Biochemical Pharmacology, Vol. 40, No. 3, pp. 551–558, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

NOVEL 6-HYDROXYCHROMAN-2-CARBONITRILE INHIBITORS OF MEMBRANE PEROXIDATIVE INJURY

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(*Received* 9 *August* 1989; *accepted* 19 *December* 1989)

Abstract—Novel 6-hydroxychroman-2-carbonitrile compounds have been synthesized, and their antiperoxidant activity against superoxide-dependent, iron-promoted mycocardial phospholipid peroxidation has been evaluated quantitatively. With few exceptions, these compounds afforded significant, concentration-dependent antiperoxidant protection to myocardial-membrane phospholipid at sub- to low-micromolar concentrations. Structure–activity correlation demonstrated that R^1-, R^2-, and R^3-methyl groups in the aromatic ring enhanced antiperoxidant activity, whereas hydrophobic groups at either R^4 or R^5 of the pyran ring compromised antiperoxidant efficacy. The most efficacious antiperoxidant synthesized contained a catechol moiety at R^4 and was some 10-fold more potent than α-tocopherol. None of the 6-hydroxychroman-2-carbonitrile antiperoxidants scavenged superoxide or inhibited the enzymatic superoxide generator, xanthine oxidase, at effective antiperoxidant concentrations. The ability of these compounds to interrupt the propagatory phase of an on-going peroxidation reaction indicated that they acted as antiperoxidants by trapping chain-carrying lipid peroxyl radicals. Since a number of the 6-hydroxychroman-2-carbonitriles were most potent antiperoxidants than a variety of known chain-breaking compounds, this new class of phenolic antioxidants may represent a novel approach to the design of therapeutics against diseases in which lipid peroxidation is a causative factor or in which lipid peroxidases serve as mediators.

Oxygen-containing free radicals are pathological agents which affect many major organs in a variety of disease states [1]. Peroxidation of membrane phospholipid polyunsaturated fatty-acid (PUFA‖) esters is a cardinal feature of oxy-radical toxicity [1, 2]. PUFA peroxidation through superoxide radical (O_2^-)-dependent, iron-promoted oxidative reactions may be a causative factor in ischemic and reperfusion heart injury [3] and in stroke-associated brain damage [4]. Mechanistically, such non-enzymatic membrane lipid oxidation is initiated when O_2^- establishes a suitable ferric iron:ferrous iron ratio for hydrogen-atom abstraction from phospholipid PUFA esters [5]. Intramolecular rearrangements of double bonds in the resultant lipid radicals yield conjugated diene intermediates which are attacked by molecular oxygen to form peroxyl radicals [6]. The highly-reactive lipid peroxyl radicals propagate the O_2^-- and iron-dependent initiation event through a 'chain reaction' in which new lipid radicals are formed along with products such as hydroperoxides and malondialdehyde (MDA) [5, 6].

Both natural products and synthetic agents have been found to suppress peroxidative PUFA injury [7, 8]. For instance, structurally-diverse compounds such as CS-045, AA-861, CV-3611, U-74006F, and L-651896 (Fig. 1, compounds **1–5**, respectively) possess oxy-radical scavenging and/or metal-chelating properties which make them effective antiperoxidants [9–13]. Since lipid peroxyl radicals are critical to the propagation of nonenzymatic phospholipid auto-oxidation and the consequent amplification of tissue injury following an oxidative insult [6, 14], compounds which trap chain-carrying peroxyls have attracted considerable chemical and pharmacologic interest [15]. The most bioactive 'chain-breaking' antiperoxidants are phenolic compounds, one of which, the α-tocopherol component of vitamin E (Fig. 1, compound **6**), is the major, naturally-occurring lipophilic scavenger of peroxyl radicals in living systems [7]. Consequently, α-tocopherol has been used as a basis for the rational design of antiperoxidants with potential commercial or therapeutic utility. Such antiperoxidants are illustrated in Fig. 1 and include compounds **1**, **2**, and **5**, Trolox™ (compound **7**) [16], and *all-rac*-2,4,6,7-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-5-hydroxy-3,4-dihydrobenzofuran [17] (compound **8**).

These considerations prompted screening of novel α-tocopherol-like 6-hydroxychromans available from synthetic studies aimed at compound **6** (Fig. 1) in a O_2^--dependent, iron-promoted peroxidation system devised by this laboratory [18]. The resultant data, summarized herein, constitute evidence that 6-hydroxychroman-2-carbonitriles of the general structure given (Fig. 2) possess appreciable antiperoxidant activity against O_2^-- and iron-dependent lipid peroxidation of the type believed to be pathogenic *in vivo* [1, 2]. The antiperoxidant

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‖ Abbreviations: PUFA, polyunsaturated fatty acid; O_2^-, superoxide anion radical; MDA, malondialdehyde; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TBA, thiobarbituric acid; ADP, adenosine diphosphate; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole, HTP, 4-hydroxymethyl-2,6-di-*tert*-butylphenol; XOD, xanthine oxidase; and SOD, superoxide dismutase.

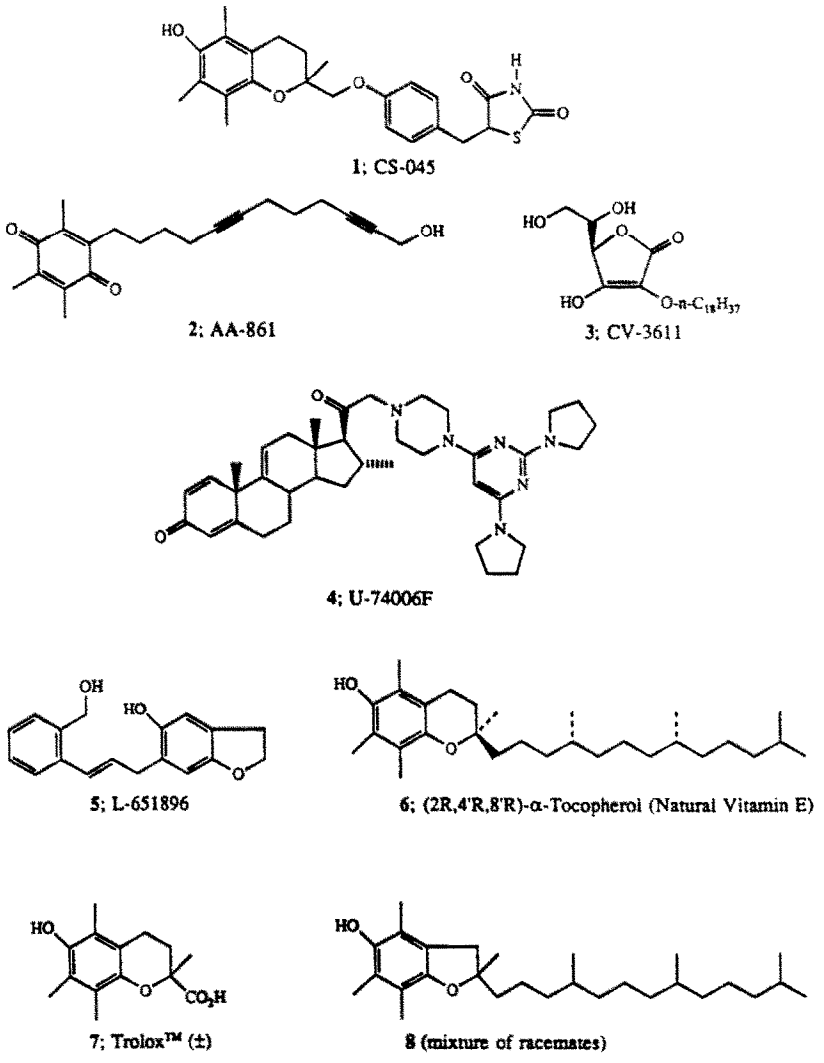


Fig. 1. Structural formulae of some known antiperoxidants discussed in the text.

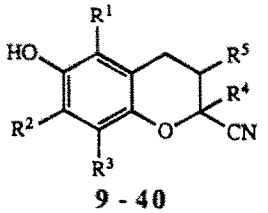


Fig. 2. General structural formula of the novel 6-hydroxychroman-2-carbonitriles, compounds **9–40**.

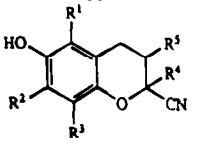
efficacy of these novel compounds appears to reflect their ability to trap chain-carrrying lipid peroxyls and act thereby as 'chain-breaking' antioxidants.

EXPERIMENTAL PROCEDURES

Materials. *N*-2-Hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES), tris(hydroxymethyl)-aminomethane (Tris), 2-thiobarbituric acid (TBA), adenosine diphosphate (ADP), xanthine, hypoxanthine, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and EDTA were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). PolyScience (Niles, IL, U.S.A.) was the source of 4-hydroxymethyl-2,6-di-*tert*-butylphenol (HTP). Xanthine oxidase (XOD; xanthine:oxygen oxidoreductase, EC 1.2.3.2; analytical preparation from bovine milk; 1 unit/mg), superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1; analytical preparation from bovine erythrocytes; 5000 units/mg), and ferricytochrome *c* (horse-heart) were from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). All-*rac*-α-tocopherol and Trolox™ were from Hoffmann-La Roche. Solvents were of analytical grade as purchased (Burdick & Jackson, Muskegon, MI, U.S.A.). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Synthetic chemistry. The syntheses of many of the 6-hydroxychroman-2-carbonitriles have been detailed recently [19]. New analogues are summarized

Table 1. 3,4-Dihydro-6-hydroxy-2H-1-benzopyran-2-carbonitriles not previously described



Compound	R^1	R^2	R^3	R^4	R^5	Method of synthesis*	Recryst. solvent†	mp	Formula‡
10	H	CH_3	CH_3	CH_3	H	A	T	164-165.5	$C_{13}H_{15}NO_2$
11	CH_3	H	CH_3	CH_3	H	A	T	162-164	$C_{13}H_{15}NO_2$
15	Br	CH_3	CH_3	CH_3	H	B	T	122-124	$C_{13}H_{14}BrNO_2$
16	CH_3	CH_3	Br	CH_3	H	B	T	164-166	$C_{13}H_{14}BrNO_2$
17	CH_3	Br	CH_3	CH_3	H	B	T-H	166-168	$C_{13}H_{14}BrNO_2$
19	$C{\equiv}C\text{-}n\text{-}C_{10}H_{21}$	CH_3	CH_3	CH_3	H	C	EtOH	80-81	$C_{25}H_{35}NO_2$
21	CH_3	CH_3	*E*-CH=CH-C_6H_5	CH_3	H	A	T	170-172	$C_{21}H_{21}NO_2$
23	CH_3	CH_3	CH_3	C_2H_5	H	A	T	149-151	$C_{15}H_{19}NO_2$
24	CH_3	CH_3	CH_3	n-C_5H_{11}	H	A	E	95-97	$C_{18}H_{25}NO_2$
25	CH_3	CH_3	CH_3	n-C_8H_{17}	H	A	H-E	95-96	$C_{21}H_{31}NO_2$
26	CH_3	CH_3	CH_3	n-$C_{12}H_{25}$	H	A	H-E	91-93	$C_{25}H_{39}NO_2$
27	CH_3	CH_3	CH_3	$(CH_2)_2CO_2H$	H	D	H-E	176-178	$C_{16}H_{19}NO_4$
28	CH_3	CH_3	CH_3	$(CH_2)_2CO_2CH_3$	H	A	T-H	101-103	$C_{17}H_{21}NO_4$
29	CH_3	CH_3	CH_3	*cyclo*-C_6H_{11}	H	A	T	180-182	$C_{19}H_{25}NO_2$
32	CH_3	CH_3	CH_3	$(CH_2)_6C_6H_5$	H	A	H-E	97-98	$C_{25}H_{31}NO_2$
34	CH_3	CH_3	CH_3	$(CH_2)_{11}OH$	H	A	T-H	91-92	$C_{24}H_{37}NO_3$
35	CH_3	CH_3	CH_3	$(CH_2)_2$-3,4-$(OCH_3)_2C_6H_3$	H	A	T	184-186	$C_{23}H_{27}NO_4$
36	CH_3	CH_3	CH_3	$(CH_2)_2$-3,4-$(OH)_2C_6H_3$	H	E	E	172-174	$C_{21}H_{23}NO_4$
38	CH_3	CH_3	CH_3	——$(CH_2)_3$——		A	T	167-172§	$C_{16}H_{19}NO_2$
39	CH_3	CH_3	CH_3	CH_3	$CH_2C_6H_5$	A	H-E	101-103§	$C_{21}H_{23}NO_2$
R-9	CH_3	CH_3	CH_3	CH_3	H	F	T-H	178-180¶	$C_{14}H_{17}NO_2$
S-9	CH_3	CH_3	CH_3	CH_3	H	F	T-H	178-180**	$C_{14}H_{17}NO_2$

*See Synthetic Chemistry Section for description of methods.
†T = toluene; H = hexane; E = ether.
‡All compounds gave C, H, N microanalytical values within ±0.4% of theory.
§Mixture of diastereomers.
||Apparently a single diastereomer of unknown relative configuration.
¶$[\alpha]_D^{25}$ -72.47° (c 1, $CHCl_3$).
**$[\alpha]_D^{25}$ +71.58° (c 1, $CHCl_3$).

in Table 1. Most of the new compounds were prepared using previously described methodology [19]: specifically, the treatment of a 2-methoxychroman with cyanotrimethylsilane in the presence of a Lewis acid (method A). The 2-methoxychroman intermediates were synthesized in acidic methanol by cyclocondensation of trimethylhydroquinone or an analogue with a vinyl ketone (in the sequences leading to compounds **10**, **11**, and **21**) or with a β-hydroxy ethylene acetal (in the sequences leading to compounds **38** and **39**). Lead compound *R*,*S*-**9** was prepared this way rather than through the original synthesis described in the patent literature [20]. The bromo analogues **15–17** were obtained by bromination (Br_2/CCl_4); of the corresponding 6-hydroxydimethylchroman-2-carbonitriles (method B). Acetylene **19** was secured via a palladium-catalyzed coupling of compound **15** with 1-dodecyne (method C). The acid **27** was obtained by saponification of ester **28** (method D). Hydrogenolysis (Pd/C) (method E) of the corresponding 2-(3,4-dibenzyloxyphenyl)ethyl-6-hydroxychroman-2-carbonitrile (itself prepared using method A) afforded catechol **36**. The enantiomers *R*-**9** and *S*-**9** were synthesized by conversion of the enantiomeric chroman-2-carboxylic acids *R*-**7** and *S*-**7** [16] to the carboxamides (carbonyldiimidazole/tetrahydrofuran followed by concentrated ammonium hydroxide) which were then dehydrated as described by Claremon and Philips [21] (method F). The dihydrobenzofuran-2-carbonitrile **41** (see Table 4) was prepared starting from the corresponding carboxylic acid [22] via dehydration of the amide with *N*-methylmorpholine-titanium tetrachloride [23], giving a colorless solid (m.p. 167–168° from hexane); Anal. Calc. for $C_{13}H_{15}NO_2$: C, 71.87; H, 6.96; N, 6.45. Found: C, 71.67; H, 6.93; N, 6.40. Further information concerning the synthesis of any compound described herein will be provided by the authors upon request.

Peroxidation reaction system. Membrane phospholipids were extracted and purified from the ventricular myocardium of adult Sprague–Dawley rats and were quantified as lipid ester [18]. Liposomes were formed by placing a known amount of myocardial phospholipid (in $CHCl_3$) into a rotating glass flask, evaporating the solvent under nitrogen, and resuspending the lipid film in 10 mM HEPES–0.145 M KCl, pH 7.4, by indirect anaerobic sonication for 15 min at room temperature [18].

Cardiac phospholiposomes were subjected to $O_2^{\cdot-}$- and iron-dependent peroxidation in a reaction containing 10 mM HEPES–0.145 M KCl, pH 7.4; 1.0 mM Fe^{3+}–1.0 mM ADP complex; 125 μg lipid/mL; 1.0 mM hypoxanthine; and 10 mUnits XOD/mL. In some cases, a test compound was also present and was added from a concentrated stock solution in ethanol such that the final ethanol concentration in the reaction did not influence peroxidation. Peroxidation was started by adding the XOD, mixing, and warming to 37° in a shaking water bath. After 60 min, peroxidation was terminated by adding 0.15 mL of 76% (w/v) trichloroacetic acid in 2.3 N HCl per mL of reac-

tion and immersing the sample in an ice-water bath.

Assessment of lipid peroxidation. Lipid peroxidation was quantified as TBA-reactive material [18]. To each 1.15 mL of acidified peroxidation reaction (above) was added 0.35 mL of a mixture containing water:BHT (7.145 M in ethanol):TBA (1.514 wt-% in 0.2 M Tris, pH 7.0) in the volume ratio 1:1:5. After thorough mixing, the samples were incubated in an 80° shaking water bath for 30 min. After this time, the tubes were plunged into an ice-water bath, and 0.5 mL of ice-cold 90% (w/v) trichloroacetic acid followed by 2.0 mL $CHCl_3$ were added. After centrifugation for 30 min at 2000 *g* (4°), the absorbance of the upper phase was read at 532 nm. MDA was prepared for the standard curve of TBA-reactivity by acid hydrolysis of 1,1,3,3-tetramethoxypropane [18]. Computer-assisted regression analysis of the standard curve was used to quantify the molar amounts of TBA-reactive material (as MDA-equivalents) in the experimental samples.

Evaluation of antiperoxidant efficacy. All experiments were run in triplicate. Compounds were evaluated initially in the peroxidation reaction system (above) at a final concentration of 100 μM, and any inhibition of peroxidation was determined as compared to a reaction without test substance conducted in parallel. The observed degree of antiperoxidant activity at 100 μM was used to establish six or more concentrations at which each compound was re-evaluated. From these latter data, concentration–response curves were generated with the assistance of RS/1 software (BBN Corp., Cambridge, MA) on an IBM PC-AT (IBM Corp., Boca Raton, FL). Antiperoxidant IC_{50} values were calculated from the respective concentration–response curves for each compound tested. In every experiment, the original lead compound *R,S*-**9** (see Tables 2–4) was run as an internal standard, and its antiperoxidant IC_{50} was used to calculate an antiperoxidant potency ratio with respect to the antiperoxidant IC_{50} values of the other compounds tested in that experiment. The antiperoxidant potency ratios in Tables 2–4 were confirmed by independent, duplicate determinations, which agreed to within 5% of each other.

Assessment of O_2^- scavenging. Prevention of the SOD-inhibitable reduction of ferricytochrome *c* by test substance was taken as evidence of its O_2^- scavenging potential [24]. The assay contained: 0.25 mM potassium phosphate buffer, pH 8.6; 10^{-4} M EDTA; 2 mM NaOH; air-saturated dimethyl sulfoxide containing 0.55 M water; and 76 μM ferricytochrome *c*. The linear rate of SOD-inhibitable cytochrome *c* reduction was monitored at 550 nm, and attenuation of this rate by test substance was considered positive evidence of the O_2^- trapping ability of the substance.

XOD activity. XOD was assayed spectrophotometrically by monitoring the conversion of xanthine substrate to uric acid at 25° [25]. The assay mixture contained: 50 mM potassium-phosphate buffer, pH 7.8; 10 μM EDTA; 4.2×10^{-9} M catalytically flavin-active XOD; and 0.5 mM xanthine. Some incubations included a test substance at concentrations indicated in the text. These conditions have been demonstrated [25, 26] to be optimal with respect to linearity of absorbance rise relative to XOD activity and obviate both interference by iron [25] and accumulation of xanthine if hypoxanthine or purine was used as substrate [27].

Table 2. Comparative antiperoxidant activities of simple, chain-breaking phenolic antioxidants

Compound	Antiperoxidant potency ratio*
R,S-**9**†	1.0
R-**9**†	1.0
S-**9**†	1.1
all-*rac*-α-Tocopherol (**6**)‡	2.0
BHT	2.0
BHA	2.0
HTP	3.0
7‡	10.0

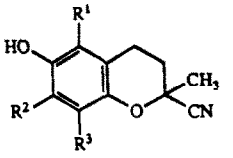
* Ratio of the antiperoxidant IC_{50} of listed compound to that of compound *R,S*-**9** run in parallel.
† Structure given in Table 1.
‡ Structure given in Fig. 1.

RESULTS AND DISCUSSION

Assessment of antiperoxidant activity. The 6-hydroxychroman-2-carbonitriles were evaluated as antiperoxidants in a O_2^-- and iron-dependent peroxidative-injury system developed by this laboratory [18]. The system is optimized to afford maximal initial rates of O_2^- production and O_2^--, iron-dependent peroxidation, the latter maintaining linearity beyond 60 min of peroxidation [6]. Under these conditions, the TBA-reactivity of the peroxidation reaction largely represents the MDA end-product of hydroperoxide formation and decomposition [18]. Preliminary experiments verified that no cyanochroman tested interfered with the analysis of peroxidation as TBA-reactivity: the cyanochromans neither influenced the TBA-reactivity of purified MDA nor were TBA-reactive themselves. Since the number of compounds studied precluded their being analyzed simultaneously, the original lead compound, *R,S*-**9** (Tables 2–4), was used as the reference compound in every experiment such that the antiperoxidant activity of any other cyanochroman tested could be expressed relative to it as a potency ratio. The absolute antiperoxidant IC_{50} (i.e. the concentration at which peroxidation was inhibited by 50%) for compound *R,S*-**9** was 4.4 ± 0.3 μM (mean ± SE; N = 10). As summarized in Table 2, the antiperoxidant potencies of this compound and its resolved isomers, *R*-**9** and *S*-**9**, compared very favorably to those of known chain-breaking phenolic antioxidants, including α-tocopherol, BHT, BHA, and HTP. Compound *R,S*-**9** was ~10-fold more potent than its direct, 2-carboxyl analog, Trolox™ (compound **7**; Fig. 1).

Effect of aromatic-ring variations on the antiperoxidant activity of 6-hydroxychroman-2-carbonitriles. As summarized in Table 3, substitution of the R^1-, R^2-, or R^3-methyl group of compound *R,S*-**9** with a hydrogen (compounds **10**, **11**, and **12**) reduced antiperoxidant activity some 4-fold. Substitution of all three methyl groups with hydrogens (compound **13**)

Table 3. Antiperoxidant activities of 6-hydroxychroman-2-carbonitriles - variations in the aromatic ring



Compound	R^1	R^2	R^3	Antiperoxidant potency ratio*
R,S-9+	CH_3	CH_3	CH_3	1.0
10	H	CH_3	CH_3	4.3
11	CH_3	H	CH_3	3.8
12+	CH_3	CH_3	H	4.3
13+	H	H	H	>14
14+	H	$C(CH_3)_3$	H	1.0
15	Br	CH_3	CH_3	1.8
16	CH_3	CH_3	Br	1.2
17	CH_3	Br	CH_3	4.0
18+	CH_3	OCH_3	OCH_3	18.5
19	$C{\equiv}C\text{-}n\text{-}C_{10}H_{21}$	CH_3	CH_3	7.3
20+	CH_3	-CH=CH-CH=CH-		6.7
21	CH_3	CH_3	E-CH=CH-C_6H_5	6.4

*Ratio of the antiperoxidant IC_{50} of listed compound to that of compound R,S-9 run in parallel.

+Compound described in reference [19]; others described in Table 1.

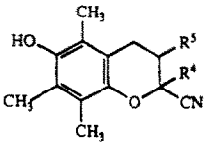
virtually abolished antiperoxidant activity. Compound **14**, containing a branched alkyl (*tert*-butyl) group at R^2, with hydrogens at R^1 and R^3, did not differ in antiperoxidant potency from the original lead. Halogen substitution at R^1 or R^3 led to modest declines in antiperoxidant activity (compounds **15** and **16**), whereas a halogen substitution at R^2 (compound **17**) greatly reduced antiperoxidant activity. As exemplified by compound **18**, methoxy substitutions of the methyl groups at R^2 and R^3 led to a significant decline in antiperoxidant efficacy. Long-chain alkynyl (compound **19**) or styryl (compound **21**) substitutions on the aromatic ring or fusion of an additional aromatic ring (compound **20**) also lessened antiperoxidant activity.

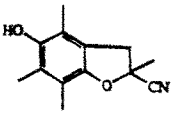
The importance of the R^1-, R^2-, R^3-methyl groups to the antiperoxidant activity of the 6-hydroxychroman-2-carbonitriles is supported by findings that methyl groups *ortho* and *meta* to the hydroxyl enhance the chain-terminating properties of phenolic compounds [28, 29]. The equipotence of compounds *R,S*-**9** and **14** as antiperoxidants is interesting in light of evidence that *ortho* alkyl groups larger than methyl sterically 'protect' the phenoxyl oxygen and thereby interfere with the chain-breaking activity of phenols [28–30]. The hydrogens at R^1 and R^3 in compound **14** might have relieved any potential steric hindrance of the *tert*-butyl group.

Effect of pyran-ring substitutions on the antiperoxidant activity of 6-hydroxychroman-2-carbonitriles. The phytyl side-chain of α-tocopherol at R^4 of the pyran ring enhances the retention of vitamin E within liposomes and membranes [31, 32]. This fact, along with the data in Table 3, prompted us to investigate the influence of various hydrophobic groups at R^4 and R^5 in the pyran ring on the antiperoxidant efficacy of 6-hydroxychroman-2-carbonitriles having a fully-methylated phenolic ring (Table 4).

Replacement of the 2-methyl group of compound *R,S*-**9** with hydrogen (compound **22**) reduced antiperoxidant efficacy only slightly. Straight-chain, saturated alkyl moieties of increasing lengths above an ethyl group (compound **23**) at R^4 led to a progressive decline in antiperoxidant potency (compounds **24**, **25**, and **26**). A short-chain carboxylic-acid (compound **27**) substituent at R^4 was associated

Table 4. Antiperoxidant activities of 6-hydroxychroman-2-carbonitriles - variations in the pyran ring



Compound	R^4	R^5	Antiperoxidant potency ratio*
R,S-9+	CH_3	H	1.0
22+	H	H	1.7
23	C_2H_5	H	0.7
24	$n\text{-}C_5H_{11}$	H	2.3
25	$n\text{-}C_8H_{17}$	H	8.6
26	$n\text{-}C_{12}H_{25}$	H	>10
27	$(CH_2)_2CO_2H$	H	3.3
28	$(CH_2)_2CO_2CH_3$	H	0.7
29	cyclo-C_6H_{11}	H	3.0
30+	C_6H_5	H	5.7
31+	$(CH_2)_2C_6H_5$	H	6.3
32	$(CH_2)_6C_6H_5$	H	3.8
33+	$(CH_2)_3OH$	H	1.9
34	$(CH_2)_{11}OH$	H	2.6
35	(CH_2)-3,4-$(OCH_3)_2C_6H_3$	H	1.6
36	$(CH_2)_2$-3,4-$(OH)_2C_6H_3$	H	0.2
37+	$(CH_2)_4$		1.1
38	$(CH_2)_3$		0.8
39	CH_3	$CH_2C_6H_5$	0.7
40+	CH_3	$(CH_2)_3OH$	1.4
41			2.0

*Ratio of the antiperoxidant IC_{50} of listed compound to that of compound R,S-9 run in parallel.

+Compound described in reference [19]; others described in Table 1.

with reduced antiperoxidant efficacy. A carboxylic acid-ester (compound **28**) substituent at R^4 effected a modest increase in potency. Cyclohexyl (compound **29**), phenyl (compound **30**), or alkyl (compounds **31** and **32**) and short- (compound **33**) or long- (compound **34**) chain alcohol moieties reduced the antiperoxidant efficacy. Alkyl moieties at R^4 having aromatic-ring substitutions also decreased antiperoxidant potency (compound **35**), except in the case where the aromatic system was a catechol moiety (compound **36**): the antiperoxidant potency of compound **36** was some 5-fold greater than that of the original lead, compound *R,S*-**9**, and 10-fold greater than α-tocopherol (cf. Table 2), possibly due to an additive effect of the two antioxidant moieties present in this analogue.

Since the primary (if not only) function of the phytyl side-chain of α-tocopherol is to ensure that the hydroxychroman reactive-center is retained in membranes and liposomes [31, 32], it is of note that 6-hydroxychroman-2-carbonitriles having hydrophobic moieties at R^4 (e.g. compounds **25**, **26**, and **30–35**) were significantly less potent antiperoxidants than was compound *R,S*-**9**, which has a methyl substituent at R^4. An explanation for this observation may lie with the aqueous nature of our free-radical generator, for Niki *et al.* [31] have noted that in a system with a water-soluble chemical initiator of peroxidation, phenolic compounds with hydrophobic side-chains were less effective antiperoxidants than were the identical phenols without the side-chain.

A variety of 6-hydroxychroman-2-carbonitriles with substitutions at R^5 were synthesized (compounds **37–40**, Table 4). Such changes contributed,

if anything, only modestly to antiperoxidant potency and in most cases had a detrimental effect. Likewise, compound **41**, the dihydrobenzofuran-2-carbonitrile analog of compound **8** (Fig. 1), was less potent than the corresponding 6-hydroxychroman-2-carbonitrile, compound *R,S*-**9** (Table 4), in contrast to previous observations [17].

6-Hydroxychroman-2-carbonitriles as O_2^- scavengers and XOD inhibitors. The antiperoxidant efficacy of many 6-hydroxychroman-2-carbonitriles in a XOD-dependent, O_2^--driven, iron-promoted peroxidation system prompted us to test directly whether these compounds could inhibit XOD or scavenge O_2^-. Each compound was tested at its antiperoxidant IC_{50} as well as at a concentration that inhibited peroxidation by ~100%. At concentrations that significantly reduced or even blocked cardiac phospholipid damage through XOD-dependent, O_2^--driven oxy-radical chemistry, no 6-hydroxychroman-2-carbonitrile could scavenge O_2^- [24] or inhibit XOD [25]. In these tests, known XOD inhibitors and O_2^- scavengers were effective at (or below) low-micromolar concentrations: for example, 8 μM allopurinol inhibited XOD by some 50%, and 10 nM SOD scavenged all of the O_2^- produced [26]. Thus, the antiperoxidant activity of the 6-hydroxychroman-2-carbonitriles could not have been a consequence of their limiting the O_2^- necessary for the initiation of peroxidation. This conclusion is independently supported by a recent pulse-radiolysis analysis which demonstrated that Trolox™ does not react with O_2^- [33].

6-Hydroxychroman-2-carbonitriles as 'chain-breaking' molecules. The ability of 6-hydroxychroman-2-carbonitriles to prevent the formation of lipid peroxides and their lack of XOD-inhibitory and O_2^--scavenging properties at effective antiperoxidant concentrations (above) suggested that these compounds may act mechanistically to interrupt the propagatory, chain-reaction phase of auto-oxidative lipid damage [14]. The lack of stereospecificity of their antiperoxidant effect (Table 2, compound **9**) would also suggest such a mode of inhibition with respect to non-enzymatic PUFA auto-oxidation. Accordingly, each of the various 6-hydroxychroman-2-carbonitriles was introduced at or after 30 min into an on-going peroxidation reaction at a concentration known to block hydroperoxide formation if present at the start of the reaction. This protocol for our peroxidation system was selected from consideration of data presented elsewhere [6] which demonstrate that, by 30 min, peroxyl radical–PUFA interactions are actively amplifying the O_2^--dependent initiation, and O_2^- generation from XOD is negligible.

As exemplifed by compound *R,S*-**9** (Fig. 3), the 6-hydroxychroman-2-carbonitrile antiperoxidants could prevent peroxidative lipid injury by interrupting the propagatory chain-reaction between fatty-peroxyl radicals and PUFAs. This 'chain-breaking' mode of action not only brought about a halt in the progressive formation of hydroperoxides under pro-oxidant stress, but also reduced the extent of peroxidative damage and limited the injury over time.

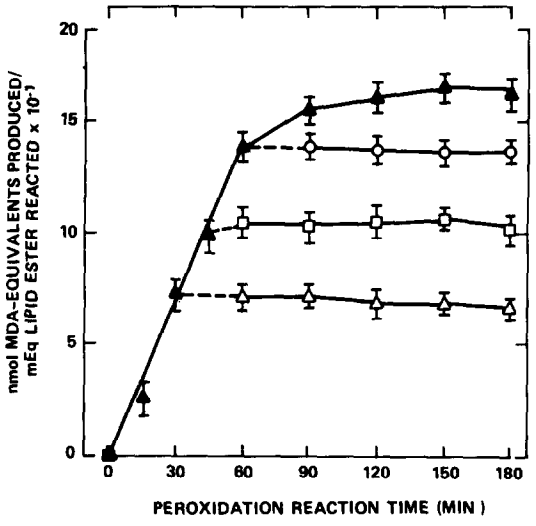


Fig. 3. Effect of compound *R,S*-**9** on the progression of peroxidative damage to myocardial phospholipid. Four identical samples of myocardial-membrane phospholipid purified from the rat heart were exposed (as liposomes) to peroxidative insult (XOD + hypoxanthine + Fe^{3+}·ADP) at 37°. Four samples of the liposome suspension alone (i.e. without XOD + hypoxanthine + Fe^{3+}·ADP) were also incubated in parallel. At 30 min (△), 45 (□), or 60 min (○), compound *R,S*-**9** (50 μM final concentration) or the equivalent volume of buffer (▲) was added to one of the four respective, on-going peroxidation reactions and to one of the four respective liposome suspensions. The net production of TBA-reactive substance (as MDA-equivalents) was calculated over a 180-min peroxidation reaction as the difference in the content of TBA-reactive substance between each peroxidized sample and its respective, non-peroxidized control. Data points are the means of four determinations ± SD.

investigation provide evidence that 6-hydroxychroman-2-carbonitriles constitute a novel class of small-molecule antiperoxidants which act as chain-breaking molecules capable of scavenging the organic peroxyl radicals which propagate lipid peroxidation. Some members of this chemical class are more potent antiperoxidants than is α-tocopherol, the major, naturally-occurring chain-breaking antioxidant. As such, the 6-hydroxychroman-2-carbonitriles represent a new approach to the design of therapeutics efficacious in disease states where lipid auto-oxidation is a pathogenic factor [34]. Since compounds which react with lipid peroxyls may suppress the enzymatic oxygenation of arachidonic acid to form bio-active lipid peroxides [35], the 6-hydroxychroman-2-carbonitriles may also be of pharmacologic interest as inhibitors of prostaglandin, leukotriene, or lipoxin biosynthesis. The biologic activity of these compounds in preventing, for example, oxidative injury to the heart-muscle cell will be the subject of future reports.

Acknowledgements—We thank L. Klevans and A. C. Sullivan for support; the Pharmacology Word Processing Unit, B. Kinback, and C. Muscara for secretarial assistance; M.

Garber, W. Lee, and R. Lopez for technical assistance; and A. Bendich and C. Burghardt for comments.

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