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PEROXYL RADICAL SCAVENGING BY A SERIES OF COUMARINS

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Sixteen plant-derived or synthetic coumarins with various hydroxyl and other substitutions were tested for their ability to scavenge alkylperoxyl radicals generated in the aqueous phase by the controlled thermolysis of 2,2′-azo-bis-(2-amidinopropane) dihydrochloride (ABAP). Protection by coumarins against inactivation of lysozyme by the radicals was assayed by measuring the loss of turbidity of suspensions of *M. lysodeikticus*. Ten of the coumarins were potent scavengers of aqueous peroxyl radicals with activities comparable to n-propyl gallate, desferrioxamine, ferrioxamine and trolox c, yielding IC_{50} values in the range 21 to 92 micromolar. The presence of 6,7-ortho-dihydroxy functions gave compounds of the greatest potency. Scavenging activity was unrelated to ability to chelate iron ions. The active coumarins are attractive candidates for evaluation as protective agents against disorders in which oxidative stress is implicated.

KEY WORDS: Coumarins, natural products, catechols, peroxyl radicals, radical scavengers, tocopherols, desferrioxamine.

INTRODUCTION

Various radical and non-radical species derived from molecular oxygen (reactive oxygen species, ROS) appear to be involved in cellular dysfunctions brought about by oxidative stress. Thus ROS have been shown to damage lipids, proteins, carbohydrates and nucleic acids (reviewed in refs. 1–3). Much effort has therefore been made to identify potential antioxidants of both synthetic and natural origin in order to provide rational therapies for these conditions.[1,4]

The coumarins comprise a large group of phenolic compounds widely distributed in nature. They possess numerous biological and pharmacological properties,[5,6] including antioxidant activity.[7–11] We recently evaluated a series of natural and synthetic coumarins for their ability to inhibit microsomal lipid peroxidation and to scavenge hydroxyl radicals, hypochlorous acid and superoxide radicals. Many of them demonstrated potent antioxidant activity *in vitro*, depending on their structures.[12]

Peroxyl radicals are key intermediates in the chain reaction of lipid peroxidation,[1,13] and agents which inhibit this process may do so by reacting with them. For example, ascorbic acid may scavenge water-soluble peroxyl radicals whereas alpha-tocopherol may react with lipid-soluble peroxyl radicals.[13–16] Moreover, there is a close chemical relationship between coumarins and tocopherols as both are benzopyrane derivatives.

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We have therefore tested the coumarins as scavengers of peroxyl radicals generated in the aqueous phase, in order to establish the structural features necessary for this property. Peroxyl radicals were obtained from the thermolysis of 2,2′-azo-bis(2-amidinopropane) (ABAP), as in ref. 17. Radicals formed by ABAP decomposition were allowed to inactivate the enzyme lysozyme: peroxyl radical scavengers can offer protection to this enzyme.[17]

MATERIALS AND METHODS

Vitamin E (±-α-tocopherol), n-propyl gallate, probucol, EDTA, lysozyme grade III and *Micrococcus lysodeikticus* (ATCC 4698) were obtained from the Sigma Chemical Company; ABAP was from Park Scientific Ltd, Northampton; diethylenetriamine-pentaacetic acid (DETAPAC) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were from Aldrich. All other chemicals and reagents were from the sources given in ref. 12. Iron-loaded desferrioxamine (ferrioxamine) was prepared by mixing freshly prepared solutions of desferrioxamine and ferric chloride in the molar ratio of 1:0.9 immediately before use, to ensure that no 'free' iron remained in the solution.

Incubations were performed in two stages, involving the generation of peroxyl radicals in the presence or absence of coumarins and lysozyme, followed by the removal of aliquots for assay of residual lysozyme activity. Reaction mixtures of 1.0 ml contained the following: 50 mM KH_2PO_4–KOH pH 7.4, lysozyme 0.68 mM, coumarin 0.1 mM and ABAP 10 mM and were incubated at 45°C (to facilitate the

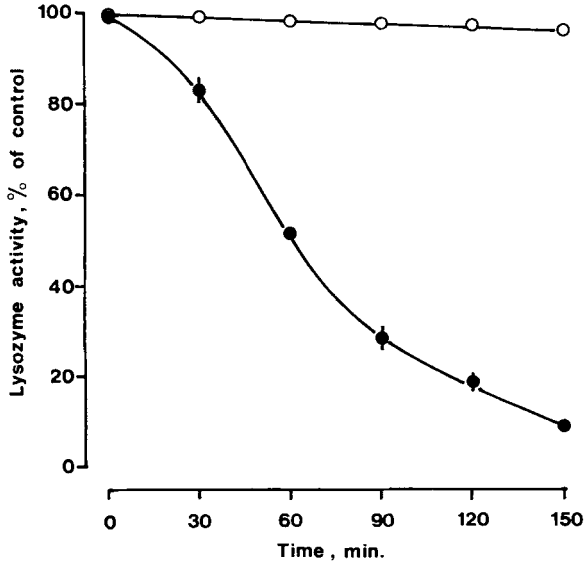


FIGURE 1 Time-dependent inactivation of lysozyme in the presence of ABAP at 45°C. Lysozyme was incubated with 10 mM ABAP (●) or with an equivalent volume of buffer, control (○), and sampled at the times shown for measurement of enzyme activity using a suspension of lyophilized *Micrococcus lysodeikticus*. Results show mean values ± sem from 6 tests.

thermal decomposition of ABAP). Coumarins were added to the glass reaction tubes in ethanol and the solvent evaporated under a N_2 stream before adding the other reagents (because ethanol itself actively scavenges peroxyl radicals).

Aliquots of 50 μL were withdrawn at different times and added to 950 μL of a suspension of *M. lysodeikticus* (0.3 mg/mL) in Dulbecco's buffer. Lysozyme activity was measured at 25°C by monitoring the loss of turbidity at 450 nm.[17] The rate of change of absorbance was recorded during the first minute.

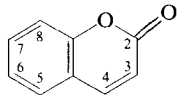
Control experiments showed that the coumarins did not themselves alter lysozyme activity, which under these conditions was 0.453 ± 0.008 absorbance units/min ($n = 20$).

RESULTS AND DISCUSSION

Peroxyl radicals were generated at a controlled rate by the thermal decomposition at 45°C of the water soluble 'azo-initiator' ABAP, and they were identified by their ability to inactivate lysozyme. Figure 1 shows the time course of enzyme inactivation under the chosen reaction conditions: in the presence of ABAP there was progressive loss of lysozyme activity, whereas in buffer alone the enzyme was stable. Accordingly 90 min incubation of lysozyme with ABAP at 45°C (yielding $71.2 \pm 2.4\%$ inactivation, $n = 6$) was selected as the optimum time point for identifying the possible protective (or enhancing) actions of test compounds.

Sixteen coumarins of natural or synthetic origin (structures given in Table I) were tested for their ability to scavenge peroxyl radicals. They were screened at 100 μM

TABLE I
Chemical structures of the coumarins used in this study



Number	Test compound Name	Substituents 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-glucosylcoumarin (esculin)	—	—	OGlu	OH	—
12	7-hydroxy-6-methoxy-8-O-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

TABLE II
Inhibition of peroxyl radical-induced inactivation of lysozyme by coumarins

Number	Test compound Name	Percent inhibition at 100 μM	IC_{50} μM	IC_{50} μM (Lip per)
1	4-hydroxycoumarin	16.4 ± 2.8*	—	—
2	7-hydroxycoumarin (umbelliferone)	57.5 ± 2.5*	92	—
3	7-methylcoumarin	6.1 ± 3.3	—	—
4	7-methoxycoumarin (herniarin)	3.4 ± 1.5	—	—
5	7-hydroxy-4-methylcoumarin (4-methylumbelliferone)	39.3 ± 2.7*	—	—
6	7-methoxy-4-methylcoumarin	0.4 ± 0.3	—	—
7	7,8-dihydroxy-6-methoxycoumarin (fraxetin)	72.1 ± 1.9*	45	3.3
8	6,7-dihydroxycoumarin (esculetin)	81.2 ± 4.8*	21	13.0
9	6,7-dihydroxy-4-methylcoumarin (4-methylesculetin)	94.8 ± 2.8*	26	8.0
10	7-hydroxy-6-methoxycoumarin (scopoletin)	84.2 ± 2.6*	54	—
11	7-hydroxy-6-O-glucosylcoumarin (esculin)	86.8 ± 3.7*	51	—
12	7-hydroxy-6-methoxy-8-O-glucosylcoumarin (fraxin)	85.2 ± 1.8*	49	—
13	5,7-dihydroxy-4-methylcoumarin	82.0 ± 3.2*	52	12.0
14	3,4-dihydrocoumarin	1.1 ± 0.7	—	—
15	7,8-dihydroxycoumarin (daphnetin)	70.4 ± 2.9*	57	18.0
16	7,8-dihydroxy-4-methylcoumarin (4-methyldaphnetin)	72.6 ± 2.6*	51	2.8
Reference compounds				
	n-Propyl gallate	97.8 ± 1.1*	38	
	Vitamin E (±-α-tocopherol)	0.7 ± 0.4	—	
	Probucol	3.0 ± 0.4	—	
	Desferrioxamine mesylate (DFO)	59.7 ± 2.4*	80	
	Iron-loaded DFO	61.8 ± 1.2*	86	
	Trolox c	90.2 ± 2.1*	43	
	DETAPAC	1.3 ± 0.7	—	
	EDTA	1.1 ± 0.5	—	

— = not determined. Results show mean ± sem for 6 tests; IC_{50} values based on tests at 4 concentrations. See reference 12 for further details concerning tests on lipid peroxidation (= "Lip per"). * indicates significant inhibition compared to control tubes, $P < 0.01$ by Student's unpaired t-test.

and were compared with various reference compounds known to scavenge peroxyl radicals (Table II). Of the coumarins, ten showed substantial activity (defined as greater than 50% inhibition of lysozyme inactivation at 100 μM), comparable to that shown by propylgallate, desferrioxamine and trolox. These 15 active compounds were then tested at various concentrations in order to obtain approximate IC_{50} values.

Vitamin E and probucol were not able to protect lysozyme. This may be because these compounds are essentially insoluble in the aqueous media used. Niki *et al.*[18] showed that phenolic compounds with hydrophobic side-chains such as α-tocopherol are less effective scavengers of peroxyl radicals generated in the aqueous phase than are identical phenols lacking the side-chain.

The 10 active coumarins all displayed IC_{50} values in the range 21 to 92 μM (Table II), and the most active were compounds **8** and **9** (containing the 6,7-dihydroxy catechol function). These were more potent than propylgallate or trolox. Other dihydroxy or hydroxy/methoxy substituted coumarins were also potent inhibitors (Table II, compounds **7**, **10**, **11**, **12**, **13**, **15 and 16**). 7-Hydroxyl substitution (but not 7-methyl or 7-methoxy) appears to confer some measure of activity.

Many of these compounds were found previously to be active inhibitors of

non-enzymic lipid peroxidation in rat liver microsomes (12), and the IC_{50} values from these tests are also shown in Table II. Comparison of the two sets of data shows that many of the coumarins exhibit high activity in both assays, but that the optimal features for the two scavenging properties are not the same. Thus high anti-lipid peroxidative activity requires dihydroxyl substitution (and loss of hydroxyl by methoxylation, or addition of a sugar residue are both unfavourable), whereas optimal scavenging of aqueous peroxyl radicals requires dihydroxyl or hydroxyl/methoxy substitutions and is indifferent to the addition of a sugar residue. Moreover, 6,7-dihydroxy-substitution appears to confer optimal activity against aqueous peroxyl radicals, whereas 7,8 substitution is optimal against membrane lipid peroxidation.

A further consideration concerns iron ion chelation. Desferrioxamine was a powerful inhibitor in the present system, with activity comparable to that of 7-hydroxycoumarin (Table II). Iron-loaded desferrioxamine possessed equivalent peroxyl radical scavenging activity (Table II), whereas the chelators EDTA and DETAPAC were both inactive. These results show that the activity of desferrioxamine is independent of its ability to chelate iron ions. Darley-Usmar *et al.*[19] have found desferrioxamine to be capable of scavenging linoleic acid-derived peroxyl radicals, and showed clearly that this is independent of iron chelation. Moreover, desferrioxamine and ferrioxamine scavenge OH· at equal rates.[20]

In conclusion, we have shown that various water soluble dihydroxylated coumarins or their glycosylated congeners are powerful scavengers of ABAP-derived peroxyl radicals generated in the aqueous phase. The fact that these coumarins interact favourably with reactive oxygen species in both aqueous and hydrophobic environments (this paper and ref. 12) makes them attractive candidates for evaluation as protective agents against disorders in which oxidative stress is implicated.

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