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Inhibition of Rat Liver Microsomal Lipid Peroxidation Elicited by 2,2-Dimethylchromenes and Chromans Containing Fluorinated Moieties Resistant to Cytochrome P-450 Metabolism

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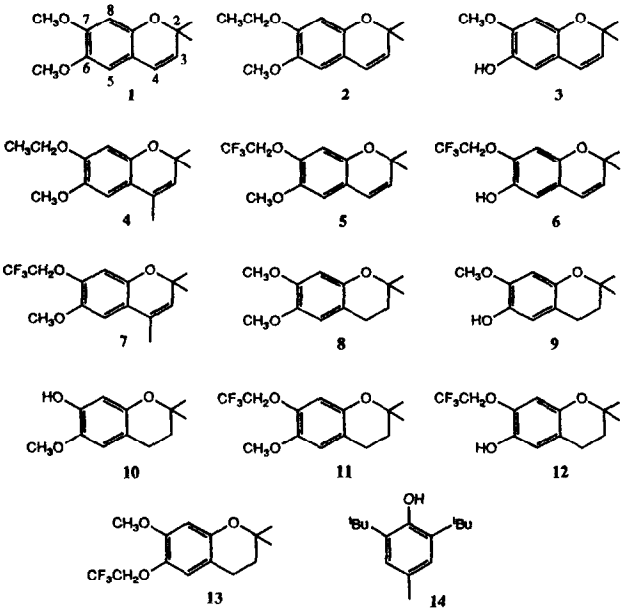
Abstract—2,2-Dimethylchromenes and chromans containing cytochrome P-450 resistant 2,2,2-trifluoroethoxy aryl substituents were synthesized and their activity as lipid peroxidation inhibitors evaluated and compared with that exhibited by the corresponding non-fluorinated derivatives. Lipid peroxidation was stimulated in rat liver microsomes by addition of Fe–ascorbate or NADPH, and determined with the TBARS (thiobarbituric acid reactive substances) test. In assays using Fe–ascorbate stimulation, only those derivatives with a OH group at C6 (i.e. **6** and **12**) elicited good inhibitory activities (IC_{50} = 6.0 and 5.3 μM, respectively). In respect to the NADPH dependent incubations, inhibitory activity of compound **11** (IC_{50} = 6.0 μM) was the highest found within the 6,7-dialkoxy derivatives tested. Results on metabolism assays with this compound showed the generation of phenol **12** (i.e. the putative active antioxidant species); on the other hand, no metabolite resulting from dealkylation at C7 was detected, thus confirming the resistance conferred by the CF_3CH_2O group to the cytochrome P-450 promoted cleavage. Finally, in assays where incubations in the presence of NADPH were prolonged up to three hours, inhibitory activity of the non-fluorinated 6,7-dialkoxychroman **8** remained constant, thus suggesting that a continued release of the species responsible for the inhibitory activity was produced. However, inhibition elicited by the fluorinated analog **11** showed a small decrease during the third hour of incubation. This decrease could be attributed to the slight inhibition of the cytochrome P-450 metabolism exerted by substrates bearing the CF_3CH_2O substituent, which would decelerate the generation of the active phenol species. Concerning the derivatives with a free hydroxyl at C6 (i.e. compounds **9** and **12**), although their inhibitory activities decreased during the period studied, that of fluorinated compound **12** was more persistent.

Introduction

Membrane lipid peroxidation is a pathophysiological event which can cause degenerative processes going from membrane denaturation to tissue damage.[1] Therefore, the search of compounds exhibiting activities as lipid peroxidation inhibitors is an active field of investigation. In this sense, a family of vitamin E analogs with an excellent peroxyl radical scavenging activity has been recently described by Battioni *et al.*[2] In the same context, we have reported the inhibitory activity of lipid peroxidation elicited by compounds with 2,2-dimethylchromene or chroman structure on NADPH dependent incubations of rat liver microsomes.[3] Among the compounds tested, those bearing a hydroxyl group at C6, i.e. chromene **3** and chroman **9** (Scheme I) were the best inhibitors, which suggested that the inhibitory effect was due to a free radical scavenger mechanism operated by phenolic species structurally related to vitamin E already present or generated during the incubation. This hypothesis was supported by metabolism assays performed on other potent inhibitors, such as dialkoxy derivatives **1** and **8**. Incubation of these compounds with rat liver microsomes in the presence of NADPH led to the identification of the corresponding hydroxy derivatives **3** and **9** as metabolites, respectively, although an important oxidative metabolism also took place at other points of the molecule, particularly at C7 and C4. Therefore, modification of the substitution pattern at these positions to reduce undesired metabolism



Scheme I. Chromans, chromenes and reference compound tested as lipid peroxidation inhibitors. Systematic numbering of relevant carbon atoms of benzopyran skeleton is indicated in compound **1**.

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would improve the efficiency of these lipid peroxidation inhibitors. Our recent finding on the resistance of the 2,2,2-trifluoroethoxy aryl moiety to the cytochrome P-450 promoted metabolism in rat liver microsomes,[4] led us to assay this fluorinated substituent for the above purposes.

In this paper we report our results on the inhibitory effect on rat liver microsomal lipid peroxidation caused by fluorinated 2,2-dimethylchromans and chromenes. This study was carried out in incubations where lipid peroxidation was enzymatically stimulated by NADPH or chemically stimulated with Fe(II) and ascorbate, and results were compared with those obtained using the respective non-fluorinated derivatives. Identification of primary metabolites formed during the incubations with selected fluorinated substrates (i.e. compounds **5** and **11**) were performed to confirm the metabolic resistance of the fluorinated substituent under our assay conditions. Finally, assays with derivatives **8**, **9**, **11** and **12** under prolonged incubation times to assess the persistency of the inhibitory activity were also carried out.

Results and Discussion

Evaluation of lipid peroxidation inhibition elicited by several fluorinated and non-fluorinated 2,2-dimethylchromans and chromenes in NADPH induced or Fe–ascorbate stimulated incubations of rat liver microsomes is shown in Table 1. In all cases the thiobarbituric acid-reactive substances (TBARS) production test was the method used for this evaluation. Values for BHT were also determined for comparison purposes. It has been reported that fluorimetric determination of TBARS could be interfered with by compounds already present in the medium or generated during the incubation with Fe–ascorbate.[5–7] Accordingly, incubations with hydroxychroman **9** (2 and 10 μM concentrations) were carried out and TBARS production measured by fluorimetry was compared with thiobarbituric acid–malondialdehyde (TBA–MDA) complex contents determined by HPLC. Results obtained did not show differences between both methods; therefore, the fluorimetric one was used all through this study for simplicity reasons.

Our hypothesis that the inhibition of rat liver microsomal lipid peroxidation in NADPH incubations caused by a family of 2,2-dimethylchromenes and chromans was due to a free radical scavenger mechanism analogous to that operating in vitamin E mode of action[3] has found additional support with the results reported herein. Thus, only those derivatives with a free hydroxy group (i.e. compounds **3**, **9** and **10**) elicited potent inhibitory effects when lipid peroxidation was induced by Fe–ascorbate (Table 1). These effects were three to six-fold lower than those obtained in NADPH dependent assays. A similar relationship was obtained with BHT (**14**), a compound tested for comparison purposes, which indicates that the observed discrepancies were due to intrinsic differences among both assays. On the other hand, the absence of dealkylative metabolism in Fe–ascorbate incubations justify that 6,7-dialkoxy derivatives such as compounds **1** and **8** were inactive as lipid peroxidation inhibitors under these conditions.

Table 1. Inhibitory effects of 2,2-dimethylchromans and chromenes **1**–**13** on lipid peroxidation of rat liver microsomes incubated with NADPH or Fe–ascorbate, expressed as IC_{50} values for the inhibition of TBARS production[a]

	IC_{50} TBARS production (μM)	
Compound	NADPH	Fe-ascorbate
1	11.4[b]	(35%)[c]
2	69.4	N.D.[d]
3	1.4[b]	3.8
4	39.2	N.D.
5	55.4	N.D.
6	3.7	6.0
7	108	N.D.
8	15.0[b]	(17%)
9	0.3[b]	1.9
10	3.4[b]	19.0
11	6.0	(5%)
12	3.9	5.3
13	240	(0%)
14	0.8[b]	2.7

[a]For experimental details, see Experimental Section. Values for compound **14** were determined for comparison purposes.
[b]Values taken from Ref. 3.
[c]Values in parentheses represent inhibition percentages calculated at a concentration 0.5mM.
[d]Not determined.

As it occurred with incubations with NADPH,[3] hydroxychroman **9** was the strongest inhibitor in assays with Fe–ascorbate (IC_{50} = 1.9 μM), and its activity was again higher than that of BHT (IC_{50} = 2.7 μM). This inhibitory activity could also be compared to that elicited by other chromanols studied elsewhere.[8] On the other hand, the fact that the inhibition caused by **9** is an order of magnitude higher than that of compound **10** (as it happened in NADPH dependent incubations), points out the importance of a free hydroxy group at C6 for eliciting a strong antioxidant effect. In this context, the effectiveness of vitamin E analogs bearing a free OH group at C6 as lipid peroxidation inhibitors is well documented in the literature.[9]

Nevertheless, metabolism studies carried out previously with compounds **1** and **8** showed the formation, besides the corresponding hydroxy derivatives **3** and **9**, respectively, which would account for the antioxidant activity of the parent substrates in NADPH dependent incubations, of other metabolites.[3] In particular, although a product resulting from dealkylation at C7 was observed in both cases, chromene **1** gave rise to the corresponding (*Z*/*E*)-3,4-diol derivatives whereas chroman **8** underwent hydroxylation at C4. Therefore, to improve either the potency or the persistency of the lipid peroxidation inhibition effect it was convenient to protect these sensitive positions, in particular C7, from the cytochrome P-450 associated metabolism. In this context, encouraging

results observed by us on the resistance of the CF_3CH_2O aryl moieties to the cytochrome P-450 promoted dealkylations[4] led us to synthesize the fluorinated derivatives **5–7** and **11–13** and test them as lipid peroxidation inhibitors. In the case of compound **7** an additional protection of C4 by introducing a methyl group was also sought.

Fluorinated chromenes

Results on the inhibitory activity of fluorinated chromenes in incubations in the presence of NADPH (Table 1) show that compound **5** elicited an activity comparable to that exhibited by the non-fluorinated analog **2**. To confirm that the presence of a CF_3CH_2O-substituent could prevent the dealkylation metabolism at the position where it is attached without interfering with other cytochrome P-450 mediated oxidations, in particular that at C6, NADPH dependent metabolism of chromene **5** was studied and results obtained are shown in Scheme II.

Thus, the HPLC-Diode Array Detector (HPLC-DAD) analysis of the organic extract from the above incubations showed the presence of two polar metabolites. One of them coeluted with the *Z/E* isomeric mixture of diols **15** obtained from the epoxidation of chromene **5** with dimethyldioxirane,[10] followed by reaction with water. The presence of this diol mixture as metabolites was confirmed by GC-MS analysis. The second metabolite was identified by HPLC-DAD and GC-MS as hydroxychromene **6**, a compound arising from the dealkylation of **5** at C6. These metabolites together with unreacted substrate accounted for over 95% of the amount of compound **5** added to the incubation mixture (HPLC, external standard method). It is worth noting that the presence of a metabolite originated from the dealkylation of the fluorinated substituent (i.e. hydroxychromene **16**) could not be detected under the conditions assayed. These results indicate that the fluorinated substituent effectively protects C7 towards metabolic deactivation. However, this protection does not improve the inhibitory activity of chromene **5** in comparison with that of the non-fluorinated analog **2**. This fact could be explained by the extended metabolism occurring at the C3–C4 double bond in these molecules.[11] On the other hand, as shown in Table 1, introduction of a methyl substituent at C4 did not afford conclusive results, since the increase in inhibitory activity obtained for the non-fluorinated chromenes (cf. compounds **4** and **2**) was not observed for the respective fluorinated derivatives (**7** and **5**).

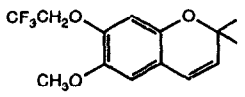
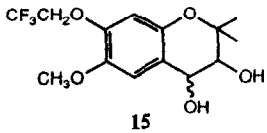
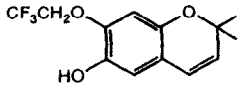
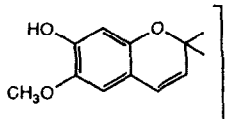
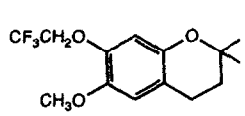
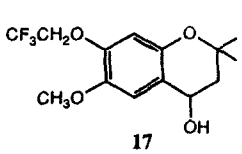
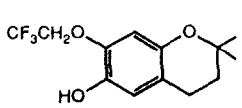
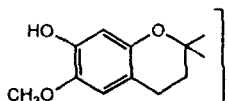
Among the fluorinated chromenes assayed, the 6-hydroxy derivative **6** exhibited the highest inhibitory activity in incubations with NADPH (IC_{50} = 3.7 μM). Therefore, its detection as a metabolite in the incubations of **5** with NADPH (see above) could justify the inhibitory activity elicited by this 6,7-dialkoxychromene. In respect to the incubations with Fe–ascorbate, compound **6** also exhibited a high inhibitory activity (IC_{50} = 6.0 μM).

Fluorinated chromans

Results on the inhibitory activity of fluorinated chromans in incubations in the presence of NADPH are also shown in Table 1 and Figure 1. The first observation is that the inhibitory activity elicited by chroman **11** (IC_{50} = 6.0 μM) is 9-fold higher when compared to that produced by the fluorinated chromene **5** (IC_{50} = 55.4 μM). In addition, comparison of the inhibitory activities of **11** and the non-fluorinated analog **8** indicates that the presence of the fluorinated substituent at C7 improves the lipid peroxidation inhibition effect. Since chroman **11** did not show inhibitory activity *per se* (cf. incubations with Fe–ascorbate, Table 1), a metabolism study of this compound in the presence of NADPH was carried out to identify the putative antioxidant species responsible for the observed activity.

Accordingly, the HPLC-DAD analysis of the organic extract from the above incubations showed the presence of two polar metabolites with a UV spectrum similar to that of **11** (Scheme II). Likewise, the GC-MS analysis of this extract showed three peaks in addition to that of chroman **11**, which exhibited molecular ions and fragmentation patterns related to the structure of the incubated substrate. One of them was identified as hydroxychroman **12**. A second peak was assigned to compound **17**. In this case, the presence of a molecular ion at m/z 306 and the fragmentation pattern supports the proposed structure. A further confirmation of the presence of this hydroxylated metabolite was the third peak, which was identified as chromene **5**. In this sense, it has usually been observed that 2,2-dimethylchromans bearing a hydroxy substituent at C4 undergo dehydration when injected onto a GC system. The amounts of metabolites **12** and **17** formed together with that of unreacted substrate accounted for over 97% of compound **11** added to the incubation mixture (HPLC, external standard method). Finally, the presence of a metabolite originated from the dealkylation of the fluorinated substituent (i.e. hydroxychroman **10**) could not be detected, which suggested that the metabolism protection conferred by the fluorinated substituent could account for the higher inhibitory effect observed for compound **11** in comparison with that elicited by the non-fluorinated chroman **8**. This assumption found additional support from the comparison of inhibitory activities between fluorinated chromans **11** and **13**. In this latter compound, exhibiting an IC_{50} value 40-fold higher than that of **11**, protection against dealkylation at C6 leaves C7 as the unique position to give rise to a free hydroxy group, which would originate less efficient peroxyl radical scavenger species.

Concerning the fluorinated hydroxy derivative **12**, incubations with NADPH afforded the highest activity within the fluorinated compounds assayed (IC_{50} = 3.9 μM). This result supports the assumption that **12** could be the species responsible for the potent inhibitory activity elicited by the 6,7-dialkoxychroman **11**. In respect to the incubations in the presence of Fe–ascorbate, hydroxy fluorinated derivative **12** exhibited an inhibitory activity (IC_{50} = 5.3 μM) which confirmed that this compound is a lipid peroxidation inhibitor *per se*. Conversely, 6,7-dialkoxylated chroman **11** did not exhibit inhibitory activity under these conditions.

	SUBSTRATE	METABOLITES		
	 5	 15	 6	[ 16]
UV (λ_{max})	225, 270 and 320 nm	210, 225 and 290 nm	210, 240 and 320 nm	210, 255 and 320 nm
MS (m/z, %)	288 (M^+, 20) 273 [(M-Me)$^+$, 100] 189 [(M-CF_3CH_2O)$^+$, 25]	322 (M^+, 30) 250 [(M-$(CH_3)_2CHOH$)$^+$,100] 235 [(250-Me)$^+$, 95]	274 (M^+, 30) 259 [(M-Me)$^+$, 100]	206 (M^+, 20) 191 [(M-Me)$^+$, 100] 176 [(M-2Me)$^+$, 28]
	 11	 17	 12	[ 10]
UV (λ_{max})	205, 220 and 295 nm	205, 225 and 295 nm	205, 225 and 295 nm	205, 225 and 295 nm
MS (m/z, %)	290 (M^+, 30) 235 [(M-$(CH_3)_2CCH$)$^+$, 100]	306 (M^+, 17) 288 [(M-H_2O)$^+$, 12] 273 [(M-H_2O-Me)$^+$, 100] 251 [(M-$(CH_3)_2CCH$)$^+$, 40]	276 (M^+, 40) 221 [(M-$(CH_3)_2CCH$)$^+$, 100]	208 (M^+, 30) 153 [(M-$(CH_3)_2CCH$)$^+$, 100]

Scheme II. Spectroscopic (UV-diode array) and mass spectra (GC-MS, electron impact) data of the metabolites generated in the incubation of fluorinated substrates **5** and **11** with rat liver microsomes in the presence of NADPH. Compounds in parentheses were not detected under the conditions assayed.

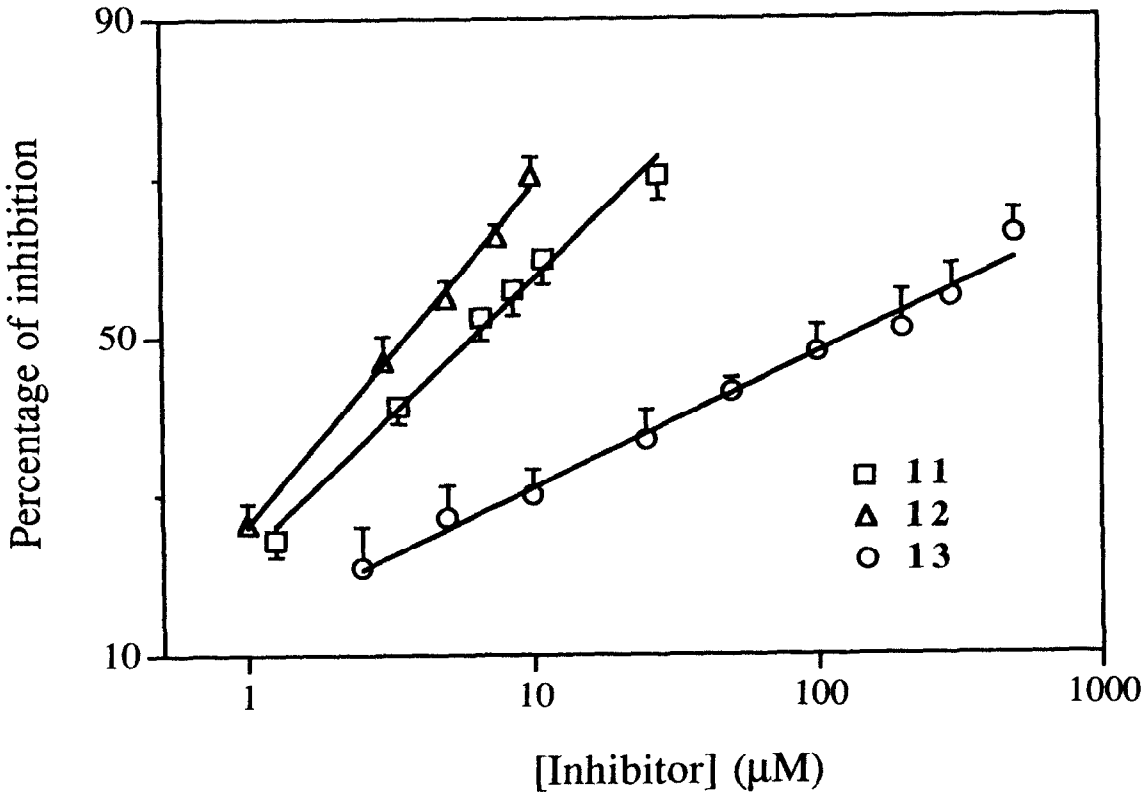


Figure 1. Inhibition of NADPH-induced lipid peroxidation elicited by fluorinated chromans **11**, **12** and **13** in rat liver microsomes. The results represent the average of three different experiments ± SD

Persistency of the lipid peroxidation inhibitory effect

From the above results it can be derived that the 2,2-dimethylchromans assayed, fluorinated or non, were better lipid peroxidation inhibitors than their corresponding 2,2-dimethylchromene analogs. Therefore, four of these chromans (i.e. compounds **8**, **9**, **11** and **12**) were chosen to study the persistency of the inhibitory activity. These inhibitors were independently incubated for three hours in the presence of NADPH using concentrations close to their respective IC_{50} values and results obtained are shown in Figure 2. For the case of the non-fluorinated substrates, while the inhibitory effect elicited by chroman **8** remained constant, activity of hydroxychroman **9** decreased significatively after the second and third hours of incubation. This decrease could be due to the deactivating metabolism suffered by the compound in the incubation medium. On the other hand, the fact that activity of dialkoxychroman **8** remained essentially unaltered through all the experiments could be due to the constant release of the active antioxidant species of **8**, i.e. the metabolite **9**. In this sense, the concentration of the hydroxy derivative **9** generated was estimated to exceed its IC_{50} value.[3] Hence, compound **8** could be envisaged as an inhibitor of lipid peroxidation with a potential slow-release mode of action.

Concerning the fluorinated derivatives, the inhibitory effect caused by fluorinated chroman **11** was slightly higher during the second hour of incubation, which suggests that a continuous generation of an active species, i.e. the hydroxy derivative **12** (cf. Scheme II), could take place. The identification of this compound as a metabolite of substrate **11** supports this hypothesis. The significant decrease of the inhibitory activity observed during the third hour could be due to a deceleration of the dealkylation metabolism leading to the formation of the active antioxidant species. This assumption is based on our finding that substrates bearing a CF_3CH_2O aryl moiety exerted a slight inhibition of the cytochrome P-450 promoted dealkylation metabolism.[4] It is worth noting that the above decrease is not significant when compared to the inhibition percentage measured for the first hour of incubation ($P > 0.005$). Therefore, this fluorinated chroman appears also to be a potent lipid peroxidation inhibitor with slow-release mode of action.

On the other hand, as observed with hydroxychroman **9**, inhibitory activity of compound **12** decreased during the second and third hours of incubation. Nevertheless, this decrease was less pronounced than that obtained for **9**, which could indicate that the active species is more persistent in the incubation medium. The extent at which the fluorinated substituent participates for attaining this persistency remains to be explored.

In conclusion, results herein reported indicate that a proper combination of protection in front of oxidative metabolism by introduction of fluorinated substituents at selected positions and occurrence of free hydroxy groups at C6 (already present or potentially generated through dealkylation) led to lipid peroxidation inhibitors presenting either more persistent intrinsic activities (cf. **9** and **12**) or a more metabolism-dependent antioxidant efficiency (cf. **8** and **11**).

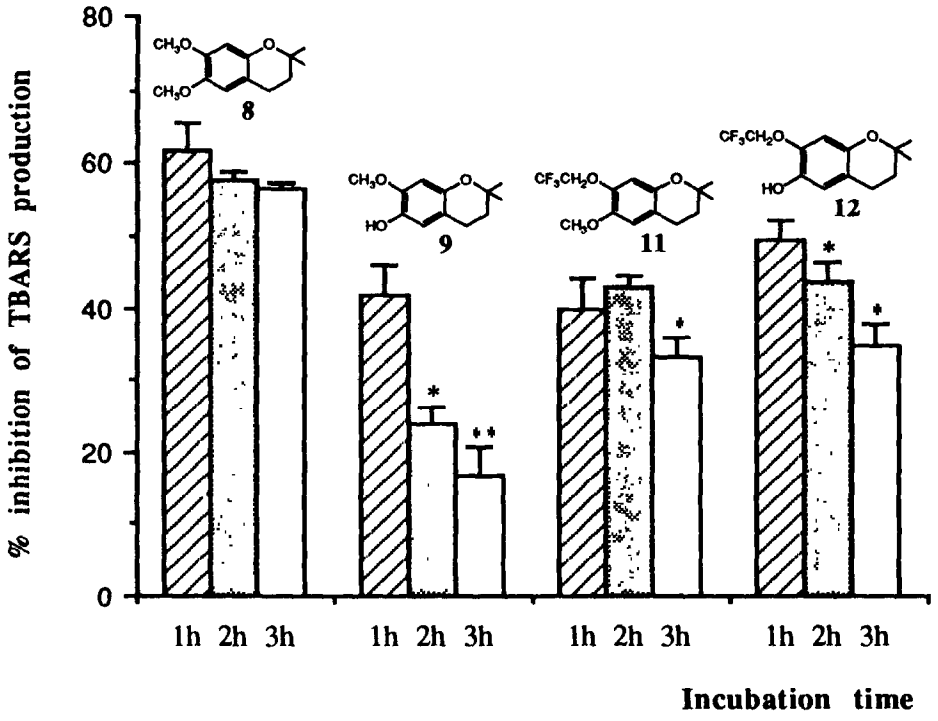


Figure 2. Effect of the incubation time on the inhibition of NADPH-induced rat liver microsomal lipid peroxidation elicited by chromans **8**, **9**, **11** and **12** at the concentrations of 15, 1, 6 and 5 μM, respectively. Values shown are the mean ± SD of sextuplicate determinations. Differences between consecutive hours are marked by asterisks: $^{*}P < 0.001$, $^{**}P < 0.005$

Experimental Section

Compounds

General. The IR spectra were recorded with a Bomen MB 120 spectrometer. The ^1H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded with a Varian Unity 300 spectrometer. All NMR spectra were performed in neutralized $CDCl_3$ solutions and chemical shifts are given in ppm downfield from tetramethylsilane. The HPLC analyses were carried out with an Applied Biosystems set constituted by a model 400 Solvent Delivery System, a model 491 Injector and 1000S Diode Array Detector (DAD). The gas chromatography-mass spectrometry (GC-MS, electron impact) analyses were carried out with a Hewlett-Packard 5995 apparatus provided with a 30 m HP-5 bonded phase capillary column. Elemental analyses were performed with a Carlo Erba 1108 instrument (Microanalysis Service, CID).

Synthesis of test compounds (Scheme I)

Preparation of 6,7-dimethoxy-2,2-dimethyl-1-2*H*-benzopyran (**1**), 6-hydroxy-7-methoxy-2,2-dimethyl-1-2*H*-benzopyran (**3**), 3,4-dihydro-6,7-dimethoxy-2,2-dimethyl-1-2*H*-benzopyran (**8**), 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1-2*H*-benzopyran (**9**), 3,4-dihydro-7-hydroxy-6-methoxy-2,2-dimethyl-1-2*H*-benzopyran (**10**) and 7-hydroxy-6-methoxy-2,2-dimethyl-1-2*H*-benzopyran (**16**, Scheme II) has been described elsewhere.[3] 2,6-Di-*tert*-butyl-4-methylphenol (BHT, **14**) was commercially available. 6-Methoxy-2,2-dimethyl-7-(2,2,2-trifluoroethoxy)-1-2*H*-benzopyran (**5**) was prepared from the corresponding benzopyran-4-one[12] as described.[13] The same procedure was used for the preparation of 7-ethoxy-6-methoxy-2,2-dimethyl-1-2*H*-benzopyran(**2**).[14]

The fluorinated chromans **11** and **13** were prepared by reduction of their corresponding benzopyran-4-ones[12] as described.[3] 3,4-Dihydro-6-methoxy-2,2-dimethyl-7-(2,2,2-trifluoroethoxy)-1-2*H*-benzopyran (**11**): ^1H NMR δ: 1.30 (s, 6H), 1.77 (t, 2H, J = 7 Hz), 2.70 (t, 2H, J = 7 Hz), 3.80 (s, 3H), 4.32 (q, 2H, J_{H-F} = 8 Hz), 6.43 (s, 1H), 6.61 (s, 1H); ^{13}C NMR δ: 22.2, 26.7, 32.7, 56.8, 67.6 (q. J_{C-F} = 36 Hz), 74.0, 106.1, 113.9, 114.9, 123.7 (q, J = 277 Hz), 143.6, 146.3, 147.7; MS, m/z (%): 290 (M^+, 30), 235 (100), 207 (5). Anal. calcd for $C_{14}H_{17}F_3O_3$: C, 57.92; H, 5.91. Found: C, 57.83; H, 5.89. 3,4-Dihydro-6,7-methoxy-2,2-dimethy-(2,2,2-trifluoroethoxy)-1-2*H*-benzopyran (**13**): ^1H NMR δ: 1.25 (s, 6H), 1.69 (t, 2H, J = 7 Hz), 2.59 (t, 2H, J = 7 Hz), 3.72 (s, 3H), 4.22 (q, 2H, J_{H-F} = 8 Hz), 6.30 (s, 1H), 6.66 (s, 1H); ^{13}C NMR δ: 21.7, 26.7, 32.7, 55 8, 69.4 (q, J_{C-F} = 36 Hz), 74.2, 101.6, 111.9, 119.6, 123.7 (q, J = 277 Hz), 140.4, 150.0, 150.3; MS, m/z (%): 290 (M^+, 40), 235 (100), 207 (5). Anal. calcd for $C_{14}H_{17}F_3O_3$: C, 57.92; H, 5.91. Found: C, 58.04; H, 6.09.

Hydroxy fluorinated compounds **6** and **12** were obtained from the corresponding benzopyran-4-one. This ketone was prepared from 2,3-dihydro-6,7-dihydroxy-2,2-dimethyl-1-4*H*-benzopyran-4-one (a generous gift from Dr Tibor Timar, Alkaloida Chem. Co., Hungary) by using the general procedure described by Camps *et al.*[12] 2,3-Dihydroxy-6-hydroxy-2,2-dimethyl-7-(2,2,2-trifluoroethoxy)-1-4*H*-benzopyran-4-one: IR (film): 3275, 1674, 1618 cm^{-1}; ^1H NMR δ: 1.40 (s, 6H), 2.64 (s, 2H), 4.40 (q, 2H, J = 8 Hz), 5.5 (br, 1H, OH), 6.37 (s, 1H), 7.38 (s, 1H); ^{13}C NMR δ: 26.5; 48.5, 66.2 (q, J = 36 Hz), 79.6, 101.6, 111.7, 115.0, 122.6 (d, J = 277 Hz), 140.1, 151.0, 155.0, 191.1; MS, m/z (%): 290 (M^+, 35), 275 (100), 235 (69). Anal. calcd for $C_{13}H_{13}F_3O_4$: C, 53.80; H, 4.51. Found: C, 53.70; H, 4.60. Reduction of this ketone with lithium aluminum hydride and further dehydration of the intermediate alcohol afforded crude chromene **6**, which was purified by preparative TLC (3:1 hexane:ethyl acetate). 6-Hydroxy-2,2-dimethyl-7-(2,2,2-trifluoroethoxy)-1-2*H*-benzopyran (**6**): IR (film): 3450, 1500 cm^{-1}; ^1H NMR δ: 1.29 (s, 6H), 4.33 (q, 2H, J = 8 Hz), 5.05 (s, 1H, OH), 5.52 (d, 1H, J = 10 Hz), 6.20 (d, 1H, J = 10 Hz), 6.60 (s, 1H), 6.35 (s, 1H); ^{13}C NMR δ: 27.6, 66.8 (q, J = 36 Hz), 76.0. 102.1, 112.9, 116.4, 121.6, 123.0 (d, J = 277 Hz), 129.9, 139.6, 144.2, 146.5; MS, m/z (%): 274 (M^+, 30), 259 (100). Anal. calcd for $C_{13}H_{13}F_3O_3$: C, 56.94; H, 4.78. Found: C, 57.02; H, 4.89. Likewise, Clemmensen reduction of the above ketone under the conditions described by Casas *et al.*[3] afforded crude chroman **12**, which was purified by preparative TLC (2:1 hexane:ethyl acetate). 3,4-Dihydro-6-hydroxy-2,2-dimethyl-7-(2,2,2-trifluoroethoxy)-1-2*H*-benzopyran (**12**): IR (film): 3275, 1627, 1529 cm^{-1}; ^1H NMR δ: 1.28 (s, 6H), 1.74 (t, 2H, J = 7 Hz), 2.65 (t, 2H, J = 7 Hz), 4.30 (q, 2H, J = 8 Hz), 5.02 (s, 1H, OH), 6.64 (s, 1H), 6.30 (s. 1H); ^{13}C NMR δ: 22.0, 26.6, 32.8, 66.8 (q, J = 36 Hz), 74.0, 102.2, 115.1, 115.3, 123.0 (d, J = 277 Hz), 139.0, 143.4, 147.1; MS, m/z (%): 276 (M^+, 40), 221 (100). Anal. calcd for $C_{13}H_{15}F_3O_3$: C, 56.52; H, 5.47. Found: C, 56.66; H, 5.53.

Benzopyrans **4** and **7** were prepared by reaction of their respective benzopyran-4-ones with excess methyl magnesium iodide in dry tetrahydrofuran followed by thermal dehydration under vacuum. Purification of reaction crudes by flash chromatography on silica gel afforded the pure compounds in good overall yields. 7-Ethoxy-6-methoxy-2,2,4-trimethyl-1-2*H*-benzopyran (**4**): ^1H NMR δ: 1.38 (s, 6H), 1.45 (t, 3H, J = 7 Hz), 1.98 (d, 3H, J = 1 Hz), 3.84 (s, 3H), 4 06 (q, 2H, J = 7 Hz), 5.28 (q, 1H, J = 1 Hz), 6.43 (s, 1H), 6.69 (s, 1H); ^{13}C NMR δ: 14.7, 18.0, 27.7, 56.9, 64.1, 75.8, 101.9, 107.9, 114.9, 125.0, 127.4, 143.0, 147.3, 148.9; MS, m/z (%): 248 (M^+, 59), 233 (100), 205 (71), 190 (16). Anal. calcd for $C_{15}H_{20}O_3$: C, 72.54; H, 8.13. Found: C, 72.47; H, 8.18. 6-Methoxy-7-(2,2,2-trifluoroethoxy)-2,2,4-trimethyl-1-2*H*-benzopyran (**7**): ^1H NMR δ: 1.37 (s, 6H), 1.98 (d, 3H, J = 1 Hz), 3.84 (s, 3H), 4.36 (q, 2H, J_{H-F} = 8 Hz), 5.35 (q, 1H, J = 1 Hz), 6.47 (s, 1H), 6.73 (s, 1H); ^{13}C NMR δ: 18.0, 27.7, 57.2, 67.5 (q, J_{C-F} = 36 Hz), 76.0, 105.5, 109.2, 118.1, 123.4 (q, J = 277 Hz), 126.5, 127.1, 144.0, 147.3, 147.4; MS, m/z (%): 302 (M^+, 28), 287 (100), 203 (60). Anal. calcd for $C_{15}H_{17}F_3O_3$: C, 59.59; H, 5.68. Found: C, 59.51; H, 5.70.

Evaluation of lipid peroxidation

Incubations. Suspensions of male Sprague–Dawley rat liver microsomes in Krebs–Ringer phosphate (KRP) buffer, pH 7.4, were prepared as described.[15] For enzymatically induced lipid peroxidation assays, microsomal protein (2 mg) in 1 mL KRP buffer was incubated during 1 h at 37 °C in the presence of 0.1 mM NADPH and 2 μL dimethyl sulfoxide (DMSO) containing the test compound at the appropriate concentration. In assays where incubations were prolonged up to three hours, 0.1 mL 2 mM NADPH was added every hour to the incubation medium. For chemically induced lipid peroxidation assays, microsomal protein (2 mg) in 1 mL KRP buffer was incubated during 30 min at 37 °C in the presence of 50 μM $FeSO_4$, 0.5 mM ascorbic acid[2] and 2 μL DMSO containing the test compound at the appropriate concentration. In both cases, incubations were stopped by addition of 4% BHT (0.1 mL).

TBARS Test. After incubations were completed, lipid peroxidation was evaluated by the production of TBARS as described.[3,15] Briefly, 0.5 mL of 3% sodium dodecylsulfate was added to aliquots of microsomal incubations (0.4 mL for those coming from enzymatically induced assays and 0.2 mL for those coming from chemically stimulated lipid peroxidation). After mixing, 2 mL of 0.1 N HCl, 0.3 mL of 10% phosphotungstic acid, and 1 mL of 0.7% 2-thiobarbituric acid (in 0.1 N NaOH) were added. The mixture was heated for 30 min at 100 °C, and TBARS were extracted with 3 mL of 1-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515-nm excitation and 555-nm emission. The values obtained, calculated as nanomoles of TBARS (malondialdehyde equivalents) per gram of liver, were expressed as percentage inhibition referring to a measurement carried out in the presence of DMSO (2 μL). Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane. Assays were performed in triplicate. The IC_{50} values were determined by plotting percent inhibition versus log [I], using at least 5 different concentrations. Figure 1 shows a representative plot for fluorinated chromans **11**, **12** and **13**.

HPLC Analysis of TBA–MDA complex. The analytical procedure used for the determination of the TBA–MDA complex was based on that reported by Bird *et al.*[5] Thus, 1 mL of the above *n*-butanol extract was evaporated to dryness and redissolved in 0.25 mL of water, and 10 μL was injected onto the HPLC system with the detector set at 546 nm. Samples were injected onto a Lichrospher 100 RP-18 column (125 x 4 mm, 5 μm, Merck), using a 95:5 water:methanol eluent solution at 1 mL/min.

Metabolism studies. Microsomal protein (2 mg) in 1 mL KRP buffer was incubated during 1 h at 37 °C in the presence of 0.1 mM NADPH and 2 μL DMSO containing the test compound at 50 μM final concentration. Incubations were stopped and treated as described.[3] Identification of metabolites was carried out by HPLC and GC-MS analysis. The HPLC analyses were performed using mixtures of methanol: formic acid/triethylamine 50 mM buffer, pH 3.5 at 1 mL/min.

Statistics. The statistical significance of the difference between means was evaluated using Student's *t*-test for independent samples.

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