 5.09 (m, 1H, H-C2 dioxolane); 5.13 (s, 2H, CH_2-Ph at C4 furanone); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 677.8 $(M+H)^+$, $C_{42}H_{44}O_8$ requires 676.79.

9c foam; 280 mg, yield 12%; TLC (hexane:Et_2O 6:4, 0.32); ^1H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $Ar\underline{CH_2}CH_2$); 1.9 (dd, 2H, CH_2, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH_3 Ar); 2.5 (t, 2H, $ArCH_2CH_2$); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.48–4.50 (d, 1H, H-C5 furanone, $J=3.4$ Hz); 4.6 (d, 2H, CH_2-Ph benzopyran); 5.01 (d, 2H, CH_2-Ph at C3 furanone); 5.10 (m, 1H, H-C2 dioxolane); 5.14 (s, 2H, CH_2-Ph C4 furanone); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 676.8 $(M)^+$, $C_{42}H_{44}O_8$ requires 676.79.

9d foam; 370 mg, yield 16%; TLC (hexane:Et_2O 6:4, 0.30); ^1H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.6–1.8 (m, 2H, $ArCH_2\underline{CH_2}$); 1.9 (dd, 2H, CH_2, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH_3 Ar); 2.5 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.6-3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.50–4.52 (d 1H, H-C5 furanone, $J=3.2$ Hz); 4.6 (d, 2H, CH_2-Ph benzopyran); 5.01 (d, 2H, CH_2-Ph al C3 furanone); 5.09 (m, 1H, H-C2 dioxolane); 5.14 (s, 2H, CH_2-Ph at C4 dioxolane); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 676.9 $(M)^+$, $C_{42}H_{44}O_8$ requires 676.79.

3,4-Dihydroxy-5*R*-2*S*-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2(*R* and *S*)yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (11a,b) and 3,4-dihydroxy-5*R*-2*R*-(6-hydroxy-oxy-2,5,7,8-tetramethyl-chroman-2(*R,S*)yl-methyl)-1,3]di-oxolan-4*S*-yl]-5*H*-furan-2-one (11c,d). The four diastereisomers were deprotected by the following general procedure: each of the above obtained **9a–d** (200 mg, 0.29 mmol each-one) were dissolved in EtOAc (10 mL) and hydrogenated in presence of 10% Pd/C (50 mg) for 3 h, at atmospheric pressure. The catalyst was then removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography (CH_2Cl_2:MeOH 9:1) to give the expected **11a–d** in good yield (90–93%).

11a oil; 106 mg, yield 90%; ^1H NMR (DMSO-d_6): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $ArCH_2\underline{CH_2}$); 1.85 (dd, 2H, CH_2, $J=4$ Hz, $J=4.4$); 1.95, 2.0, 2.03 (3s, 9H, 3 CH_3 Ar); 2.49 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.79 (d, 1H, H-C5 furanone, $J=4$ Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.40 (s, 1H, OH 6-benzo-

pyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH, at C4 furanone). MALDI-TOF MS: m/z 406.8 $(M)^+$, $C_{21}H_{26}O_8$ requires 406.4. Anal. C, H.

11b oil; 108 mg, yield 92%; ^{1}H NMR (DMSO-d_6): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $ArCH_2\underline{CH_2}$); 1.8 (dd, 2H, CH_2, $J=4$ Hz, $J=4.4$); 1.95, 2.0, 2.03 (3s, 9H, 3 CH_3 Ar); 2.49 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.77 (d, 1H, H-C5 furanone, $J=4$Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.40 (s, 1H, OH 6 benzopyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH at C4 furanone). MALDI-TOF MS: m/z 406.9 $(M)^+$, $C_{21}H_{26}O_8$ requires 406.4. Anal. C, H.

11c foam; 109 mg, yield 93%; ^{1}H NMR (DMSO-d_6): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $ArCH_2\underline{CH_2}$); 1.8 (dd, 2H, CH_2, $J=4$ Hz, $J=4.4$); 1.95, 2.0, 2.03 (3s, 9H, 3 CH_3 Ar); 2.49 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.73 (d, 1H, H-C5 furanone, $J=4$Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.42 (s, 1H, OH benzopyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH, at C4 furanone). MALDI-TOF MS: m/z 406.9 $(M)^+$, $C_{21}H_{26}O_8$ requires 406.4. Anal. C, H.

11d foam; 106 mg, yield 90%; ^{1}H NMR (DMSO-d_6): δ 1.21 (s, 3H, CH_3-C2 benzopyran); 1.7 (m, 2H, $ArCH_2\underline{CH_2}$); 1.8 (dd, 2H, CH_2, $J=4$ Hz, $J=4.4$.); 1.95, 2.0, 2.03 (3s, 9H, 3 CH_2 Ar); 2.49 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.72 (d, 1H, H-C5 furanone, $J=4$Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.42 (s, 1H, OH benzopyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH, at C4 furanone). MALDI-TOF MS: m/z 406.9 $(M)^+$, $C_{21}H_{26}O_8$ requires 406.4. Anal. C, H.

4-*O*-Benzyloxy-5*R*-2*S*-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R,S*)yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (10a,b)- and 4-*O*-benzyloxy-5*R*-2*R*-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (10c,d). 2-*O*-Octadecyl-3-*O*-benzyl-ascorbic acid (**8**) (414 mg, 0.8 mmol) was reacted with **6** (540 mg, 1.6 mmol) in the same conditions described above for **9a–d**. After work up the solvent was removed under reduced pressure to give a 1:1:1:1 mixture of the four diastereoisomers. The crude product was purified by silica gel chromatography (hexane:Et_2O 6:4) to give **10a,b** and **10c,d** as inseparable mixtures of the couple of two diastereo-isomers.

10a,b: oil; 230 mg, yield 34%; ^{1}H NMR ($CDCl_3$): δ 0.80 (m, 3H, CH_3); 1.2 (s, 3H, CH_3-C2 benzopyran); 1.22–1.26 (m, 32H, alkyl); 1.6–1.8 (m, 2H, $ArCH_2\underline{CH_2}$); 1.9 (m, 2H, CH_2); 2.01, 2.08, 2.14 (3s, 9H, 3 CH_3 Ar); 2.5 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.8–4.0 (m, 4H, -OCH_2 alkyl, CH_2-C5 dioxolane); 4.1–4.3 (m, 1H, H-C4 dioxolane); 4.48 (**a**) and 4.49 (**b**) (d×2, 1H, $J=4$ Hz, H-C5 furanone,); 4.61 (d, 2H, CH_2–Ph benzopyran); 5.06–5.08 (m, 1H, H-C2 dioxolane); 5.4 (s, 2H, CH_2-Ph at C4 furanone); 7.1–7.4 (m, 10H, Ph). MALDI-TOF MS: m/z 839.8 $(M)^+$, $C_{53}H_{74}O_8$ requires 839.14.

10c,d: foam; 310 mg, yield 46%; ^{1}H NMR ($CDCl_3$): δ 0.80 (m, 3H, CH_3 alkyl); 1.20 (s, 3H, CH_3-C2 benzopyran); 1.22–1.26 (m, 32H, alkyl); 1.6–1.8 (m, 2H, $ArCH_2\underline{CH_2}$); 1.9 (m, 2H, CH_2); 2.01, 2.08, 2.14 (3s, 9H, 3 CH_3 Ar); 2.5 (t, 2H, $Ar\underline{CH_2}CH_2$); 3.8–4.0 (m, 4H, -OCH_2 alkyl, CH_2-C5 dioxolane); 4.1–4.3 (m, 1H, H-C4 dioxolane); 4.50 (**c**) and 4.52 (**d**) (d×2, 1H, H-C5 furanone, $J=4$ Hz); 4.61 (d, 2H, CH_2-Ph benzopyran); 5.08–5.10 (m, 1H, H-C2 dioxolane); 5.4 (s, 2H, CH_2-Ph at C4 furanone); 7.1–7.4 (m, 10H, Ph). MALDI-TOF MS: m/z 840.2 $(M+H)^+$, $C_{53}H_{74}O_8$ requires 839.14.

4-*O*-Hydroxy-5*R*-2*S*-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (12a,b) and 4-*O*-hydroxy-5*R*-2*R*-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (12c,d). Compounds **10a,b** (200 mg, 0.24 mmol) and **10c,d** (280 mg, 0.33 mmol) were deprotected by the procedure described above for compounds **9a–d**. After the usual work up and silica gel chromatography purification (CH_2Cl_2:MeOH 9:1), the expected **12a,b** and **12c,d** were obtained in good yields as inseparable mixtures of the of two diastereoisomer.

12a,b: oil; 142 mg, yield 90%; ^{1}H NMR (DMSO-d_6): δ 0.84 (t, 3H, $J=6$ Hz, CH_3, alkyl); 1.20 (s, 3H, CH_3-C2 benzopyran); 1.22–1.26 (m, 32H, CH_2 alkyl); 1.6–1.8 (m, 2H, Ar-CH_2-$\underline{CH_2}$); 1.9 (dd, 2H, $J=5$ and 4.4 Hz, -CH_2-); 1.95, 1.99, 2.03 (3 s, 9H, 3 CH_3 Ar.); 2.44–2.5 (m, 2H, Ar-$\underline{CH_2}$-CH_2-); 3.83–3.92 (m, 4H, CH_2-C5 dioxolane, -OCH_2 alkyl); 4.31–4.38 (m, 1H, -CH, H-C4 dioxolane); 4.82 (**a**) and 4.84 (**b**) (d, 1H, $J=2.2$ Hz, H-C5 furanone); 4.96–5.01 (m, 1H, H-C2 dioxolane); 7.40 (sbr, 1H, OH benzopyran); 10.9 (sbr, 1H, OH at C4 furanone). MALDI-TOF MS: m/z 659.8 $(M+H)^+$, $C_{39}H_{62}O_8$ requires 658.9. Anal. C, H.

12c,d: foam; 148 mg, yield 94%; ^{1}H NMR (DMSO-d_6): δ 0.84 (t, 3H, $J=6$ Hz, CH_3, alkyl); 1.20 (s, 3H, CH_2-C2 benzopyran); 1.22–1.26 (m, 32H, CH_2, alkyl); 1.6–1.8 (m, 2H, Ar-CH_2-$\underline{CH_2}$); 1.9 (dd, 2H, $J=5$ and 4.4 Hz, -CH_2-); 1.95, 1.99, 2.03 (3 s, 9H, 3 CH_3 Ar); 2.44–2.5 (m, 2H, Ar-$\underline{CH_2}$-CH_2); 3.83–3.92 (m, 4H, CH_2-C5 dioxolane, -OCH_2 CH_2 alkyl); 4.31–4.38 (m, 1H, -CH-, H-C4 dioxolane); 4.78 (d, 1H, $J=2.2$ Hz, H-C5 furanone); 4.99–5.01 (m, 1H, -CH-, H-C2 dioxolane); 7.42 (sbr, 1H, OH benzopyran); 11.1 (sbr, 1H, OH at C4 furanone). MALDI-TOF MS: m/z 658.9 $(M)^+$, $C_{39}H_{62}O_8$ requires 658.9. Anal. C, H.

Chemical stability

Solutions of compounds **11a–d** were prepared dissolving an aliquot of compounds in water at pH 3, 5 and 8 to give a final concentration of about 10^{-3} M. The solution was maintained at 37 °C and aliquots were withdrawn every 2 h for the initial 12 h of incubation and successively every 12 h for 4 days. The disappearance of the compounds was monitored by HPLC analysis using the method 1. Conjugates half-lives were from 1 to 3 days.

Molecular modeling

Computational studies have been conducted on a Silicon Graphics Indigo 2 and O2 work stations using the SYBYL[20] and SPARTAN[21] software packages. A systematic conformational search was conducted on compounds **11(a,b)** *cis* and **11(c,d)** *trans* with SYBYL and all the conformers obtained were energy minimized using MAXIMIN2 (Tripos force field, Powell method[22]). Semiempirical quantum-mechanic calculations were performed using the MOPAC (AM1) program[23] under SYBYL. The conformations having closer distances between C5 proton at the furanone ring and C3 proton and C2 methyl at the chromanyl residue were studied with ab initio Hartree–Fock methods,[24] geometry was optimized with STO-3G basic set to determine the position of the three ring systems.

Biology

Preparation of microsomal membranes

Microsomal membranes were isolated from adult male Wistar rats as previously reported.[25] Microsomal protein were determined by Bradford method.

Lipid peroxidation. Lipid peroxidation was initiated by 2-2′-azobis(2-amidinopropane)dihydrochloride (AAPH) as previously described.[10] The reaction was carried out with 25 mM AAPH at 37 °C under air for 15 min. Lipid peroxidation was measured by the appearance of malondialdeyde (MDA) formation. MDA was extracted and analyzed by HPLC as indicated.[26] Briefly, aliquots of 1 mL of membrane suspension were mixed with 0.5 mL of TBA solution (two parts 0.4% TBA in 0.2 M HCl and one part distilled water) and 0.07 mL of 0.2 BHT in 95% ethanol. Samples were then placed in a 90 °C water bath for 45 min. After incubation, the TBA–MDA adduct was extracted with isobutyl alcohol. The isobutanol extract was mixed with methanol (2:1) prior to injection in HPLC system. The column was packed with Supelcosil LC-18 material, 3 μm particle size, in a 15 cm×4.6 mm cartridge format (Supelco, Bellefonte, PA). A 2 cm cartridge precolumn containing 5 μm LC-18 Supelcosil packing was used. The mobile phase was a 1:1 (v:v) mixture of methanol and double distilled water, with the addition of tetrabutyl ammonium dihydrogen phosphate (0.06%, w:v), as an ion pairing reagent. The TBA–MDA adduct was detected by a fluorimeter set at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. At a flow rate of 1 mL/min, the retention time of TBA–MDA adduct was 5 min. MDA concentration was calculated from a calibration curve generated from a peak height of the MDA standard, prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane.[27] The concentration necessary to inhibit MDA formation by 50% (IC_{50}) was calculated from linear plots (concentration versus percent of control) of at least three different concentrations.

Pharmacology

Ischemia-reperfusion in isolated rabbit heart

All experimental protocols were approved by the Animal Care Committee of the University of Milan and were in accordance with the Italian guidelines for the laboratory animals which conform with the European Communities Directive of November 1986 (86/609/EEC).

Male New Zealand White rabbits (BMG-Allevamento, Cividate al Piano, BG, Italy) weighing 2.2–2.5 kg were used for these experiments. The hearts were excised and perfused retrogradely at 37 °C through the aorta as previously described by Henry et al.[27] and slightly modified by Berti et al.[28] The perfusion medium (Krebs Henseleit) contained (in mM): NaCl 118, KCl 2.8, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5. The pH of the perfusate was 7.4 after a period of equilibration with a 5% CO_2 and 95% O_2 gas mixture, and the rate of perfusion was maintained at 20 mL/min with a roller pump (Minipuls 3, Gilson, Villiers-Le Bel, France). Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were measured with two HP-1280C pressure transducers (Hewlett-Packard, Waltham, MA, USA) connected with a Hewlett-Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter (with a small latex balloon on the top) inserted in the left ventricular cavity. The balloon was filled slowly with saline until left ventricular end-diastolic pressure (LVEDP) stabilised in the range of 4–6 mmHg. Left ventricular developed pressure (LVDP; peak left ventricular systolic pressure minus LVEDP) was also evaluated. The hearts were electrically paced at a frequency of 180 beats/min with rectangular impulses (1 ms duration, voltage 10% above threshold) by a Grass stimulator (mod. S-88; Grass Instr., Quincy, MA, USA). CPP of 55–60 mmHg was obtained with a flow of 20 mL/min (preischemic period). Ischemia was induced by reducing the flow rate to 1 mL/min for 40 min (ischemic period; CPP = 12–14 mmHg). A normal flow rate (20 mL/min) was then restored, and the perfusion was continued for 20 min (reperfusion period). The compound under investigation, **11a–d** was tested in groups of five hearts each. In particular, **11a–d** (100–300 μM) was perfused through the hearts for a period of 30 min before reduction of coronary flow.

Creatine kinase determination

The heart perfusates were collected every 150 s in an ice-cooled beaker before flow reduction and during reperfusion and the activity of creatine kinase (CK) was evaluated according to the method of Bergmeyer et al.[29] by a specific kit (Amersham-Italia, MI, Italy). The amount of the enzyme was determined on a Lambda16-spectrophotometer (Perkin–Elmer Italia, Monza, MI, Italy) and expressed as mU/min/g/wet tissue.

Data analysis

In all experiments, differences between control and treated groups were analyzed for statistical significance using a one-way analysis of variance (ANOVA) and

Tukey–Kramer test for multiple comparisons. A value of $p < 0.05$ was considered significant. In all figures, results are expressed as mean±standard error of the mean (SEM). The areas under the curve (AUCs) were assessed by using a computerized program Microcal Origin.[30]

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