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Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 5: Pulse Radiolysis Studies on the Antioxidant Action of 7,8-Diacetoxy-4-methylcoumarin

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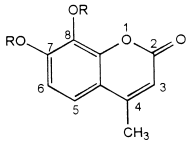
Abstract—7,8-Dihydroxy-4-methylcoumarin (**1**, DHMC) and 7,8-diacetoxy-4-methylcoumarin (**2**, DAMC) were shown to possess radical scavenging property and strongly inhibit membrane lipid peroxidation. Although free polyphenolic compounds are known to be antioxidants, the antioxidant action of the acetoxy compound DAMC was intriguing. Hence, pulse radiolysis studies were undertaken to explain the antioxidant action of DAMC. Accordingly, DAMC and DHMC were separately reacted with the system generating azide radicals and the resulting transient spectra were recorded. The spectra so obtained in both the cases demonstrated peak at 410 nm, characteristic of phenoxyl radical. The rate constants for the formation of phenoxyl radical from DHMC and DAMC were 34×10^8 M^{-1} s^{-1} and 6.2×10^8 M^{-1} s^{-1}, respectively. We propose that the free radical mediated oxidation of DAMC initially produces a radical cation that loses an acetyl carbocation to yield the phenoxyl radical. It is possible to conclude that the mechanism of the antioxidant action of DAMC follows the pathway similar to that of DHMC involving the formation of a stable phenoxyl radical.

Introduction

Halliwell has defined an antioxidant as a substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.[1] Accordingly, normal physiological constituents of the mammalian cells, such as proteins (superoxide dismutase, glutathione peroxidase, etc.), uric acid, creatinine, polyamine, retinol, etc., have been demonstrated to have antioxidant activity. A large number of chemicals (naturally occurring as well as synthetic), foreign to mammalian cells have also been accredited as potent antioxidants.[2,3] Among them, phenolics that include flavones, coumarins, xanthones, etc., have attracted considerable attention.[4,5] Studies on many of their derivatives have suggested that the catechol moiety in their structure may be important in imparting the antioxidant activity.[6,7] Our earlier work [8] highlighted the superb antioxidant action of dioxygenated 4-methylcoumarins including DHMC and DAMC.



1 R=H (DHMC)
2 R=$COCH_3$ (DAMC)

The antioxidant action of DAMC which lacked free catecholic hydroxyl groups was intriguing. Hence pulse radiolysis studies were undertaken to seek explanation. The results indicated that phenoxyl radical was formed from both DHMC and DAMC as a result of azide

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radical scavenging. It was possible to conclude that the antioxidant mechanism of DAMC followed the pathway similar to that of DHMC.

Materials and Methods

DHMC and DAMC were synthesized in our laboratory by the well-known Pechmann condensation.[9] Xanthine and xanthine oxidase were procured from SRL, Mumbai, India. K_2HPO_4, KH_2PO_4, sodium azide and acetonitrile of high purity were purchased from the local suppliers. Pulse radiolysis set up, established at the Bhabha Atomic Research Centre (BARC), Trombay, Mumbai was used.

Pulse radiolysis studies

Reactions of specific one-electron transfer agent (oxidant), namely azide radicals, separately with DHMC and DAMC were studied using the pulse radiolysis facility at Chemistry Division, BARC, Mumbai. The experiments consisted of irradiation of the samples with a very short (50 ns) high energy electron pulses (7 MeV), whereby high concentration of transient radicals are produced in a very short time and the species detected in microsecond to millisecond time scale following changes in light absorption before, during and after the pulse of irradiation over the wavelength range 280–750 nm. In the spectral region, where the product radical and the parent radical overlap, a difference absorbance (ΔOD) is obtained as ($\Delta OD = (\varepsilon_t - \varepsilon_p)cl$), where ε_t and ε_p are the molar extinction coefficients of the transient and parent radicals, respectively, c the concentration and l the optical path length (1 cm). From the time variation of the light absorption at a particular wavelength, preferably absorption maximum, the kinetic parameters such as rate constant for the reaction and the life time of resultant radicals were estimated. The absorbed dose was determined using aerated thiocyanate solutions by following the absorbance at 472–500 nm due to $(SCN)_2^{-\cdot}$ ion radical. For these studies, azide radicals were chosen, as they are known to react selectively by electron transfer and thus mimic reactions of many biological oxidants like peroxyl radicals.[10,11] The reactions of DHMC and DAMC with azide radical were studied by the pulse radiolysis of N_2O saturated aqueous solutions containing 0.1 M sodium azide and 100 μM DHMC or DAMC at pH 7. In case of studies with DAMC, solutions also contained 10% acetonitrile as DAMC was not soluble in water.

Scavenging of superoxide radical by DAMC and DHMC

Superoxide scavenging activity. Superoxide scavenging was assayed by measuring the rate of removal of superoxide generated by the reaction of xanthine oxidase and xanthine, as determined by the reduction of cytochrome C at 550 nm.

The reaction mixture (total volume of 1.0 ml) consisted of phosphate buffer (50 mM, pH 7,4), xanthine (2.5 mM) and ferricytochrome C (0.3 mM). The reaction was initiated by adding xanthine oxidase (0.1 unit) and the superoxide production was quantified by measuring the reduction of cytochrome C. The test chemicals (DAMC/DHMC) were included at a concentration of 100 μM in the reaction mixture mentioned above when the reduction of cytochrome C was inhibited, which would be proportional to scavenging of superoxide radical.

Effect of DAMC/DHMC on xanthine oxidase activity

Xanthine oxidase was assayed by the method described by Berg Meyer.[12] The enzyme activity was examined in the presence as well as absence of the test compounds. The results (data not shown) indicated that neither DAMC nor DHMC had any inhibitory action on the activity of xanthine oxidase.

Measurement of oxidation potential of test compounds

Cyclic voltammetric measurements were carried out using a BAS, CV-50 W electrochemical analysing system. A mixed solvent system comprising of DMSO: CH_3CN (1:9) was employed for the CV studies with 0.1 M $NaClO_4$ as supporting electrolyte. A three electrode configuration was used, comprising of platinum disk working electrode, platinum wire counter electrode and $Ag/AgNO_3$ reference electrode. The reversible one electron ferrocene/ferrocenium couple in the same solvent system was found to have a $E_{1/2}$ of 0.113V.

Results and Discussion

Various pathological conditions including atherosclerosis, cancer, inflammation, arthritis and regressive changes in ageing appear to have etiological relation to the active oxygen-induced and free radical-mediated oxidation of biomolecules.[13,14] The search has been going on at a rapid pace for newer antioxidants that react easily with free radicals and thus protect neighbouring structures from oxidative damage.[13] Our earlier reports demonstrated that DHMC and DAMC were endowed with superb antioxidant properties on three counts: (i) efficient scavenging of the oxygen radicals;[8] (ii) prevention of the formation of ADP-perferryl leading to the cessation of the formation of the oxygen radicals;[15] and (iii) inhibition of cytochrome P-450-linked mixed function oxidases.[16]

In order to understand chemical properties of oxygen radicals and related species and mechanism of their action which are responsible for oxidation reactions in biological systems leading to various ailments and ageing, it is important to measure the rates for radical reactions. Two techniques are usually used for these measurements, i.e. stop-flow method and pulse radiolysis method. The stop-flow methods are often used where the rates of radical reactions are too slow to be measured conveniently by pulse radiolysis, yet too fast to be measured by standard biochemical techniques. In the present study, pulse radiolysis studies were undertaken to seek explanation for the antioxidant action of DAMC and DHMC. The spectra of transient species

obtained from DHMC and DAMC, produced on their reaction with azide radicals were recorded (Figs 1 and 2). It showed absorption from 200 to 600 nm with maximum absorption at 410 nm and another broad peak for absorption at 470–480 nm and simultaneous bleaching in the region where the parent compound (DHMC) absorbed (324 nm[16]). In analogy with the reaction of phenols, the transient species is attributed to the coumarin-phenoxyl radical formed by the electron transfer followed by proton transfer to azide radical.[17,18] It decays by second order kinetics due to radical–radical reactions. The rate constant (Fig. 3) for the formation of the transient species was determined to be 34×10^8 M^{-1} s^{-1} from the slope of the linear plot for change in the rate of formation of the transient species at 410 nm as a function of DHMC concentration from 20 to 140 μM. Similar experiments with DAMC also revealed the formation of a transient species showing identical absorption with reduced yield, it also decayed by second order kinetics, indicating the formation of similar phenoxyl radical. However the rate constant for the formation of the radical in the case of DAMC was lower and was found to be 6.2×10^8 M^{-1} s^{-1}. DAMC does not show any oxidation wave up to +1.0 V, while DHMC underwent irreversible oxidation beyond +0.6 V, thereby implying that the oxidation step of DAMC by azide radical is slower as compared to that of DHMC.

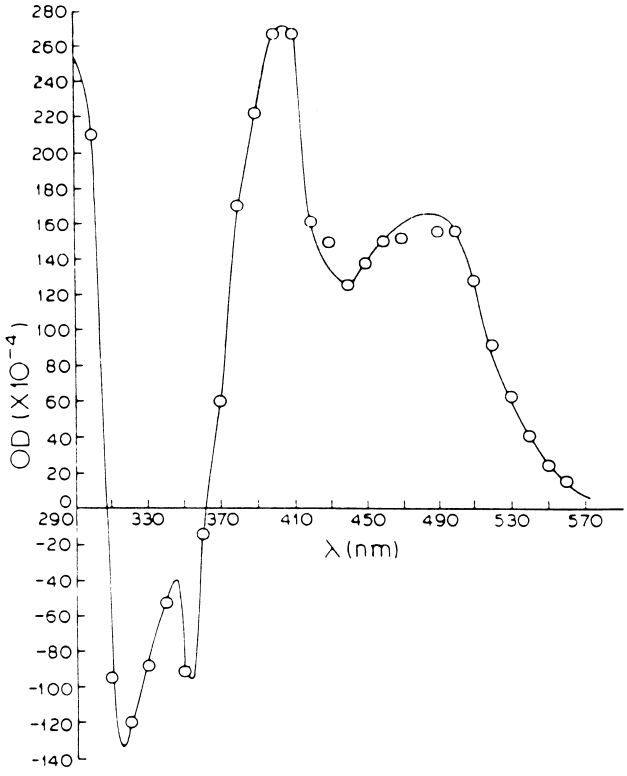

Figure 1. Transient spectra of DHMC.

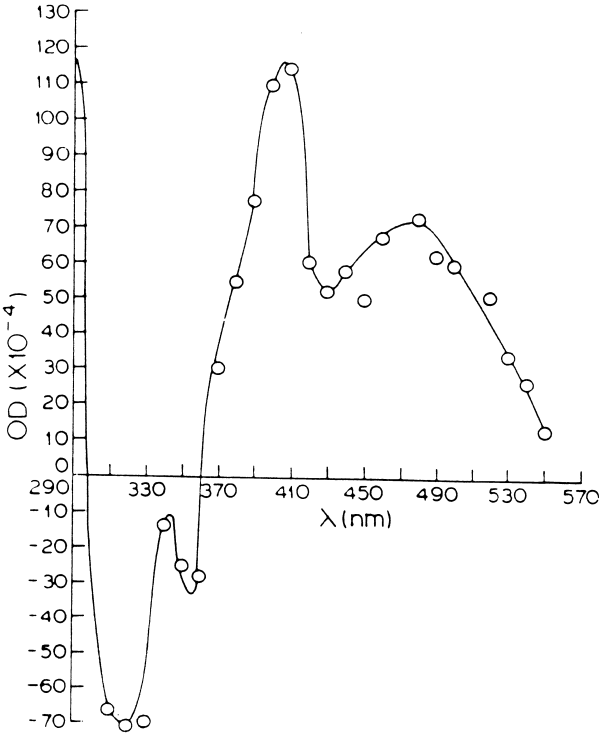

Figure 2. Transient spectra of DAMC.

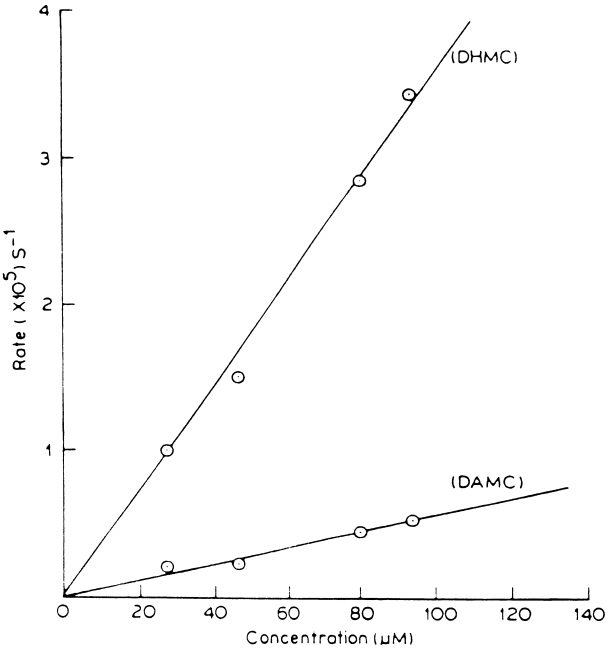

Figure 3. Measurement of rate constant for the formation of phenoxyl radical.

Table 1. Scavenging of superoxide radical by DHMC and DAMC[a]

	Cytochrome C reduced (μmol/min)	Superoxide scavenging (no. of folds)
Control	2730	0
DHMC	633	4.31
DAMC	793	3.44

[a] Superoxide radical generated during the reaction catalysed by xanthine oxidase is utilised for the assay of scavenging action of the test compounds. Details are given under Materials and Methods. The values expressed are mean of three different experiments with variation < 5%.

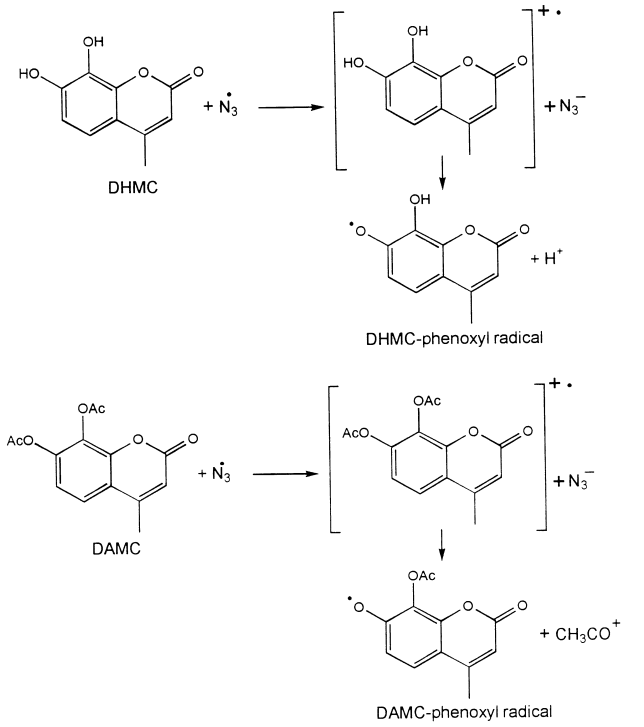

Figure 4. Comparison of free radical ($\dot{N}_3$) interaction with DHMC and DAMC.

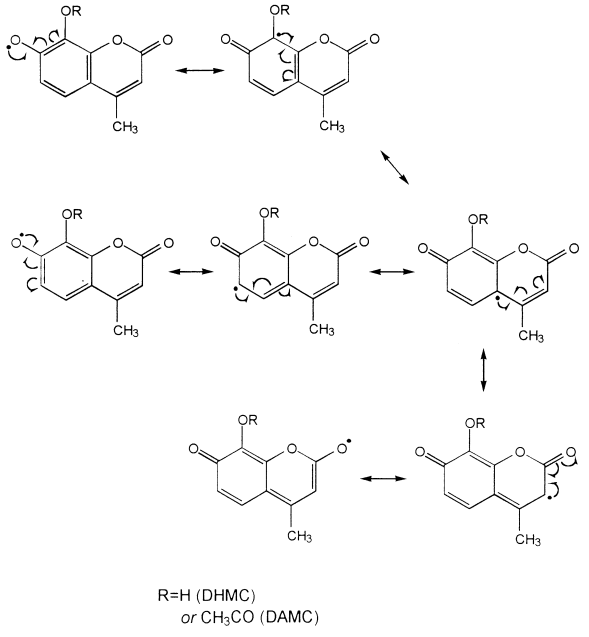

Figure 5. Extensive resonance stabilization of the coumarin phenoxyl radical obtained from DHMC and DAMC.

Thus the results of pulse radiolysis study have thrown light on the mode of generation of phenoxyl radical from DAMC (although not containing free phenolic hydroxyl group) consequent to the interaction with an initiating radical such as azide radical. It is possible that reactive oxygen species (ROS) can interact with DAMC in a manner similar to that of DHMC resulting in the efficient scavenging of ROS both by DHMC and DAMC. The results tabulated in Table 1 confirm this notion, DAMC was found to scavenge superoxide radicals very efficiently. The aforementioned results suggest that the free radical mediated oxidation of DAMC may initially produce a radical cation that may lose an acetyl carbocation to produce phenoxyl radical (Fig. 4). This is justified by the lower rate constant for the phenoxyl radical from DAMC. It is possible to conclude from these studies that the antioxidant mechanism of DAMC follows the pathway similar to that of DHMC (Fig. 4) and involves the formation of an extensively resonance-stabilised coumarin-phenoxyl radical (Fig. 5).

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References

1. Halliwell, B. *Free Rad. Res. Commun.* **1990**, *9*, 1.
2. Block, G. *Nutr Revs.* **1992**, *50*, 207.
3. Kahl, R. *Toxicol.* **1984**, *33*, 185.
4. Barclay, R. C.; Baskin, K. A.; Dakin, K. A.; Locke, S. J.; Vinquist, M. R. *Can. J. Chem.* **1990**, *68*, 2258.
5. Sato, T.; Kawamoto, A.; Tamura, A.; Tatsumi, Y.; Fuji, T. *Chem. Pharm. Bull.* **1992**, *40*, 721.
6. Yones, M.; Siegers, C. P. *Planta Medica* **1981**, *43*, 240.
7. Husain, S. R.; Gillard, J.; Cillard, P. *Phytochemistry* **1987**, *26*, 2489.
8. Raj, H. G.; Parmar, V. S.; Jain, S. C.; Goel, S.; Poonam; Himanshu; Malhotra, S.; Singh, A.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 833.
9. Parmar, V. S.; Bisht, K. S.; Jain, R.; Singh, S.; Sharma, S. K.; Gupta, S.; Malhotra, S.; Tyagi, O. D.; Vardhan, A.; Pati, H. N.; Berghe, D. V.; Vlietinck, A. J. *Indian. J. Chem.* **1996**, *34*, 1013.
10. Alfassi, Z. B.; Schuler, R. H. *J. Phys. Chem.* **1985**, *89*, 3359.
11. Buxton, G. V. In *Radiation Chemistry: Principles and Applications*; Farhataziz; Rodgers, M. A. J., Eds.; VCH: New York, 1987: pp 321; *Chem. Abstr.* **1987**, *107*, 124271.
12. Berg Meyer, H. U., Ed. In *Methods of Enzymatic Analysis*; 2nd ed.; 1974; Vol. 2, pp 644–649.
13. Diplock, A. T. *Food Chem. Toxicol.* **1996**, *34*, 1013.
14. Scott, G. *Chemistry in Britain* **1995**, 879.
15. Raj, H. G.; Sharma, R. K.; Garg, B. S.; Goel, S.; Singh, A.; Parmar, V. S.; Jain, S. C.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 2205.
16. Raj, H. G.; Parmar, V. S.; Jain, S. C.; Goel, S.; Singh, A.; Tyagi, Y. K.; Jha, H. N.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 1895.
17. Priyadarsini, K. I. *Free Radical Bio. Med.* **1997**, *23*, 838.
18. Priyadarsini, K. I.; Guha, S. N.; Rao, M. N. A. *Free Radical Biol. Med.* **1998**, *24*, 933.