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Mercapto-Substituted Perylenequinonoid Pigments. A New Type of Singlet Oxygen Sensitizer

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ABSTRACT

Mercapto-substituted hypocrellin B derivatives (MHBDs) have recently been prepared and characterized. Their significantly enhanced red absorptivities longer than 600 nm and singlet oxygen-generating function may qualify them as promising photodynamic therapy (PDT) agents. The generation of singlet oxygen during photoirradiation of MHBDs was detected as an oxidation product of a sterically hindered amine (tetramethylpiperidine oxide;TEMPO) by ESR spectroscopic techniques. Sodium azide inhibited the ESR signal intensity in a dose-dependent manner with a quenching rate constant of $2.65\times10^8\ M^{-1}s^{-1}$ *and* $3.13\times10^8 M^{-1}s^{-1}$ *for compound 6 and compound 7 in* $CHCl_3$*, respectively. Deuterated solvents, known to increase the lifetime of singlet oxygen, augmented the ESR signal intensity. The rate of production of* $^1O_2$ *was dependent not only upon the concentration of MHBDs and the time of irradiation but also on the oxygen content of the reaction mixture. The hyperfine splitting constant and g-value of the photoproduct of TEMP-singlet oxygen and TEMPO were found to be identical, this indicates that the nitroxide species detected by ESR spectroscopy generated by reacting TEMP with photogenerated* $^1O_2$ *is TEMPO. © 1997 Elsevier Science Ltd*

Keywords: mercapto-substituted, hypocrellin B derivatives, TEMP, ESR, singlet oxygen.

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INTRODUCTION

Considerable attention has been paid to the photodynamic therapy (PDT) of human malignancies. The curative or palliative PDT treatment of a variety of forms of cancer, using hematoporphyrin derivative (HPD) as sensitizer, has also been achieved clinically within the last two decades [1, 2]. However,this treatment is always accompanied by prolonged normal tissue photosensitivity, and the PDT efficiency of the presently used HPD is also limited by its poor red absorptivity in tissue [3]. These disadvantages have led to attempts to develop more efficient PDT photo-sensitizers, since improvement in PDT treatment depends, to a large extent on the photosensitizer used currently, most of the perylenequinonoid pigments (PQPs), as a new generation of PDT agents, are receiving much attention.

Perylenequinonoid pigments, which possess some unique chemical and biological properties, are a new class of potent singlet oxygen generator [4]. Because singlet oxygen is now generally considered to be an active PDT intermediate, this property characterizes PQPs as promising PDT photosensitizers [4, 5]. Hypocrellins, newly identified members of natural PQPs, which are also traditional Chinese medicinal herb ingredients, have been successfully employed clinically in the PDT treatment of some diseases (such as white lesion of the vulva, keloid, vitiligo, psorasis, tinea capitis and lichen amyloidisis) without observing prolonged photosensitivity [5]. Besides the above-mentioned promising applications of PQP in PDT of cancer, they are also potent inhibitors of some viruses, especially human immunodeficiency virus (HIV) and protein kinase C (PKC) [6–12].

Nevertheless, as in the case of HPD, natural PQPs themselves do not exhibit absorptivity longer than 600 nm, and this limits their PDT applications at present. Preparation of certain new mercapto-substituted hypocrellin B derivatives (MHBDs) has been reported in a previous paper. In this paper, absorption spectral properties and evidence for the generation of singlet oxygen during the photoirradiation of MHBDs are discussed.

MATERIALS AND METHOD

Materials

HA and HB were extracted from fungus sacs of *Hypocrella bambusae* and were purified by recrystallization from acetone. Compounds 1–9 (Fig. 1) were synthesized according to the method of a previous paper [23]. Subsequently, 2,2,6,6-Tetramethylpiperidine (TEMP),2,2,6,6-tetramethylpiperidine oxide (TEMPO),9,10-diphenyl anthracene (9,10-DPA) were obtained from

Aldrich Chemical Company. Sodium azide, deuterated chloroform and other solvents, all of analytical grade, were purchased from Beijing Chemical Plant, Beijing, China. The other compounds were obtained at the highest obtainable purity. The solutions were purged with oxygen, air or argon according to experimental requirements. The required high-purity solvents were prepared by further purification of the commercial products, and no impurities were detected by absorption and/or fluorescence spectroscopies.

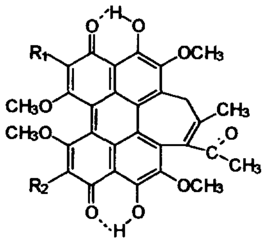
Quantum yield of $^1O_2$ generation

The quantum yield of $^1O_2$ generation for compounds 1–9 was determined using the 9,10-DPA bleaching method established by Diwu and Lown [4]. The reactions were followed spectrophoto-metrically by observing the decrease in the 374 nm absorption peak of 9,10-DPA (where the sensitizers used have the lowest absorptivity) as a function of irradiation time.The photooxidations of DPA sensitized by compounds 1–9 were carried out on a 'merry-go-round', where the sample was illuminated by 579 nm light, obtained from the combination of a medium-pressure sodium lamp with a narrow band interference filter. The spin trapping of $^1O_2$ by TEMP was used as an alternative method to determine the formation of $^1O_2$. Sample and TEMP were injected quantitatively into specially made quartz capillaries for ESR analysis and were illuminated with 450W medium-pressure sodium lamp. A long pass filter was employed to eliminate light of wavelength less than 470 nm. The capillaries were sealed to isolate the sample from the environment. Anaerobic samples were made in cuvettes which allowed purging of the reaction volume with purified argon. Measurements of the ESR spectra were carried out on a Bruker ER-300 EPR spectrometer operating at room temperature.(X band: microwave frequency, 9.5GHz).The commercial TEMPO radical, which is an oxidation product of TEMP, was used as a standard. Both the standards and experimental samples were dissolved in the same solvent and were analyzed under similar experimental conditions. Typically, the reaction solution consists of 3.4×10^{-5} M sample and 10 mM TEMP. These two methods gave consistent results.

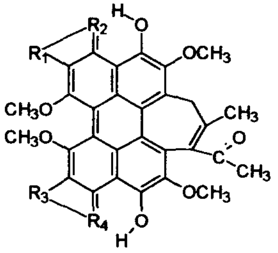
RESULTS AND DISCUSSION

Absorption characteristics of MHBDs

In general, the absorption spectra of PQPs in the visible region consist of three strong, broad absorption bands (Ia, IIa, IIIa). The shorter wavelength absorption band is assigned to a π–π^* transition, while the longer wavelength



Compounds 1-8



Compound 9

Compound	R_1	R_2	R_3	R_4
HB	H	H	-	-
1	OH(H)	H(OH)	-	-
2	$SCH_2COOH(H)$	$H(SCH_2COOH)$	-	-
3	SCH_2COOH	SCH_2COOH	-	-
4	$S(CH_2)_7CH_3(H)$	$H(S(CH_2)_7CH_3)$	-	-
5	$S(CH_2)_7CH_3$	$S(CH_2)_7CH_3$	-	-
6	$S(CH_2)_{11}CH_3(H)$	$H(S(CH_2)_{11}CH_3$	-	-
7	$S(CH_2)_{11}CH_3$	$S(CH_2)_{11}CH_3$	-	-
8	$SCH_2CH_2NH_2(H)$	$H(SCH_2CH_2NH_2)$	-	-
9	SCH_2CH_2-N(H-O)		H-O(SCH_2CH_2-N)	

Fig. 1. Structures of mercapto-substituted Hypocrellin B derivatives.

is considered to be related to intramolecular proton/electron transfer. Compared with the absorption spectrum of HB, the visible absorption bands of mercapto-substituted Hypocrellin B derivatives (MHSDs) shift towards longer wavelengths, two long-wavelength absorption bands merge as a shoulder peak for compounds 1–8, but two new long-wavelength absorption bands appear for compound 9 (Fig. 2 and Table 1), which is useful for PDT applications because red light can penetrate biological tissue much more efficiently than UV or visible light of short wavelength.

Formation of singlet oxygen by MHBDs

Quantum yields of singlet oxygen

It has been observed that singlet oxygen is involved in many photooxygenatios sensitized by the PQPs and their complexes [13, 14]. There are two

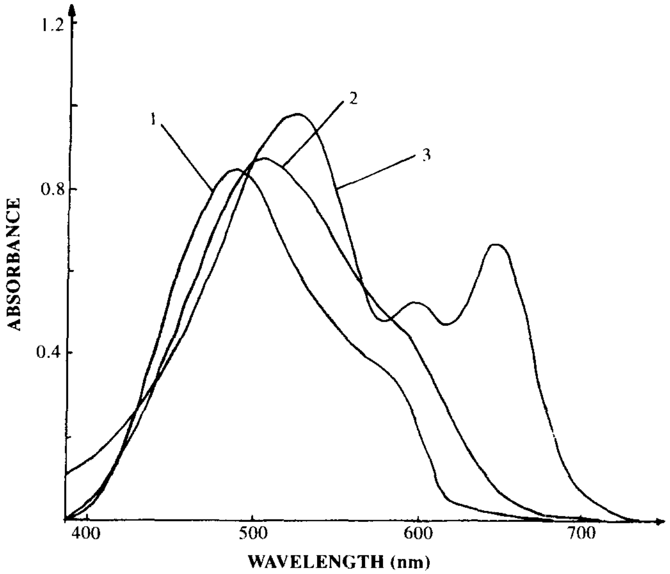


Fig. 2. UV-VIS absorption spectra of MHBDs line 1—compound 2, line 2—compound 3, line 3–compound 9.

methods available to characterize singlet oxygen, i.e. direct singlet oxygen luminescence (at 1270 nm) measurement and indirect chemical trapping [15]. Diwu and Lown [4] have measured the quantum yield of singlet oxygen generation by HA to be 0.84 (in benzene) by the 9,10-DPA photobleaching method. In order to determine the quantum yield of singlet oxygen generation by MHBDs, the 9,10-DPA bleaching method was adopted and HA was used as reference. During the measurement,the absorbance at 579 nm of the samples examined was adjusted to be the same. Figure 3 shows the rates of 9,10-DPA bleaching photosensitized by MHBDs and HA as a function of the irradiation time at 579 nm in chloroform. From Fig. 3, the $^1O_2$ yield for MHBDs in chloroform could be calculated and the results were listed in Table 1. However, no bleaching of 9,10-DPA was detected in the dark or in the irradiated sample without sensitizer or oxygen. These results show that singlet oxygen can be generated from MHBDs on photosensitization.

The generation of $^1O_2$ by MHBDs was further confirmed by the spin trapping of TEMP. The use of TEMP as a detector of singlet oxygen has been proposed by Lion *et al.* [16]. Although these authors detected a nitroxide radical, they did not identify the product of the TEMP–$^1O_2$ reaction. In order to test the hypothesis that singlet oxygen converts TEMP to TEMPO, TEMP was allowed to react with $^1O_2$ generated by photoactivation of some well-known sensitizers such as Rose Bengal and fluorescence. The characteristic ESR spectral pattern of three equal intensity lines was observed when

TABLE 1

Absorption spectral parameters and quantum of singlet oxygen of MHBDs

Compound	*$\lambda_{max}(nm)$*	*$\varepsilon_{max}(CHCl_3)$*	*$\phi;{}^1O_2$*
HB	467, 540(sh), 582(sh)	1.3×104, 6.5×103, 5.2×10^3	0.76
1	488	3.98×10^3	0.67
2	492, 580(sh)	7.94×10^3, 5.01×10^3	0.38
3	516	7.95×10^3	0.29
4	500, 586(sh)	8.53×10^3, 6.02×10^3	0.37
5	520, 635(sh)	8.88×10^3, 5.65×10^3	0.27
6	508, 600(sh)	$9.43\times 10_3$, $6.53\times 10_3$	0.35
7	535, 655(sh)	9.50×10^3, 6.45×10^3	0.25
8	515	8.23×10^3	0.44
9	532, 596, 652	2.72×10^4, 8.54×10^3, 1.34×10^4	0.87

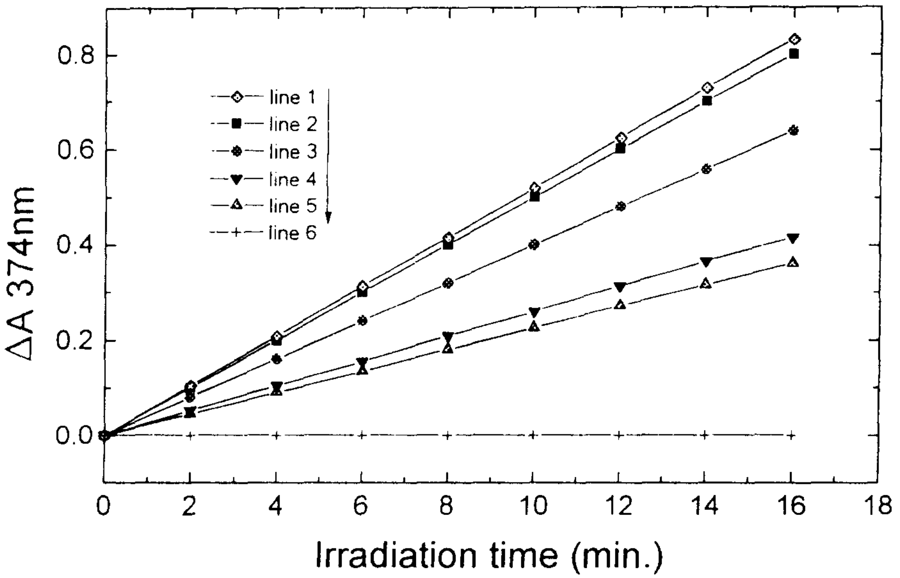


Fig. 3. Photosensitized 9,10-DPA bleaching measured at 374 nm in oxygen-saturated $CHCl_3$ with no sensitizer, or oxygen-free, or dark (line 6), compound 9 (line 1), HA (line 2) (in benzene), compound 1 (line 3), compound 2 (line 4) and compound 3 (line 5) as a function of irradiation time.

oxygen-saturated solutions of these sensitizers were irradiated in the presence of TEMP at room temperature. These spectra were compared with the spectrum of commercial TEMPO radical. The hyperfine splitting constant and g-value were found to be identical for both the photosensitized oxidation product of TEMP by these sensitizers and commercial TEMPO ($\alpha_N = 13.6$ G,g = 2.0056) indicating that ${}^1O_2$ converts TEMP to TEMPO.

As shown in Fig. 4, the ESR spectrum of three equal intensity lines, characteristic of a nitroxide radical, was recorded when the odygen-saturated solution of MHBDs and TEMP was irradiated at room temperature. Control experiments indicated that MHBDs, oxygen and light are all essential for the production of TEMPO, indicating that the formation of the nitroxide radical is a photodynamic process. The quantum yields of generation of $^1O_2$ by MHBDs can be calculated from ESR experiments using HA as reference, (also listed in Table 1) the results obtained from ESR or the 9,10-DPA bleaching method are nearly consistent.

It can be seen from Table 1 that $^1O_2$ generating quantum yields of MHBDs are lower than those of the parent PQPs (except for compound 9); this phenomenon might relate to the bulky side chains in compounds 2–8. It has been demonstrated that PQPs produce $^1O_2$ by energy transfer of excited triplet PQPs to ground state oxygen and therefore the reduction in the $^1O_2$-generating quantum yield as a result of mercapto-substitution suggests that the quantum yield of triplet formation of PQPs and/or the interaction efficiency between the triplet sensitizer (PQP) and oxygen resulting in $^1O_2$ are descreased by a side chain substitution effect, which may trigger and accelerate S_1-S_0 radiationless transition via 'loose bolt' and/or 'free roto' effects [17]. Because of the cyclic structure at positions 5/8 of compound 9, the $^1O_2$ quantum yield of compound 9 is higher than that of HA, indicating side chain substitution should exert much effect on the production of $^1O_2$ by MHBDs.

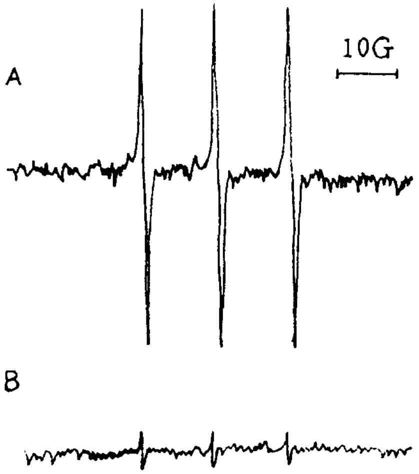


Fig. 4. A: The ESR spectrum produced by irradiation of an oxygenated solution containing compound 2 or compound 7 (0.1mM) and TEMP (15mM). B: same as A, but in the presence of NaN_3 (instrumental settings: microwave power, 5.05 mw; modulation amplitude, 1.05 G; receiver gain, 2×10^4).

Effect of deuterium

The effect of solvent deuteration on the lifetime of singlet oxygen can be used as a powerful diagnostic tool in investigating the role of singlet oxygen in various photochemical and photobiological processes [18]. In order to further investigate the role of singlet oxygen in the formation of TEMPO radical from TEMP, the effect of deuterium on the yield of TEMPO was studied during photoirradiation of MHBD in chloroform; compounds 3 and 9 were selected as representive structures. When samples containing compound 3/ compound 9 (20 um) and TEMP (40 um) were irradiated separatley in an oxygen-saturated solution of $CDCl_3$, the ESR signal intensity of TEMPO radical increased 2 times/1.5 times faster than when the solvent was $CHCl_3$ for compound 3 and compound 9 respectively. These results are in accord with the result of Hall and Chignell [19], who monitored the emission intensity of singlet oxygen at 1270 nm in water and deuterium oxide systems. The increased TEMPO accumulation in deuterated solvent is anticipated. These results further support the contention that photoirradiation of KHBDs generate singlet oxygen, which in turn reacts with TEMP to form a TEMPO radical.

Effect of azide

Sodium azide is commonly used to inhibit singlet oxygen dependent reaction If TEMPO radical is formed by reacting TEMP with $^1O_2$, generated during irradiation of MHBDs, the intensity of the TEMPO ESR signal should be inhibited by adding various amounts of sodium azide to the reaction mixture. In order to estimate the quenching rate constant, different concentrations of sodium azide were used to establish a competition reaction between NaN_3 and TEMP for the available $^1O_2$. The quenching rate constant was estimated according to the following dynamic quenching equation [20]:

$$\phi_{TEMPO} = \frac{K_T[T]}{K_T[T] + K_d + K_q[Q]} \tag{1}$$

$$\frac{\phi_0}{\phi} = \frac{K_T[T] + K_d + K_q[Q]}{K_T[T] + K_d} = 1 + \frac{K_q}{K_T[T] + K_d}[Q] \tag{2}$$

where K_T is the rate for TEMP, K_d is the decay rate and K_q is the rate for NaN_3. Φ_0/Φ is the ration of the ESR signal intensity of TEMPO radical in the absence and presence of quencher (sodium azide);$[T]$ is the concentration of TEMP and $[Q]$ is the concentration of the quencher.

Figure 5 shows the Stern-Volmer plot for the effect of NaN_3. As shown in this figure, the Stern-Volmer plot for NaN_3 quenching is linear up to at least

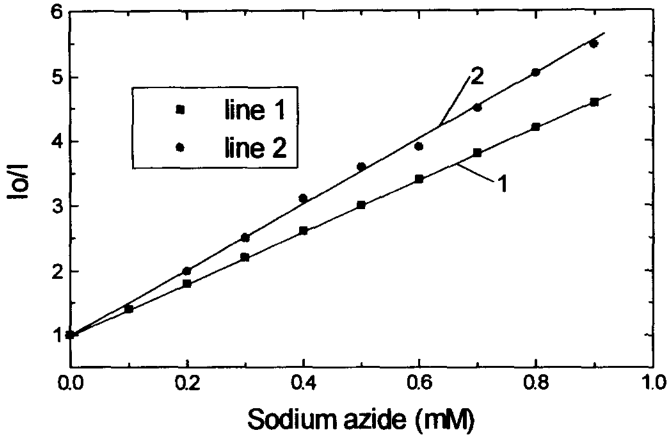


Fig. 5. Stern-Volmer quenching of the ESR signal intensity of TEMPO by NaN_3. Performed by adding various amounts of NaN_3 to a series of oxygen-saturated $CHCl_3$ solutions containing compound 7 (line 1) or compound 6 (line 2) (0.2mM) and TEMP (15mM).

2 mM NaN_3, and the bimolecular constant for NaN_3 quenching was calculated to be 2.65×10^8 M^{-1} s^{-1} for compound 6 and $3.13 \times 10^8 M^{-1}s^{-1}$ for compound 7 using $K_T = 4 \times 10^7 M^{-1}$ s^{-1} determined by Lion *et al.* [21] for a related amine and 160 us for $^1O_2$ in chloroform [22].

CONCLUSION

The results of these studies provide evidence for the generation of singlet oxygen ($^1O_2$) during photoirradiation of the MHBDs: (a) Oxidation of TEMP to TEMPO; (b) deuteurium effects on the generation of TEMPO; and (c) the quenching effect of NaN_3 on singlet oxygen generation. As discussed above, the new MHBDs, because of their significantly enhanced red absorptivities and $^1O_2$—generating functions, may be a new kind of potential PDT agent. Although our data suggest that MHBDs could produce singlet oxygen, it does not preclude the possibility of other reaction pathways, such as superoxide anion, hydrogen peroxide; the roles of other reactive oxygen species are currently being studied.

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