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INTERACTIONS OF A SERIES OF COUMARINS WITH REACTIVE OXYGEN SPECIES

SCAVENGING OF SUPEROXIDE, HYPOCHLOROUS ACID AND HYDROXYL RADICALS

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Abstract—Sixteen plant-derived or synthetic coumarins with various hydroxyl and other substitutions were tested for their ability to inhibit lipid peroxidation and to scavenge hydroxyl radicals, superoxide radicals and hypochlorous acid. Seven unsubstituted or monosubstituted coumarins were essentially inactive in all tests except for ability to scavenge $OH^{\cdot}$ with rate constants $\geq 1 \times 10^9\ M^{-1}.\ sec^{-1}$. Of the remaining nine, six containing dihydroxy substitutions were effective inhibitors of Fe^{3+}-ascorbate-dependent microsomal lipid peroxidation ($IC_{50} < 20\ \mu M$), with *ortho*-dihydroxy + one additional substitution optimal ($IC_{50} < 10\ \mu M$). *ortho*-Dihydroxylated coumarins were pro-oxidant (enhanced $OH^{\cdot}$ generation) in the Fe^{3+}–EDTA–H_2O_2 deoxyribose system but decreased $OH^{\cdot}$ generation in the Fe^{3+}–ascorbate–H_2O_2 deoxyribose system, indicating that these compounds can both chelate iron ions and also readily donate electrons for redox cycling of Fe^{3+}. The *meta*-dihydroxycoumarin did not show this behaviour, but was an effective scavenger of hypochlorous acid, a property shared by only one other compound. Several other coumarins with one or more hydroxyl substituents were also capable of effectively removing superoxide anions (IC_{50} 3.7–72 μM), although some could not be quantified due to direct rapid reduction of cytochrome *c*. We conclude that several compounds, notably 5,7-dihydroxy-4-methylcoumarin, possess beneficial biochemical profiles of interest in relation to pathophysiological processes dependent upon reactive oxygen species.

The coumarins (also known as benzopyrones) consist of fused benzene and α-pyrone rings, and form a large class of phenolic compounds occurring in green plants, as well as in fungi and bacteria [1]. They have been reported to have multiple biological activities (reviewed in Ref. 2), although these have not been evaluated systematically. It is to be expected that coumarins might affect the formation and scavenging of reactive substances derived from oxygen (reactive oxygen species, ROS‡) and influence processes involving free radical-mediated injury, as can some other plant phenolics such as flavonoids [3, 4].

For example, there is evidence that the naturally occurring prototypical compound coumarin (1,2-benzopyrone) can reduce tissue oedema and inflammation [5], and the pharmacokinetics of coumarin and its derivatives 7-hydroxycoumarin and 7-hydroxycoumarin gluconate have been studied in man [6]. Coumarin and 7-hydroxycoumarin inhibit prostaglandin biosynthesis [7], which involves fatty acid hydroperoxy intermediates. Furthermore, esculetin (6,7-dihydroxycoumarin) and various other related coumarin derivatives (e.g. fraxetin, daphnetin) are recognised as inhibitors not only of the lipoxygenase and cyclo-oxygenase pathways of arachidonate metabolism [8–10], but also of neutrophil-dependent superoxide anion generation [11]. The latter property is also demonstrable with cloricromene, a synthetic coumarin derivative with antithrombotic and vasodilator activity [12].

These facts prompted us to investigate the activities of a series of naturally occurring and related synthetic coumarins on the formation and scavenging of various ROS of relevance to the inflammatory process.

MATERIALS AND METHODS

Reagents. Dimethylsulphoxide, butylated hydroxytoluene, deoxyribose, hypoxanthine, cytochrome *c* type III, xanthine oxidase, xanthine, allopurinol, guaiacol, superoxide dismutase, catalase, horseradish peroxidase type II, α_1-antiprotease, pig pancreatic elastase, elastase substrate, 4-hydroxycoumarin, 7-methoxy-4-methylcoumarin, esculetin, esculin, fraxin, umbelliferone and myricetin were purchased from the Sigma Chemical Co. (Poole, U.K.). Fraxetin, 7-methylcoumarin and 7-methoxycoumarin were from the Aldrich Chemical Co. (Gillingham, U.K.). Scopoletin, 4-methylesculetin and 4-methylumbelliferone were from Roth. Daphnetin, 4-methyldaphnetin, 3,4-dihydrocoumarin and 5,7-dihydroxy-4-methylcoumarin were from Apin Chemicals (Abingdon, U.K.). Oxyphenbutazone was from Geigy (Horsham, U.K.), and desferrioxamine methanesulphonate was from CIBA (Horsham, U.K.). All other reagents

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‡ Abbreviations: ROS, reactive oxygen species; TBA, thiobarbituric acid.

were of the highest quality available from BDH (Poole, U.K.).

Microsomal lipid peroxidation. Microsomes were isolated from homogenized livers of freshly killed Wistar rats (either sex, 150–250 g) and their peroxidation in the presence of iron ions and ascorbic acid was measured by the thiobarbituric acid (TBA) method, essentially as described by Quinlan *et al.* [13]. Reaction mixtures were 1.0 mL, containing 0.25 mg microsomal protein, 10 μL coumarin dissolved in ethanol (or an equivalent volume of ethanol for the controls) and 10 mM KH_2PO_4-KOH buffer, pH 7.4. Peroxidation was started by adding $FeCl_3$ and ascorbic acid (final concentrations: 100 μM), followed by incubation at 37° for 20 min. After this, the extent of peroxidation was assessed using the TBA test: 1.0 mL of TBA (1%, w/v in 0.05 M NaOH) and 1.0 mL 2.8% (w/v) trichloroacetic acid were added, the tubes heated at 100° for 15 min, and after cooling and extraction of the chromogen into 3 mL *n*-butanol, the absorbance at 532 nm was read. Microsomal membranes were used within 2 weeks of preparation, and their protein content was measured using the Folin–Ciocalteau reagent with bovine serum albumin as standard.

Studies of hydroxyl radical formation of scavenging. Hydroxyl radicals were generated by incubating the following reagents at the indicated final concentrations in 1.0 mL 10 mM KH_2PO_4-KOH buffer pH 7.4 at 37° for 60 min: 1.42 mM H_2O_2, $FeCl_3$–EDTA (20 μM, 100 μM) and 2.8 mM deoxyribose [14]. The iron salt was premixed with chelator before addition to the reaction mixture. The extent of deoxyribose degradation by the formed hydroxyl radicals was measured directly in the aqueous phase by the TBA method as described above, except that butanol extraction was omitted [15].

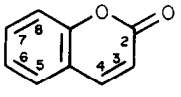
Preliminary testing showed that some coumarins exerted $OH^{\cdot}$-scavenging properties. The rate constants for this reaction were measured by incubating various concentrations of coumarin in the presence of ascorbic acid (100 μM), $FeCl_3$–EDTA (100 μM, 104 μM) and H_2O_2 (1 mM), as described [16].

To measure pro-oxidant actions of coumarins (enhancement of $OH^{\cdot}$ formation), incubations were performed as above, using a fixed concentration of coumarin (100 μM).

Solutions of iron salts, H_2O_2 and ascorbate were freshly made up just before use. Coumarins were added to the glass reaction tubes in ethanol, and the solvent evaporated under a N_2 stream before adding the other reagents. This was necessary because ethanol is itself a powerful scavenger of hydroxyl radicals.

Scavenging of hydrogen peroxide. H_2O_2 was measured spectrophotometrically by the guaiacol-peroxidase reaction which produces a brown colour measured at 436 nm [17]. Reaction mixtures of 1.0 mL contained 150 mM KH_2PO_4-KOH buffer pH 7.4, 50 μL guaiacol solution (made by adding 100 μL pure guaiacol to 50 mL distilled water) and 10 μL horseradish peroxidase (5 mg/mL in the same buffer), as described in [17], and the reaction was initiated by adding H_2O_2. Coumarins were preincubated at concentrations up to 1 mM with 0.1 or 1.0 mM H_2O_2 for 30 min at 25°, and then the remaining H_2O_2 was measured using the peroxidase system. Appropriate blanks using an equivalent volume of the ethanol solvent for the coumarins were also included, thus providing the baseline

Table 1. Chemical structures of the coumarins used in this study



Test compound		Substituents				
Number	Name	4	5	6	7	8
1	4-Hydroxycoumarin	OH	—	—	—	—
2	7-Hydroxycoumarin (umbelliferone)	—	—	—	OH	—
3	7-Methylcoumarin	—	—	—	CH_3	—
4	7-Methoxycoumarin (herniarin)	—	—	—	OCH_3	—
5	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	CH_3	—	—	OH	—
6	7-Methoxy-4-methylcoumarin	CH_3	—	—	OCH_3	—
7	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	—	—	OCH_3	OH	OH
8	6,7-Dihydroxycoumarin (esculetin)	—	—	OH	OH	—
9	6,7-Dihydroxy-4-methylcoumarin (4-methylesculetin)	CH_3	—	OH	OH	—
10	7-Hydroxy-6-methoxycoumarin (scopoletin)	—	—	OCH_3	OH	—
11	7-Hydroxy-6-*O*-β-D-glucosylcoumarin (esculin)	—	—	OGlu	OH	—
12	7-Hydroxy-6-methoxy-8-*O*-β-D-glucosylcoumarin (fraxin)	—	—	OCH_3	OH	OGlu
13	5,7-Dihydroxy-4-methylcoumarin	CH_3	OH	—	OH	—
14	3,4-Dihydrocoumarin	—	—	—	—	—
15	7,8-Dihydroxycoumarin (daphnetin)	—	—	—	OH	OH
16	7,8-Dihydroxy-4-methylcoumarin (4-methyldaphnetin)	CH_3	—	—	OH	OH

values for H_2O_2 against which the effects of removal by reaction with coumarins could be compared.

In the case of those coumarins which interfered with horseradish peroxidase (i.e. those like scopoletin which are themselves substrates [18, 19]), interaction with H_2O_2 to generate O_2 was measured using an oxygen electrode. In these experiments, samples of the reaction mixtures of coumarins (100 μM) with hydrogen peroxide [20] were injected immediately after mixing in phosphate buffer containing excess catalase into the sample compartment of a Clark-type oxygen electrode (25°, stirring; Hansatech Ltd, King's Lynn, U.K.).

Effects of coumarins as scavengers of superoxide anion radicals. Superoxide anions were generated by preparing a mixture of hypoxanthine and xanthine oxidase. Reaction mixtures of 1.0 mL contained the following: 50 mM KH_2PO_4-KOH pH 7.4, 1 mM EDTA, 100 μM hypoxanthine and 100 μM cytochrome *c*, type III. Reaction was started by adding 0.066 U of xanthine oxidase (freshly diluted in 100 μL of the above phosphate buffer), and the rate of cytochrome *c* reduction was measured at 550 nm in a recording spectrophotometer at 25° [21]. The results are expressed as percentage inhibition of cytochrome *c* reduction. The activity of the generating system alone was 0.166 ± 0.004 $O.D._{550}$ U/min.

Control experiments were performed to determine whether the coumarins themselves directly reduce cytochrome *c* or inhibit xanthine oxidase. Thus they were added to solutions containing 100 μM cytochrome *c* in phosphate buffer, and the $O.D._{550}$ was measured. Their action on xanthine oxidase was tested by measuring uric acid formation under the conditions given in the previous paragraph but with xanthine as substrate and absorbance measured at 295 nm (15 min incubation, 25°). Results are expressed as percentage inhibition of uric acid production. The activity of the generating system alone was 0.329 ± 0.006 $O.D._{295}$ U/min.

Assay of hypochlorous acid generation and its scavenging by coumarins. HOCl was prepared immediately before use by adjusting a solution of NaOCl to pH 6.2 with dilute sulphuric acid, and its concentration was measured spectrophotometrically at 235 nm, using a molar extinction coefficient of 100. For the assay, α_1-antiprotease (final concentration 0.77 mg/mL) was mixed with the drug dissolved in phosphate-buffered saline at the final concentration stated and then HOCl was added to yield a concentration of 75 μM. The final reaction volume was 35 μL in phosphate-buffered saline, pH 7.4 [22]. This reaction mixture was incubated at 25° for 1 hr; 2 mL of buffer was then added, followed by 30 μL of a 0.1% (w/v) solution of pig pancreatic elastase in the same buffer. After incubation for a further 30 min, elastase substrate (80 μL of 2.5 mg/mL *N*-succinyl-ala-ala-ala-*p*-nitroanilide in phosphate-buffered saline) was added and the elastase activity remaining in the solution was then determined by measuring the linear rate of change of absorbance at 410 nm. Results are expressed as the percentage inhibition of elastase activity. Activity of elastase under these conditions was 0.027 ± 0.002 $O.D._{410}$ U/min without addition of inhibitor antiprotease.

RESULTS

Sixteen coumarins with varying degrees of substitution (Table 1) were tested for their ability to

Table 2. Inhibition of microsomal lipid peroxidation by coumarins

Test compound		% inhibition at 100 μM	IC_{50} (μM)
Number	Name		
1	4-Hydroxycoumarin	0.6 ± 0.4	—
2	7-Hydroxycoumarin (umbelliferone)	3.2 ± 1.1	—
3	7-Methylcoumarin	0.1 ± 0.1	—
4	7-Methoxycoumarin (herniarin)	3.0 ± 1.7	—
5	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	6.7 ± 1.6	—
6	7-Methoxy-4-methylcoumarin	0.9 ± 0.4	—
7	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	100	3.3
8	6,7-Dihydroxycoumarin (esculetin)	100	13.0
9	6,7-Dihydroxy-4-methylcoumarin (4-methylesculetin)	100	8.0 (7.2*)
10	7-Hydroxy-6-methoxycoumarin (scopoletin)	11.6 ± 3.1	—
11	7-Hydroxy-6-*O*-glucosylcoumarin (esculin)	0	—
12	7-Hydroxy-6-methoxy-8-*O*-glucosylcoumarin (fraxin)	0	—
13	5,7-Dihydroxy-4-methylcoumarin	100	12.0
14	3,4-Dihydrocoumarin	0	—
15	7,8-Dihydroxycoumarin (daphnetin)	100	18.0
16	7,8-Dihydroxy-4-methylcoumarin (4-methyldaphnetin)	100	2.8
Reference compound			
Butylated hydroxytoluene (BHT)		100	0.7

Results show means ± SEM for six tests; IC_{50} values based on values at six concentrations.

* This IC_{50} value was measured after adding 100 μM butylated hydroxytoluene to the TBA reagent to suppress any peroxidation that might occur during the heating of the complete assay mixture.

—, Not determined.

Table 3. Effect of coumarins on iron ion-dependent hydroxyl radical generation

Reaction conditions	Test compound (100 μM)	Deoxyribose degradation (A_{532} after 60 min incubation)
$FeCl_3$ alone		0.021 ± 0.001
$FeCl_3$–EDTA alone		0.046 ± 0.001
$FeCl_3$–EDTA + H_2O_2 (full reaction mixture, RM)		0.119 ± 0.002
RM + **1**	4-Hydroxycoumarin	0.117 ± 0.002
RM + **2**	7-Hydroxycoumarin (umbelliferone)	0.105 ± 0.003*
RM + **3**	7-Methylcoumarin	0.106 ± 0.003*
RM + **4**	7-Methoxycoumarin (herniarin)	0.108 ± 0.002*
RM + **5**	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	0.108 ± 0.002*
RM + **6**	7-Methoxy-4-methylcoumarin	0.110 ± 0.002*
RM + **7**	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	1.340 ± 0.007†
RM + **8**	6,7-Dihydroxycoumarin (esculetin)	0.197 ± 0.002†
RM + **9**	6,7-Dihydroxy-4-methylcoumarin (4-methylesculetin)	0.192 ± 0.002†
RM + **10**	7-Hydroxy-6-methoxycoumarin (scopoletin)	0.109 ± 0.002*
RM + **11**	7-Hydroxy-6-*O*-glucosylcoumarin (esculin)	0.108 ± 0.001*
RM + **12**	7-Hydroxy-6-methoxy-8-*O*-glucosylcoumarin (fraxin)	0.110 ± 0.001*
RM + **13**	5,7-Dihydroxy-4-methylcoumarin	0.110 ± 0.001*
RM + **14**	3,4-Dihydrocoumarin	0.125 ± 0.002
RM + **15**	7,8-Dihydroxycoumarin (daphnetin)	0.365 ± 0.004†
RM + **16**	7,8-Dihydroxy-4-methylcoumarin (4-methyldaphnetin)	0.406 ± 0.002†
Reference compounds		
RM + myricetin		0.958 ± 0.007†
RM + ethanol		0.111 ± 0.001*

Results show means ± SEM for six tests.
* Indicates value significantly decreased from control (RM), $P < 0.05$ by Student's unpaired *t*-test.
† Indicates value significantly increased, $P < 0.05$.

inhibit non-enzymatic lipid peroxidation in rat liver microsomes stimulated by $FeCl_3$–ascorbate. All the compounds were initially tested at 100 μM, and the IC_{50} values (concentrations required to inhibit by 50%) were determined for those coumarins possessing significant inhibitory activity (defined as greater than 35% inhibition at 100 μM), as shown in Table 2. Those coumarins with two free hydroxyl groups such as compounds **7**, **8**, **9**, **13**, **15** and **16** were highly active inhibitors, whereas replacement of hydroxyl with methoxy or glucose caused loss of inhibitory activity (compare **10**, **11** with **8**). None of the compounds interfered with the TBA test, since they did not alter the colour formation if they were added at the end of the incubation with the TBA reagents instead of being included in the reaction mixtures. Possible formation of peroxides leading to additional colour formation during the heating step (see Materials and Methods and Ref. 23) did not influence the results: for example, similar IC_{50} values for 4-methylesculetin (**9**) were obtained when butylated hydroxytoluene was added as a precautionary measure before heating the TBA test reagents (Table 2).

The effects of coumarins on iron ion-dependent hydroxyl radical generation were tested in several ways. Mixtures of $FeCl_3$–EDTA and H_2O_2 generate $OH^{\cdot}$ at a relatively slow rate by Fenton chemistry [24], and can be useful for identifying compounds capable of accelerating hydroxyl radical formation. On the other hand, mixtures of $FeCl_3$–ascorbate and H_2O_2 generate $OH^{\cdot}$ at a more rapid rate, and are used to assess the ability of compounds to scavenge hydroxyl radicals and for determining the rate constants for such reactions [16].

Table 3 shows a representative set of results obtained by adding coumarins under conditions of slow generation of hydroxyl radicals. All *ortho*-dihydroxy coumarins (compounds **7**, **8**, **9**, **15** and **16**) accelerated hydroxyl radical formation (recorded as enhanced deoxyribose damage). Most of the other coumarins caused small but significant reductions in $OH^{\cdot}$ generation. Control experiments showed that none of the coumarins affected the measurement of deoxyribose degradation (they had no effect when added to the TBA reagents), nor did they react with $OH^{\cdot}$ to give TBA-reactive material (omission of the deoxyribose from the reaction mixtures completely abolished chromogen formation). In the presence of ascorbic acid (conditions of rapid hydroxyl radical formation), the stimulatory effects of compounds **7**, **8**, **9**, **15** and **16** was much less pronounced (data not shown).

The rate constants for reaction with hydroxyl radicals for those 10 coumarins which effectively scavenged $OH^{\cdot}$ are shown in Table 4; compound **1** was not active. The rate constants were high, and all lay between the values obtained for ethanol and dimethylsulphoxide (Table 4).

It is now known that "site-specific" damage to deoxyribose occurs if the Fe^{3+}–ascorbate–H_2O_2-induced generation of hydroxyl radicals is performed in the absence of EDTA, since omission of the chelator allows iron ions to bind directly to the

Table 4. Scavenging of hydroxyl radicals by coumarins

Test compound		Rate constant for the reaction with $OH^{\cdot}$
Number	Name	($M^{-1} . sec^{-1}$)
1	4-Hydroxycoumarin	not active
2	7-Hydroxycoumarin (umbelliferone)	6.1×10^9
3	7-Methylcoumarin	4.0×10^9
4	7-Methoxycoumarin (herniarin)	7.1×10^9
5	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	6.7×10^9
6	7-Methoxy-4-methylcoumarin	2.4×10^9
10	7-Hydroxy-6-methoxycoumarin (scopoletin)	7.2×10^9
11	7-Hydroxy-6-*O*-glucosylcoumarin (esculin)	5.2×10^9
12	7-Hydroxy-6-methoxy-8-*O*-glucosylcoumarin (fraxin)	6.4×10^9
13	5,7-Dihydroxy-4-methylcoumarin	2.9×10^9
14	3,4-Dihydrocoumarin	2.1×10^9
Reference compounds		
Ethanol		1.4×10^9
Dimethylsulphoxide		8.2×10^9

Results based on six tests at each of five concentrations.

Table 5. Effect of coumarins on iron ion-dependent site-specific deoxyribose damage in the H_2O_2–$FeCl_3$–ascorbate system (iron ion chelation)

Test compound		% inhibition
Number	Name	at 100 μM
1	4-Hydroxycoumarin	1.4 ± 0.5
2	7-Hydroxycoumarin (umbelliferone)	3.0 ± 0.8
3	7-Methylcoumarin	1.9 ± 0.7
4	7-Methoxycoumarin (herniarin)	0.2 ± 0.5
5	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	2.7 ± 0.9
6	7-Methoxy-4-methylcoumarin	0.4 ± 0.3
7	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	31.1 ± 0.5†
8	6,7-Dihydroxycoumarin (esculetin)	25.8 ± 1.8†
9	6,7-Dihydroxy-4-methylcoumarin (4-methylesculetin)	9.5 ± 0.8*
10	7-Hydroxy-6-methoxycoumarin (scopoletin)	3.7 ± 1.7
11	7-Hydroxy-6-*O*-glucosylcoumarin (esculin)	1.9 ± 0.6
12	7-Hydroxy-6-methoxy-8-*O*-glucosylcoumarin (fraxin)	2.8 ± 0.8
13	5,7-Dihydroxy-4-methylcoumarin	2.4 ± 0.6
14	3,4-Dihydrocoumarin	1.4 ± 0.5
15	7,8-Dihydroxycoumarin (daphnetin)	15.5 ± 0.8†
16	7,8-Dihydroxy-4-methylcoumarin (4-methyldaphnetin)	15.8 ± 0.9†
Reference compounds		
Desferrioxamine		58.3 ± 1.3†
Ethanol		1.2 ± 0.4

Results show means ± SEM for six tests.
*,† Indicate statistically significant inhibition, $P < 0.05, 0.01$, by Student's unpaired *t*-test.

deoxyribose [25, 26]. The only molecules which can inhibit deoxyribose degradation in the absence of EDTA are those which have iron ion-binding capacity and which can withdraw the iron ions and render them inactive or poorly active in Fenton reactions [26]. The possible chelating effects of coumarins in this system are shown in Table 5. Under these conditions, none of the coumarins which were shown to scavenge $OH^{\cdot}$ in the Fe^{3+}–EDTA–ascorbate system caused significant inhibition of $OH^{\cdot}$ generation (i.e., they could not effectively remove the iron ions). On the other hand, the five coumarins which were pro-oxidant in the presence of Fe^{3+}–EDTA produced striking inhibition of site-specific deoxyribose degradation induced by iron ions, as did the established chelator desferrioxamine (Table 5). Thus these five *ortho*-dihydroxy coumarins appear to be effective chelators of iron ions.

The coumarins were tested as possible scavengers of hypochlorous acid (Table 6). This was measured

Table 6. Effect of coumarins as scavengers of hypochlorous acid

Reaction conditions	Test compound (1 mM unless indicated)	% inhibition of elastase activity
Buffer + elastase		—
Buffer + α_1-antiprotease + elastase		96.1
Buffer + α_1-antiprotease + HOCl + elastase (full reaction mixture, RM)		6.9
RM + **1**	4-Hydroxycoumarin	6.7
RM + **2**	7-Hydroxycoumarin (umbelliferone)	14.0
RM + **3**	7-Methylcoumarin	17.9
RM + **4**	7-Methoxycoumarin (herniarin)	2.8
RM + **5**	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	0
RM + **6**	7-Methoxy-4-methylcoumarin	3.3
RM + **7**	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	35.9
+ **7**	at 0.5 mM	13.4
RM + **8**	6,7-Dihydroxycoumarin (esculetin)	8.4
RM + **9**	6,7-Dihydroxy-4-methylcoumarin (4-methylesculetin)	6.1
RM + **10**	7-Hydroxy-6-methoxycoumarin (scopoletin)	7.3
RM + **11**	7-Hydroxy-6-*O*-glucosylcoumarin (esculin)	0
RM + **12**	7-Hydroxy-6-methoxy-8-*O*-glucosylcoumarin (fraxin)	0
RM + **13**	5,7-Dihydroxy-4-methylcoumarin	64.0
+ **13**	at 0.5 mM	54.5
+ **13**	at 0.1 mM	25.8
RM + **14**	3,4-Dihydrocoumarin	0
RM + **15**	7,8-Dihydroxycoumarin (daphnetin)	6.1
RM + **16**	7,8-Dihydroxy-4-methylcoumarin (4-methyldaphnetin)	0
Reference compound		
RM + oxyphenbutazone		91.1
+ oxyphenbutazone at 0.5 mM		86.0
+ oxyphenbutazone at 0.1 mM		35.9

Results show the mean values obtained from duplicate tests at each concentration.

The reagents were added in the order listed as RM; test compounds were added before HOCl for incubation prior to adding elastase (see Materials and Methods). The activity of the elastase alone was 0.027 $O.D._{410}$ U/min. Control experiments showed that none of the test compounds affected elastase activity or the ability of α_1-antiprotease to inhibit it.

in terms of their ability to protect α_1-antiprotease from inactivation by HOCl, with the antiprotease measured in terms of its inhibitory effect on elastase: incubation of antiprotease with elastase almost totally abolished its catalytic activity, whereas pre-incubation of the antiprotease with HOCl substantially decreased its elastase-inhibitory activity (Table 6). Inclusion of all but two of the coumarins at final concentrations of up to 1 mM failed to protect the antiprotease, showing that they are not effective scavengers of hypochlorous acid under these reaction conditions. The two exceptions were fraxetin (**7**) and, in particular, 5,7-dihydroxy-4-methylcoumarin (**13**). This latter compound was also protective at the lower concentrations of 0.5 and 0.1 mM, although it was somewhat less active than the reference standard, oxyphenbutazone (a powerful scavenger of hypochlorous acid). Control experiments showed that compounds **7** and **13** did not inhibit elastase directly, nor did they interfere with the ability of antiprotease to inhibit elastase.

We then investigated the ability of the coumarins to scavenge superoxide anions (Table 7). Using a mixture of hypoxanthine and xanthine oxidase at pH 7.4, the generated superoxide anions can be detected by their ability to reduce ferricytochrome *c* to ferrocytochrome *c* [27]. Any added compound that is itself able to react with O_2^- should decrease the rate of reduction of cytochrome *c*, unless the compound itself reacts with the cytochrome. In this regard, of the coumarins studied, esculetin (**8**), 4-methylesculetin (**9**) daphnetin (**15**) and 4-methyldaphnetin (**16**) themselves reduced cytochrome *c* at the concentrations tested (10^{-4} and 10^{-5} M). At lower concentrations these compounds neither reduced cytochrome *c* themselves nor scavenged O_2^- generated by the hypoxanthine–xanthine oxidase system. Fraxetin (**7**) at 10^{-4} M also reduced cytochrome *c*, but did not do so at 10^{-5} M; at the latter concentration it proved to be an effective scavenger of superoxide anions (Table 7). Of the other coumarins, compounds **2**, **5**, **10** and **13** also markedly scavenged O_2^- at 10^{-4} M, and their IC_{50} values were determined (Table 7). The remainder of the compounds (**1**, **3**, **4**, **6**, **11**, **12** and **14**) were essentially inactive in these tests.

Control experiments were performed to find out whether those coumarins which apparently scavenged O_2^- might in fact have done so by inhibiting xanthine oxidase (Table 8). This was determined by measuring their ability to affect the generation of uric acid from xanthine (see Materials

Table 7. Scavenging by coumarins of superoxide generated by the hypoxanthine–xanthine oxidase system

Test compound		% inhibition at 100 μM	IC_{50} (μM)
Number	Name		
1	4-Hydroxycoumarin	4.2 ± 1.0	—
2	7-Hydroxycoumarin (umbelliferone)	100	12.0
3	7-Methylcoumarin	9.1 ± 1.9	—
4	7-Methoxycoumarin (herniarin)	15.8 ± 0.7	—
5	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	100	19.0
6	7-Methoxy-4-methylcoumarin	10.8 ± 1.6	—
7	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	67.4 ± 1.6*	3.7
8	6,7-Dihydroxycoumarin (esculetin)	NT	—
9	6,7-Dihydroxy-4-methylcoumarin (4-methylesculetin)	NT	—
10	7-Hydroxy-6-methoxycoumarin (scopoletin)	100	72.0
11	7-Hydroxy-6-*O*-glucosylcoumarin (esculin)	21.6 ± 1.2	—
12	7-Hydroxy-6-methoxy-8-*O*-glucosylcoumarin (fraxin)	6.4 ± 2.5	—
13	5,7-Dihydroxy-4-methylcoumarin	92.9 ± 3.2	31.0
14	3,4-Dihydrocoumarin	1.1 ± 0.8	—
15	7,8-Dihydroxycoumarin (daphnetin)	NT	—
16	7,8-Dihydroxy-4-methylcoumarin (4-methyldaphnetin)	NT	—
Reference compound			
Superoxide dismutase (70 U/mL)		94.0 ± 0.5	4.0 U/mL

Results show means ± SEM for six tests at each concentration. For determination of IC_{50} values, 4–6 concentrations were used.

* This coumarin (fraxetin, **7**) reduces cytochrome *c* at 10^{-4} M; the value shown was obtained using 10^{-5} M.

NT, not tested because the coumarin itself reduces cytochrome *c* at 10^{-4} and 10^{-5} M.

—, Not determined.

Table 8. Effect of coumarins on xanthine oxidase

Test compound		% inhibition at 10^{-4} M
Number	Name	
2	7-Hydroxycoumarin (umbelliferone)	14.0 ± 3.9
5	7-Hydroxy-4-methylcoumarin (4-methylumbelliferone)	15.1 ± 0.8
7	7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	1.9 ± 0.6*
10	7-Hydroxy-6-methoxycoumarin (scopoletin)	4.1 ± 2.1
13	5,7-Dihydroxy-4-methylcoumarin	9.3 ± 2.1
Reference compound		
Allopurinol		96.3 ± 0.3

Results show means ± SEM for six tests.

* Fraxetin (**7**) was tested at 10^{-5} M as this was the concentration used for inhibition of superoxide generation (see Table 7).

and Methods). The five compounds had a very weak inhibitory action on xanthine oxidase at 10^{-4} M, insufficient to account for their substantial superoxide-scavenging activities. Similarly, none of the coumarins at concentrations up to 1 mM were capable of reacting directly with H_2O_2 according to the guaiacol-peroxidase method (used to test most of the compounds) or according to the release of oxygen from hydrogen peroxide monitored directly by an oxygen electrode (this alternative method was used to test compounds **7**, **8**, **9**, **10**, **15** and **16** which were found to react directly with peroxidase).

DISCUSSION

Our systematic survey of 16 naturally occurring plant-derived or synthetic coumarins shows that they have multiple interactions with ROS and that these are structure dependent. The results are summarized in Table 9. There is considerable evidence for the involvement of ROS (e.g. those generated by activated phagocytes) in processes associated with inflammation and tissue damage (reviewed in Ref. 20). It would therefore be logical to investigate whether the "active" coumarins (see below) might interact beneficially with such pathological processes.

Table 9. Major classes of synthetic and natural coumarins, according to their interactions with ROS

Test compound		Lipid peroxidation (Table 2)	$OH^{\cdot}$ generation/ scavenging (Table 3/4/5)	HOCl scavenging (Table 6)	Inhibition of $O_2^{\cdot-}$ generation (Table 7)	Effect on XO (Table 8)	Reduction of cytochrome *c* (Table 7)
Number	Substitution pattern						
Inactive (non-redox active) coumarins							
1	4-Hydroxy	Inactive	Inactive	Inactive	Inactive	NT	No
14	3,4-Dihydro	Inactive	Inactive	Inactive	Inactive	NT	No
Inactive in all tests except ability to scavenge hydroxyl radicals							
3	7-Methyl	Inactive	Scavenger +	Inactive	Inactive	NT	No
4	7-Methoxy	Inactive	Scavenger ++	Inactive	Inactive	NT	No
6	7-Methoxy-4-methyl	Inactive	Scavenger +	Inactive	Inactive	NT	No
11	7-Hydroxy-6-*O*-glucosyl	Inactive	Scavenger ++	Inactive	Inactive	NT	No
12	7-Hydroxy-6-methoxy-8-*O*-glucosyl	Inactive	Scavenger ++	Inactive	Inactive	NT	No
Inactive against lipid peroxidation, HOCl scavenging and cytochrome *c* reduction but able to scavenge hydroxyl radicals and superoxide (moderate electron donors?)							
2	7-Hydroxy	Inactive	Scavenger ++	Inactive	Active ++	Inactive	No
5	7-Hydroxy-4-methyl	Inactive	Scavenger ++	Inactive	Active ++	Inactive	No
10	7-Hydroxy-6-methoxy	Inactive	Scavenger ++	Inactive	Active +	Inactive	No
Coumarins which strongly inhibit lipid peroxidation and which are pro-oxidant and reduce cytochrome *c* (electron donating) but which do not scavenge HOCl. (Actions on superoxide not known)							
8	6,7-Dihydroxy	13.0 μM	Pro-oxidant +	Inactive	?	NT	Very fast
9	6,7-Dihydroxy-4-methyl	8.0 μM	Pro-oxidant +	Inactive	?	NT	Very fast
15	7,8-Dihydroxy	18.0 μM	Pro-oxidant +	Inactive	?	NT	Very fast
16	7,8-Dihydroxy-4-methyl	2.8 μM	Pro-oxidant ++	Inactive	?	NT	Very fast
Atypical compounds: both inhibit peroxidation and superoxide generation, but also scavenge HOCl							
Case i (reduces cytochrome *c* and is pro-oxidant)							
7	7,8-Dihydroxy-6-methoxy	3.3 μM	Pro-oxidant +++	+	Active +++	Inactive	Yes
Case ii (does not reduce cytochrome *c* and is a hydroxyl radical scavenger)							
13	5,7-Dihydroxy-4-methyl	12.0 μM	Scavenger +	++	Active +	Inactive	No

NT, not tested.

Compounds **1**, **3**, **4**, **6**, **11**, **12** and **14** were either inactive in all tests (**1** and **14**) or else solely active as scavengers of hydroxyl radicals. These compounds will not be considered further, although they might be valuable as negative controls in tests of the biological properties of the active compounds.

Compounds **2**, **5** and **10** were able both to scavenge hydroxyl radicals with high rate constants and to scavenge superoxide anions generated in the hypoxanthine–xanthine oxidase system. This latter property was not due to direct reduction of cytochrome *c* or inhibition of xanthine oxidase (see Table 9). These compounds also decrease the amounts of superoxide generated by human polymorphonuclear leukocytes (M. Payá and J. R. S. Hoult, unpublished results). We attribute this to scavenging of the superoxide anion radicals as they do not inhibit the NADPH oxidase directly. Thus, these compounds might be expected to reduce tissue damage consequent to the activation of the superoxide-producing NADPH oxidase in stimulated leukocytes.

The remainder of this discussion concerns the other six coumarins (**7**, **8**, **9**, **13**, **15** and **16**), all of which have two hydroxyl groups (and in some cases other substituents), and which possess additional interesting properties.

The dihydroxylated coumarins were all active as inhibitors of lipid peroxidation, with *ortho*-dihydroxy being more favourable than *meta*-dihydroxy (compare **9** and **16** with **13**). Addition of a further 4-methyl substituent increases potency (**9** vs **8** and **16** vs **15**), presumably by enhancing lipid solubility. Loss of the dihydroxyl function by substitution of one –OH with methoxy or glucosyl is detrimental (**8** vs **10** and **11**). These structural requirements for antiperoxidative activity are similar to those already reported for the flavonoids [28, 29], a related class of plant-derived phenolic benzopyrone derivatives.

However, the mechanism of this potentially protective effect has not yet been established. One possibility is chelation of ferric ions used to initiate peroxidation. For example, desferrioxamine has been shown to inhibit iron ion-dependent lipid peroxidation [30]. For this reason, we tested the ability of these coumarins to remove iron ions from deoxyribose (Table 5); however, of the six compounds effective against lipid peroxidation, two (**9** and **13**) did not appear to possess substantial iron chelation ability. An alternative possibility is that these coumarins possess a favourable electronic distribution for reacting quickly with intermediate lipid peroxyl radicals and sufficient lipid solubility to partition effectively in lipid bilayers. Our preliminary tests suggest that they are indeed capable of the rapid inactivation of alkylperoxy radicals (unpublished experiments).

ortho-Dihydroxy substitution is also required for pro-oxidant activity (enhanced generation of hydroxyl radicals) in the Fe^{3+}–EDTA–H_2O_2 deoxyribose assay (Table 3). This may arise by electron donation from the catechol enabling redox cycling of ferric to ferrous ions so as to maintain a supply of electrons for the Fenton reaction, as suggested previously for ascorbate and catechol-flavonoids (see Ref. 14 for further discussion). *meta*-Substituted coumarin **13** did not exert pro-oxidant activity under these conditions. A similar explanation could be offered to explain the differing abilities of the *ortho*- and *meta*-substituted dihydroxycoumarins to reduce cytochrome *c* (Table 9).

It is notable that pro-oxidant activity of the coumarins is only observed under particular reaction conditions: if the compounds are tested in the Fe^{3+}–ascorbate–H_2O_2 system (Table 5), then the pro-oxidant compounds behave as scavengers, inhibiting iron ion-induced hydroxyl radical-dependent damage to deoxyribose. The *ortho*-dihydroxycoumarins are notably more effective in this regard than is the *meta*-substituted compound **13** or the other non-dihydroxylated compounds, and this is consistent with the finding that *ortho*-hydroxyl substitution favours bidentate chelation of iron ions [31]. We therefore interpret the results to mean that the *ortho*-dihydroxylated coumarins are effectively removing iron ions from the deoxyribose (i.e. are acting as ferric chelators), as does desferrioxamine.

Only two of the compounds tested here (**7** and **13**) possessed significant ability to scavenge hypochlorous acid in the sub-millimolar range (Table 6). Thus there is not sufficient information to determine what structural features are important for this activity. However, it is of interest that compound **13**, the *meta*-substituted 5,7-dihydroxy-4-methylcoumarin, was the most potent as it possesses an array of potentially beneficial characteristics: it inhibits lipid peroxidation with satisfactory potency, it is not pro-oxidant but can chelate ferric ions, and it can scavenge superoxide anions. It would thus be of special interest to characterize this molecule in terms of its anti-inflammatory profile.

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