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Photogeneration of reactive oxygen species from ketocoumarins

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Abstract

Photosensitising properties of three ketocoumarins, viz., 3-benzoyl-7-methoxycoumarin (BMC), 5,7-dimethoxy-3-(1-naphthoyl)coumarin (DMNC) and 7-diethylamino-3-thenoylcoumarin (DETC) are studied. Photogeneration of singlet oxygen is monitored by both optical (RNO bleaching) and EPR (TEMPL spin trapping) methods. Relative to rose bengal (RB), singlet oxygen generating efficiencies of BMC, DMNC and DETC are derived to be 0.69, 0.07 and 0.06, respectively. These ketocoumarins are also found to photogenerate superoxide anion radical ($O_2^{-\bullet}$) as probed by EPR-spin trapping method using 5,5-dimethyl-1-pyrroline-*N*-oxide as a spin trap and also by optical spectroscopy using SOD-inhibitable cytochrome *c* reduction assay. The production of $O_2^{-\bullet}$ is enhanced in the presence of electron donors such as EDTA, DETAPAC and NADH. Our results indicate that BMC possesses high ability to generate reactive oxygen species. Both $O_2^{-\bullet}$ (Type I) and $^1O_2$ (Type II) paths are involved in BMC photosensitisation.

Keywords: Ketocoumarins; Reactive oxygen species; Singlet oxygen; Superoxide anion radical

1. Introduction

Currently there is vast interest in the studies on photogeneration of reactive oxygen species (ROS) in relation to photodynamic therapy (PDT) [1–3]. ROS involved in the PDT may be singlet oxygen ($^1O_2$) produced by energy transfer from the triplet state of the sensitisers to oxygen (Type II) or superoxide anion radical ($O_2^{-\bullet}$) formed by electron transfer from the sensitisers, upon exposure to light. The ability of porphyrins and phthalocyanines as photosensitisers to produce $^1O_2$ has been studied extensively [4–6]. Recently photoinduced cytotoxic activity of furocoumarins and coumarin conjugates of pyrrole and imidazole containing distamycin analogue has been described [7]. Coumarins and thiocoumarins are also found to act as photosensitisers in relation to the generation of singlet oxygen [8]. The photodynamic action of furocoumarins appears to involve both reactive oxygen species ($^1O_2$, $O_2^{-\bullet}$) and radical species formed by electron transfer from or to photoexcited furocoumarins [9]. Nevertheless, studies on photoexcitation of ketocoumarins in relation to generation of ROS are limited. We report here the results of study on three 3-ketocoumarins with high singlet–triplet intersystem crossings [10], for their photodynamic efficiencies to generate singlet oxygen and superoxide radical anion. The effect of electron donors such as ethylenediaminetetraacetic acid (EDTA) and reduced nicotinamide adenine dinucleotide (NADH) on the efficiencies of production of ROS by these ketocoumarins is also presented.

2. Experimental section

2.1. Chemicals

N,N-Dimethyl-4-nitrosoaniline (RNO), 1,4-diazabicyclo[2,2,2]octane (DABCO), diethyltriaminopentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and rose bengal (RB) were obtained from Aldrich. 2,2,6,6-Tetramethylpiperidinol (TEMPL) was obtained from Merck. Imidazole, ethylenediaminetetraacetic acid (EDTA) and sodium azide were purchased from S.D. Fine Chemicals. Superoxide dismutase (SOD) and catalase were purchased from Sigma while reduced nicotinamide adenine dinucleotide (NADH) was obtained from Boehringer Mannheim. Dimethyl sulphoxide (HPLC grade) from Qualigens fine chemicals was used as such and 5,7-dimethoxy-3-(1-naphthoyl)coumarin (DMNC) was obtained from Eastman

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Kodak, USA. Doubly distilled water was used for all the experiments. Imidazole was used after repeated crystallisation from doubly distilled water.

2.2. Preparation of ketocoumarins

BMC and DETC were prepared following the reported procedure [10]. Typically 0.01 mole of salicylaldehyde and 0.01 mole of β-ketoester were dissolved in 20 ml of warm alcohol. Then 30 drops of piperidine were added and the mixture was heated at reflux on a steam bath for about an hour. The reaction mixture was cooled in an ice bath and the product ketocoumarin was collected, washed and recrystallised. The purity of the compounds was checked by recording the ^{1}H NMR spectra on a Jeol GSX 400 spectrometer with tetramethylsilane as an internal standard. 7-Diethylamino-3-thenoylcoumarin (DETC) was prepared starting from β-diethylaminosalicylaldehyde and ethyl 2-thenoylacetate and recrystallised from alcohol (m.p. 140–141°C). NMR ($CDCl_3$): 1.25 (6H, t, 2× CH_2CH_3) 3.45 (4H, q, 2× CH_2CH_3), 6.2 (1H, dd, H-6), 6.5 (1H, d, H-8), 7.36 (1H, d, H-5), 7.13, 7.68, 7.77 (ABX system H-4′, 5′ and 3′ respectively) 8.09 (1H, s, H-4). *p*-Methoxy salicylaldehyde and ethyl benzoylacetate were used as starting materials for the preparation of 3-benzoyl-7-methoxycoumarin (BMC) which was recrystallised from alcohol (m.p. 152–153°C). NMR ($CDCl_3$): 3.93 (3H, s, $-OCH_3$), 6.88 (1H, d, H-8), 6.92 (1H, dd, H-6), 7.26–7.62 (4H, m, H-5,3′, 4′, 5′), 7.85–7.88 (2H, m, H-2′,6′), 8.1 (1H, s, H-4). DMNC was also purified by recrystallisation from alcohol. NMR ($CDCl_3$): 3.88 (3H, s, $-OCH_3$), 3.91 (3H, s, $-OCH_3$), 6.29 (1H, d, H-8), 6.45 (1H, d, H-6), 7.47–8.49 (7H, m, naphthyl protons), 8.58 (1H, s, H-4).

2.3. Light source

A 150-W xenon lamp with a UV cut off filter combination of 10 cm of potassium iodide solution (1:100) plus 1 cm of pyridine was used for irradiation. The reaction mixture in a quartz cuvette placed at a distance of 12 cm from the light source, was continuously stirred during irradiation. The illumination was generally carried out in an open cuvette in equilibrium with atmosphere.

2.4. Singlet oxygen detection

The detection of singlet oxygen was performed by the method developed by Kraljic and El Mohsni [11]. The sensitisers were exposed to light in the presence of imidazole (10 mM) and RNO (50 mM) in a 50 mM phosphate buffer (pH = 7.4). Bleaching of RNO by the transannular peroxide intermediate, formed by the reaction between the photogenerated singlet oxygen ($^1O_2$) and imidazole, was monitored spectrophotometrically at 440 nm, absorption maximum of RNO. The formation of $^1O_2$ was also monitored in the presence of $^1O_2$ acceptor such as DABCO and sodium azide.

The rate of disappearance of quencher (A) obeys the following equation [12]

$$-d[A]/dt = (I_{ab}\Phi_{^1O_2})k_r[A]/k_d$$

where k_r is the rate constant for chemical quenching of $^1O_2$ by A. k_d is the rate constant for deactivation of $^1O_2$ by the solvent and I_{ab} is the intensity of light absorbed by the sensitiser. The slope of the first-order plot is $I_{ab}\Phi_{^1O_2}(k_r/k_d)$. The slope was calculated by fitting the experimental data to a second order polynomial. Experiments were carried out with each sensitisers and the well known sensitiser RB under identical conditions. Taking the $^1O_2$ quantum yield of RB [13] as 0.76, quantum yields for DETC, DMNC and BMC were determined from the relative ratios of the slopes, corrected for molar absorption and photon energy [14]. The interference of $O_2^{-\bullet}$ and H_2O_2 in the RNO bleaching was eliminated by adding SOD and catalase.

2.5. Superoxide detection

The formation of $O_2^{-\bullet}$ was detected by using the SOD inhibitable cytochrome *c* reduction method [15]. Solutions of photosensitisers were illuminated in the presence of ferricytochrome *c* (50 μM) in 50 mM phosphate buffer (pH = 7.4) and the reduction was monitored spectrophotometrically at 550 nm using $\Delta OD_{550} = 20\,000\ M^{-1}\ cm^{-1}$ for the reduced-oxidised cytochrome *c* [16].

2.6. EPR measurements

The reaction mixture in an aqueous flat cell was irradiated and EPR observations were made using a varian E-112 spectrometer. The following parameters were used for measurements: microwave power 20 mW, modulation amplitude 0.1 mT, modulation frequency 100 kHz.

2.6.1. Detection of singlet oxygen

The detection of $^1O_2$, was carried out essentially according to the method of Lion et al. [17] and Moan and Wold [18] by converting it to an EPR detectable nitroxide free radical. Reaction mixture (1 ml) containing 0.01 M TEMP and 0.1 mM of sensitisers was irradiated and increase of EPR signal intensity was followed at different times of irradiation.

2.6.2. Detection of superoxide anion

EPR spin trapping experiments were used to determine the production of $O_2^{-\bullet}$ on photoirradiation of coumarins. Solutions of coumarins (5 mM) were irradiated in the presence of 50 mM DMPO, in DMSO. EPR observations were made by introducing the irradiated solution into a flat aqueous cell. Experiments were repeated to monitor the signal intensity at different intervals of irradiation time. The superoxide anion radical was trapped by DMPO, yielding a DMPO-OOH spin adduct with characteristic EPR spectrum.

3. Results and discussion

3.1. Singlet oxygen generation

The rate of bleaching of RNO as a function of irradiation time of coumarins DETC, DMNC and BMC is shown in Fig. 1. Rate of bleaching by RB is also included in this figure. Fig. 1 gives the raw data for absorption changes at 440 nm vs. irradiation time. The ratio of the slopes of RB to each sensitisers was corrected for light absorption taking into account of the molar absorption and photon energy [14]. After this correction, the relative yields are found to be 0.06, 0.07 and 0.69 for DETC, DMNC and BMC, respectively. Thus BMC is found to be a good singlet oxygen generator.

To confirm the production of singlet oxygen, experiments were carried out in the presence of specific singlet oxygen quenchers such as DABCO and sodium azide. The rate of bleaching of RNO, studied in the presence of equimolar amounts of imidazole and DABCO (10 mM) when BMC is photolysed, is given in Fig. 1. The ratio of the slopes of RNO bleaching in the presence and absence of DABCO from Fig. 1 is derived to be nearly half (0.47). Since the rate of quenching of $^1O_2$ by DABCO is comparable to imidazole [19], the bleaching rate of RNO is decreased about half while performing the bleaching experiment with equimolar amount of imidazole and DABCO (10 mM). The replacement of DABCO by NaN_3 (0.1 mM) at a concentration of 100 times lower yielded the same amount of inhibition (Fig. 1). This is because the quenching rate of azide with $^1O_2$ is 100 times larger than either DABCO or imidazole [19]. These results confirm the generation of $^1O_2$ during the photosensitization process.

The effect of solvent, D_2O on the yield of $^1O_2$ was also studied by taking BMC as the example. The life time of $^1O_2$ is about ten times longer in deuterated solvent than in protonated solvent due to the retardation of collisional deactivation of $^1O_2$ in D_2O [20]. Photobleaching of RNO in the presence of BMC was monitored in 50 and 85% D_2O. An increased rate of photobleaching of RNO by a factor of 1.6 and 1.1 in 85% and 50% D_2O, respectively, was observed.

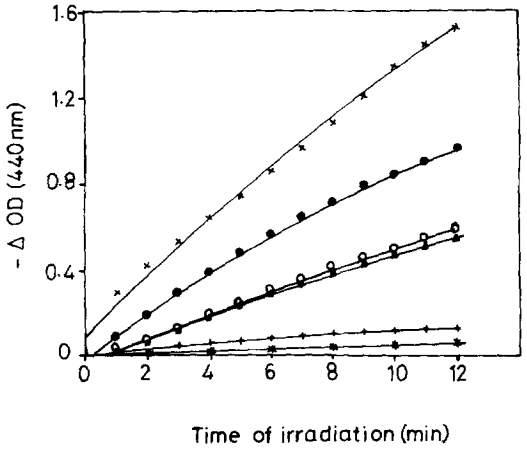


Fig. 1. Photosensitised RNO bleaching measured at 440 nm in the presence of imidazole (10 mM) in 50 mM phosphate buffer (pH = 7.4) with RB (×), BMC (●), DMNC (+) and DETC (*) as a function of illumination time in minutes. Inhibition of photosensitised RNO bleaching by BMC in the absence (●) and in the presence of (▲) 0.1 mM sodium azide and in the presence of 10 mM DABCO (○).

The observed photobleaching of RNO by the coumarins in air saturated solutions indicates that they may function as a type II photosensitisers. The marked differences in the sensitising properties among BMC, DMNC and DETC may be explained on the basis of intersystem crossing efficiencies of these coumarins. Since the singlet oxygen is most likely produced via the triplet quenching, a decreased Φ_{isc} can also lead to a lower yield of $^1O_2$. The linear relationship between yield of singlet molecular oxygen against the quantum yield of intersystem crossing efficiencies of these sensitisers supports the above statement. No published value of Φ_{isc} for RB in water is available, but it has been assumed to be unity like its close analogue erythrosin [13]. By extrapolation the Φ_{isc} value of DMNC was calculated to be 0.84.

Spin trapping method was also used to detect the generation of $^1O_2$ during the illumination of ketocoumarins. When a reaction mixture of TEMPL and ketocoumarins was irradiated, the EPR spectrum of three lines of equal intensity, characteristic of nitroxide free radical [TEMPOL] was detected. The hyperfine coupling constant ($A_N = 1.56$ mT) was found to be identical with that of an authentic sample. EPR signal intensity of TEMPOL produced was found to increase with increase of irradiation time as shown in Fig. 2. No EPR spectrum was observed for the solution exposed without sensitisers (inset of Fig. 2) even for prolonged irradiation.

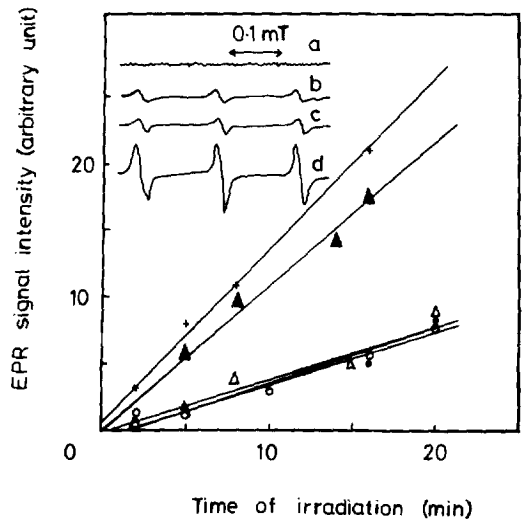


Fig. 2. The formation of TEMPOL during the photoirradiation of solutions containing RB (+), BMC (△), DMNC (△) and DETC (○) in the presence of TEMPL (20 mM) at 300 K in DMSO. Inhibitory effect of 1 mM sodium azide (●) on the intensity of TEMPOL radical during photo-irradiation of BMC. Inset: EPR spectrum of TEMPOL generated after five minute irradiation with different sensitisers. (a) no sensitiser (b) DETC, (c) DMNC, and (d) BMC. Spectrophotometer settings: microwave power 20 mW, modulation amplitude 0.1 mT, time constant 0.032 s, scan time 4 min.

Generation of $^1O_2$ during the photoirradiation, was further confirmed by studying the effect of sodium azide (Fig. 2) and DABCO on the intensities of the EPR signal by using BMC as a sensitiser. In the presence of sodium azide and DABCO, decrease in signal intensity of TEMPOL was observed, confirming the formation of $^1O_2$.

3.2. Superoxide anion generation

Generation of $O_2^{-\bullet}$ from ketocoumarins on photoirradiation was studied by EPR spin trapping experiment using DMPO as the spin trap. In an aprotic solvent such as DMSO, DMPO-OOH has a longer life time [6]. When DMPO alone was irradiated in DMSO, no EPR signal was observed (Fig. 3a). Also no EPR signal was observed in darkness. However, a twelve line EPR spectrum was observed when (5 mM) solution of BMC was photolysed in the presence of DMPO (50 mM) in air saturated DMSO solutions (Fig. 3b). The intensity of the EPR signal was found to increase with increase of illumination time. By using computer simulation method, the hyperfine coupling constants of the spin adduct was analysed as a primary nitrogen triplet ($a_N = 1.28$ mT) split by a secondary proton ($a_H^{\beta} = 1.03$ mT) which in turn is further split by a secondary proton ($a_H^{\gamma} = 0.125$ mT). The simulated EPR spectrum shows a close match with the experimental spectrum (Fig. 3c). These hyperfine coupling constants are consistent with previously reported values for the DMPO-OOH adduct in DMSO [21]. Formation of DMPO-OOH adduct was also confirmed by the addition of superoxide dismutase (SOD), which is an effective scavenger of $O_2^{-\bullet}$ radical. The addition of SOD prior to illumination prevents the generation of spin adduct which is shown in Fig. 3d. The DMPO-OOH adduct was detected when the other sensitisers DMNC and DETC were also photolysed in the presence of DMPO (50 mM) under identical conditions.

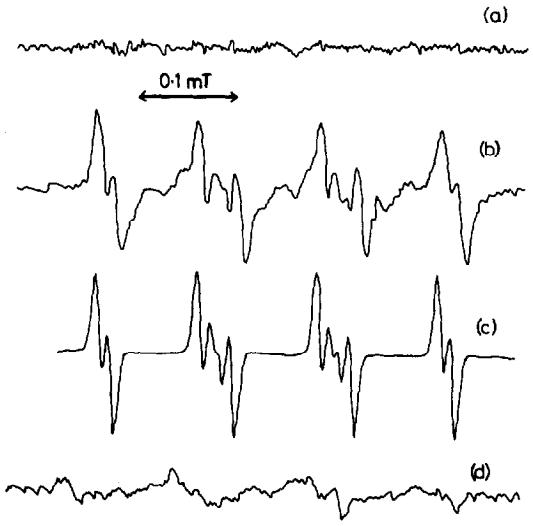


Fig. 3. The EPR spectra of DMPO-OOH adduct in air saturated DMSO solution containing BMC (5 mM) and DMPO (50 mM) (a) in dark (b) after 5 min irradiation (c) the simulated EPR spectrum of DMPO-OOH adduct using $a_N = 1.28$ mT; $a_H^{\beta} = 1.03$ mT; $a_H^{\gamma} = 0.125$ mT and (d) after 5 min irradiation in the presence of SOD. Spectrophotometer settings: microwave power 20 mW, modulation amplitude 0.1 mT, time constant 0.032 s, scan time 4 min and receiver gain 3.2×10^4.

Generation of $O_2^{-\bullet}$ radical was also confirmed by the SOD inhibitable cytochrome *c* reduction assay. Rate of reduction of ferricytochrome *c* at pH 7.4, when air saturated solutions of the sensitisers were photolysed in the presence of 50 μM cytochrome *c* in phosphate buffer, is given in Fig. 4. DMNC, DETC and BMC were found to reduce cytochrome *c* with different efficiencies. The rate of superoxide generation was arrived to be 0.167, 0.0213 and 0.00456 μM/s for BMC, DMNC and DETC, respectively. Addition of SOD (10 μg/ml) was found to inhibit the cytochrome *c* reduction.

3.3. Effect of electron donors

Fig. 4 shows effect of EDTA on the rate of cytochrome *c* reduction when air saturated solution of the BMC was photolysed in the presence of cytochrome *c* in phosphate buffer (pH = 7.4). The electron donor, EDTA enhanced the rate of cytochrome *c* reduction when BMC was used as a sensitiser. Similar enhancement in $O_2^{-\bullet}$ radical generation was observed for DMNC and DETC. The related electron donor DETAPAC was also found to involve in the electron transfer process but the enhancement of generation of $O_2^{-\bullet}$ was smaller than for EDTA. In the presence of electron donors such as EDTA and DETAPAC, the pathway of $^1O_2$ generation can effectively switch over into the production of species $S^{-\bullet}$ due to the interaction of electron donor with the triplet state of the sensitiser, as shown in Scheme 1. In the presence of oxygen the latter species, $S^{-\bullet}$, can yield $O_2^{-\bullet}$ radical.

Additionally, it is possible for an electron donor such as, NADH, to directly interact with $^1O_2$ to generate $O_2^{-\bullet}$ [22]. Hence, the effect of the coenzyme, NADH, on the SOD-inhibitable cytochrome *c* reduction rates, was also studied. Fig. 5 clearly shows that BMC is found to be more efficient in the reduction of cytochrome *c* in the presence of NADH than DMNC which in turn is more efficient than DETC. The rate of superoxide anion generation in the presence of NADH

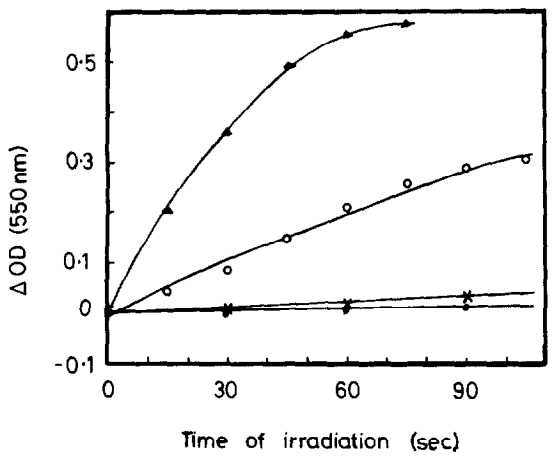
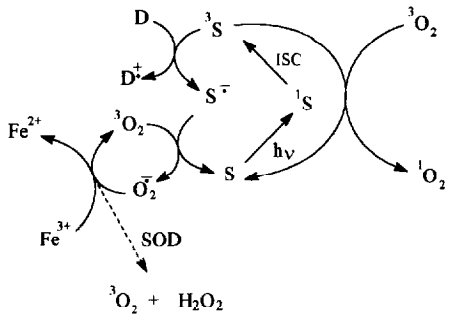


Fig. 4. Photosensitised superoxide generation measured as the rate of cytochrome *c* reduction from BMC (○), DMNC (×) DETC (●), and BMC + EDTA (△).



Scheme 1.

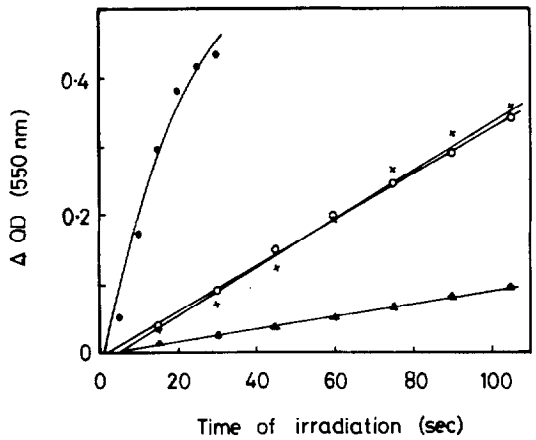


Fig. 5. Photosensitised cytochrome *c* reduction in the presence of NADH with sensitisers, BMC (●), DMNC (○) and DETC (△). Inhibition of photosensitised cytochrome *c* reduction in the presence of sodium azide (+). The number in the parentheses indicates the percentage azide inhibition.

was found to be 0.872, 0.175 and 0.0469 μM/s for BMC, DMNC and DETC, respectively. This follows the order of generation of $^1O_2$, i.e., BMC > DMNC > DETC. Addition of specific singlet oxygen quencher such as sodium azide retards the rate of cytochrome *c* reduction when BMC is photolysed in the presence of NADH (Fig. 5). The rate of cytochrome *c* reduction could be inhibited to 0.104 μM/s (88% inhibition) in the presence of sodium azide. This result confirms the interaction between $^1O_2$ and NADH.

We may conclude that both $^1O_2$ and $O_2^{-\bullet}$ radical are formed during the irradiation of ketocoumarins. This conclusion has been reached by RNO bleaching method, TEMPL spin trapping method, SOD inhibitable cytochrome *c* reduction assay and EPR spin trapping experiments. Our results indicate that, of the three ketocoumarins studied, BMC possesses high ability to generate reactive oxygen species.

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