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Reaction Kinetics between Thiobases and Singlet Oxygen Studied by Direct Detection of the $^1O_2$ Luminescence Decay†

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Thiobase derivatives have received important investigations due to their wide usage as phototherapeutic agents and their potential carcinogenic side effects as immunosuppressants. The substitution of oxygen atom by the sulfur atom makes the ultraviolet absorption of thiobases redshifted and absorbs UVA light (>300 nm), resulting in unusual high quantum yield of triplet state to generate the singlet oxygen ($^1O_2$) through photosensitization. As a type of reactive oxygen species, $^1O_2$ is highly reactive toward thiobases. Herein, we report the measurements of reaction rate constants between different thiobases and $^1O_2$ in different solvents through the direct detection of $^1O_2$ luminescence decay kinetics at 1270 nm. The rate constants of thiouracils with $^1O_2$ are five times smaller than that of thioguanine with $^1O_2$, which suggests that thiopurines are more reactive than thiopyrimidines and thus less suitable to be a photosensitive drug on the application of photodynamic therapy. Additionally, the rate constants of thiobases and $^1O_2$ were found to be obviously influenced by the solvent polarity. With the increase of solvent polarity, the rate constants of thiobases and $^1O_2$ decrease.

Key words: Singlet oxygen, Thiobase, Rate constant, Transient absorption spectroscopy

I. INTRODUCTION

Thiobases, formed through replacement of oxygen atom by sulfur atom in exocyclic carbonyl group of DNA or RNA bases, have been widely used as immunosuppressant, anticancer, and anti-inflammatory drugs for organ transplant patients [1]. As a result of sulfur substitution, the absorption spectra shift from the UVC region in the normal bases out to the UVA region in the thiobases, leading to a dramatic change in the photochemical properties of the DNA and RNA monomers [2–6]. These unusual photochemical properties can cause extensive oxidative damage to DNA [4] and proteins [7], which will lead to phototoxic side effects and skin cancer [6]. Additionally, the substitution of sulfur could cause the heavy atom effect and the enhancement of the spin-orbit and vibronic coupling between the singlet and triplet manifold [8]. As a result, thiobases will populate from singlet excited state to triplet state in high quantum yield via efficient intersystem crossing (ISC) upon UVA irradiation [9].

Meanwhile, the thiobases on triplet state will collide with molecular oxygen to produce reactive oxygen species (ROS) through energy transfer [2–5, 10–12]. The major ROS generated upon UVA irradiation of thiobases was identified to be singlet oxygen ($^1O_2$). $^1O_2$ is the main cytotoxic substance of type II photochemical reaction of photodynamic therapy (PDT), which is a promising therapeutic modality for combating cancer [13–17]. In the treatment of cancer with PDT, $^1O_2$ can lead to oxidative damage to the treated cells, resulting in rapid necrosis or delayed apoptosis of those diseased tissues [18]. However, except the reactions with target cellular components including unsaturated lipids, amino acid residues and nucleic acids, the highly reactive $^1O_2$ can also interact with the photosensitizer and result in the photobleaching of thiobases [18]. For example, $^1O_2$ can react readily with 6-thioguanine (6tGua) and 4-thiouracil (4tUra) to generate guanine-6-sulfonate (G^{SO3}) [19] and uracil-6-sulfonate (U^{SO3}) [20] respectively as we reported before, and the whole process is summarized in Scheme 1. These oxidation products could not form stable or normal base pairs with any normal DNA base, which will strongly block DNA replication and transcription and then become a major factor increasing the risk for skin cancer [6, 21, 22].

Although considerable research efforts have been devoted to the stable products or mechanism of the reaction between $^1O_2$ and thiobases, information on reaction kinetics of these reactions is quite deficient. The kinetics rate constants are very important in evaluating the reaction priority of singlet oxygen in complicated biological system. Therefore, measurement of the rate constants of the reactions between $^1O_2$ with thiobases is

†Dedicated to Professor Kopin Liu on the occasion of his 70th birthday.

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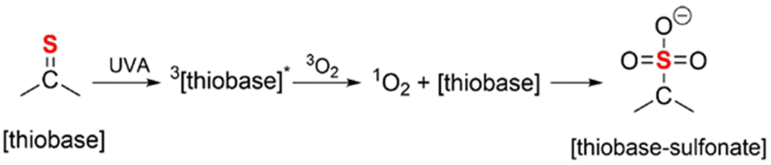
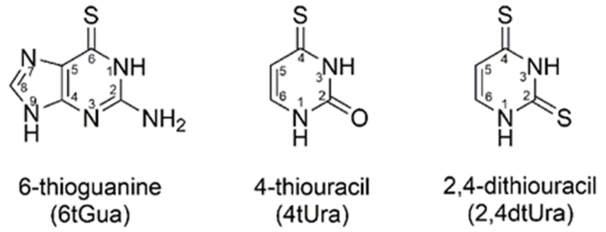


Scheme 1 Illustration of the thiobases photochemistry upon UV excitation.



Scheme 2 Molecular structures of thiobases.

highly desirable. In this work, we focus our study on the reaction kinetics of $^1O_2$ with thiobases, *i.e.*, thioguanine (6-thioguanine, 6tGua) and thiouracils (including 4-thiouracil, 4tUra and 2,4-dithiouracil, 2,4dtUra) shown in Scheme 2. We carried out a series of experiments to produce singlet oxygen by irradiating 6tGua, 4tUra or 2,4dtUra respectively with 355 nm laser. The 1270 nm near-infrared (NIR) luminescence from $^1O_2$ is monitored to obtain the quenching kinetics of singlet oxygen and then the reaction rates of thiobases with singlet oxygen. Our results reveal that the reaction rate constant of thiouracil with $^1O_2$ is smaller than that of thioguanine by a factor of five, which implies that thiouracil is more suitable as a photosensitizer drug during PDT due to its more stability in biological systems. In addition, it is found that solvent effect also has noticeable influence on the reaction rate constants of singlet oxygen with thiobases.

II. EXPERIMENTS

A. Materials

Guanine (J&K, 98%), uracil (Sigma-Aldrich, 98%), 6-thioguanine (Alfa Aesar, 98%), 4-thiouracil (JK, 95%), 2,4-dithiouracil (Sigma-Aldrich, 98%), deuterium oxide (J&K, 99.8%), and acetonitrile (Sigma-Aldrich, 99.9%) were used as received without further purification. Saturated solutions of 6tGua, 4tUra, 2,4dtUra in five different solvents, *i.e.*, deuterium oxide (D_2O), acetonitrile (CH_3CN), and their mixed solution with different volume ratio (2:1, 1:1, and 1:2), were prepared under darkness condition to avoid light induced oxidation. Several concentration gradients of the three samples are obtained from the dilution of their saturated solutions. The sample solutions were bubbled with O_2 for 15 min prior to testing and then maintained under the constant flowing oxygen condition to make sure the experimental conditions were not affected by the concentration of O_2. Note that each kinetic measurement was performed with a new sample.

B. Transient absorption spectroscopy

Nanosecond time-resolved UV-Vis transient absorption spectra were measured using a laser flash photolysis setup Edinburgh LP920 spectrometer combined with a Nd:YAG laser. All sample solutions in 1 cm×1 cm quartz cuvettes, were excited at 355 nm with the laser energy of 10 mJ/pulse. The third harmonic (355 nm) of a Continuum Surelite II Nd:YAG laser (10 Hz, FWHM≈7 ns) was used to irradiate thiobases to generate $^1O_2$ and initiate the thiobases reaction with $^1O_2$. A cutoff optical filter with an 850 nm long-pass filter (Isuzu Optics, LP850) located between the sample and detector were used to cut off any stray light and scattered light with wavelengths shorter than 850 nm. Singlet oxygen phosphorescent decay traces at 1270 nm were monitored by the Edinburgh LP920 luminescence spectrometer equipped with an NIR sensitive photomultiplier tube (R5509-73, U=1500 V) cooled to −85 °C by liquid nitrogen. The data were analyzed by the online software of the LP920 spectrophotometer. The steady absorption spectra were measured by Shimadzu UV1601 spectrophotometer. The absorbance of the samples was controlled to be in the range of 0.1−0.4 at the excitation wavelength of 355 nm. All of the measurements were carried out at ambient temperature. Samples were protected from light when not being irradiated.

III. RESULTS AND DISCUSSION

A. Observation of the formation of triplet state of thiobases

FIG. 1 shows the UV-Vis absorption spectra of guanine, uracil, 6-thioguanine, 4-thiouracil and 2,4-dithiouracil. As a result of sulfur substitution, the absorption spectra shift from the UVC region in the normal bases out to the UVA region in the thiobases. Therefore, thiobases can be pumped to S_2 state ($\pi\pi^*$) upon 355 nm laser radiation [23], which then further relaxes to S_1 state ($n\pi^*$, dark state) and depopulates to triplet T_1 state through intersystem crossing [24–26].

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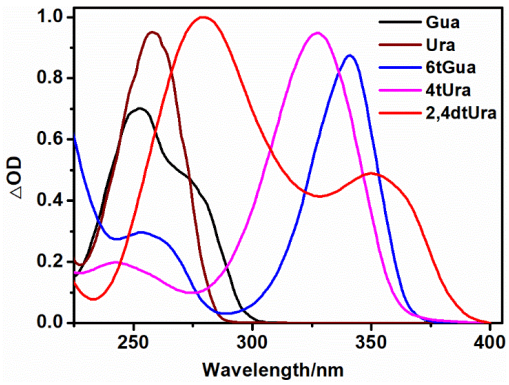

FIG. 1 UV-Vis absorption spectra of Gua, Ura, 6tGua, 4tUra, and 2,4dtUra.

FIG. 2 displays the transient absorption spectra of 4tUra in D_2O in air-saturated condition upon 355 nm excitation. Similar transient absorption signals are observed in N_2-saturated condition. A negative absorption band peaked at 327 nm due to the ground state bleaching of 4tUra is observed, which agrees with the ground state absorption spectra (FIG. 1). The positive transient absorption bands with maxima at 550 nm are ascribed to the absorption of triplet state 4tUra, which is in accordance with the Refs.[27, 28]. The decay lifetime of triplet state 4tUra is 711 ns in N_2-saturated condition, and 444 ns obtained in air-saturated condition as shown in the inset of FIG. 2. The 1.6-fold longer lifetime of triplet 4tUra in N_2-saturated condition demonstrates that the triplet state of 4tUra can be effectively quenched by molecular oxygen, which should generate singlet oxygen.

B. Reaction rate constant measurements for thiobases with singlet oxygen in deuterium oxide

Accompanied with the decay of the triplet state 4tUra, the luminescence spectra peaked at 1270 nm are observed by NIR sensitive PMT as shown in FIG. 3, which is the characteristic emission of singlet oxygen. As depicted in Scheme 1, $^1O_2$ was produced in the photosensitized process of 4tUra upon the absorption of a photon of appropriate wavelength to generate the triplet state 4tUra. There are various methods to measure $^1O_2$ indirectly via absorption, fluorescence or chemiluminescence by adding $^1O_2$ quenchers. However, the observed quencher consumption may not exactly reflect the amount of generated $^1O_2$. For example, these indirect measurements include a competitive reaction that both $^1O_2$ and superoxide anion ($O_2^{-\cdot}$) are formed through the laser flash photolysis of thiobases as reported by Hemmens *et al.* [29]. Formation of $O_2^{-\cdot}$ is possible via electron transfer from thiobases to O_2 in aqueous solution, thus would affect the measuring accuracy of $^1O_2$. In current work, the unambiguous way to measure $^1O_2$ by its characteristic phosphorescence at

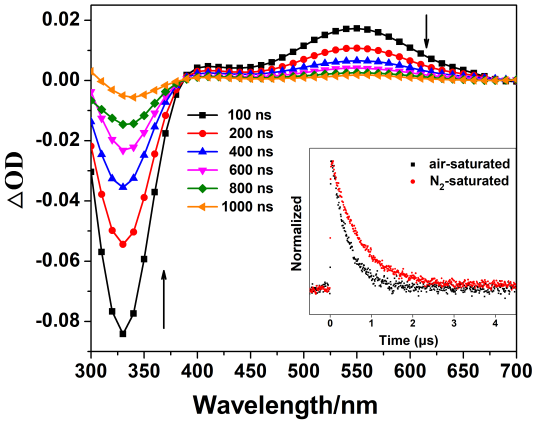

FIG. 2 Transient absorption spectra for 4-thiourcil (ΔOD_{355}=0.3) in D_2O. The spectra were recorded in air-saturated condition at time delays from 100 ns to 1000 ns using an excitation wavelength of 355 nm. Inset: singlet oxygen decay in D_2O at the specified experimental conditions. Air-saturated condition and N_2-saturated condition are shown by black and red lines, respectively.

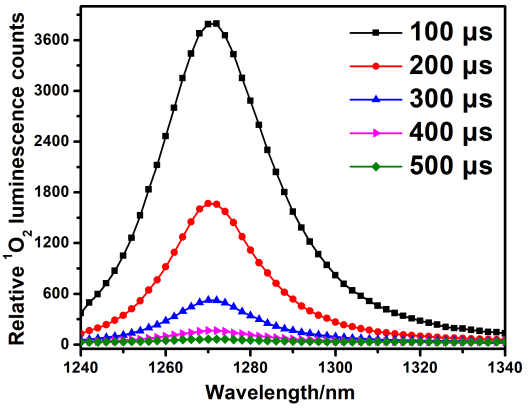

FIG. 3 Singlet oxygen luminescence spectra obtained from the 355 nm photoexcitation of 4-thiouracil in D_2O.

1270 nm is applied to determine the reaction kinetics between thiobases with $^1O_2$.

Time-resolved $^1O_2$ phosphorescence spectra peaked at 1270 nm upon 355 nm excitation of 4tUra were detected. Representative decay traces of $^1O_2$ with the decreasing concentration of 4tUra in D_2O solution under O_2-saturated condition are shown in FIG. 4. In current experiments, D_2O is used as solvent because the lifetime of $^1O_2$ in D_2O (68 μs) is much longer than the lifetime in H_2O (3 μs), which is due to that the radiationless $^1O_2$ deactivation involving collisional electronic to vibrational (E→V) energy transfer from $^1O_2$ to O−H bond in H_2O is faster than that to O−D bond in D_2O. If H_2O is used as solvent, $^1O_2$ produced by thiobases will be dominantly quenched by the solvent, resulting in the difficulty to observe the kinetics evolution of the reaction of thiobase with $^1O_2$. FIG. 4 displays the different decay profiles of $^1O_2$ phosphorescence at various 4tUra

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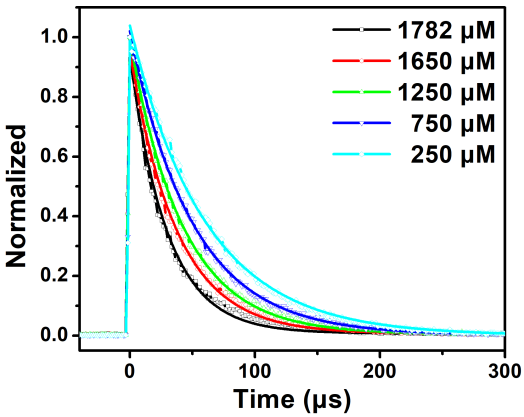

FIG. 4 Singlet oxygen phosphorescence decay traces monitored at 1270 nm obtained from the pulsed photoexcitation (355 nm, 7 ns pulse length, 10 mJ/pulse) of 4tUra at different concentrations in O_2-saturated solutions.

concentrations. It is obvious that with the increase of 4tUra concentration, the total decay lifetime of $^1O_2$ decreases. From these decay curves, the lifetime of $^1O_2$, τ, at different 4tUra concentrations can be obtained through the exponential fitting. In the current experimental conditions, the concentration of 4tUra should be much greater than that of the generated $^1O_2$. Therefore, the pseudo-first order reaction approximation can be applied for the reaction of thiobases with $^1O_2$. The reaction quenching kinetics of $^1O_2$ can be modeled as a unimolecular process given by a Stern-Volmer relationship, since the rate of $^1O_2$ decay displays a linear relationship with concentration,

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_q[\text{4tUra}] \quad (1)$$

where τ is the apparent lifetime of $^1O_2$, τ_0 is the intrinsic lifetime of $^1O_2$ in the solvent of D_2O, and k_q is the reaction rate constant of $^1O_2$ with 4tUra. Hence, k_q, can be determined from the slope of the linear dependence of the apparent lifetime $1/\tau$ versus the concentration of 4tUra (FIG. 5(a)). Extrapolation of the linear plot gives the intrinsic lifetime (τ_0), the obtained τ_0 of three thiobases are in good agreement with the previously published values [30–32], demonstrating the reliability of our experimental method. The fitted kinetic rate constant (k_q) values are presented in Table I.

Similar experiments were performed for 6-thioguanine (6tGua). The reaction rate constants for 6tGua with $^1O_2$ (k_q) are shown in FIG. 5(b) and Table I as well. The magnitude of the rate constant of 4tUra with $^1O_2$ (7.46×10^6 $(\text{mol/L})^{-1}\text{s}^{-1}$) is about five-fold smaller than that of 6tGua (3.85×10^7 $(\text{mol/L})^{-1}\text{s}^{-1}$) in D_2O solution, which indicates that thiopyrimidine is less reactive with $^1O_2$ and thus more stable than thiopurine. This observation is consistent with previous calculation [19], which showed that the reaction energy barrier of the rate-determining step for 6tGua with $^1O_2$

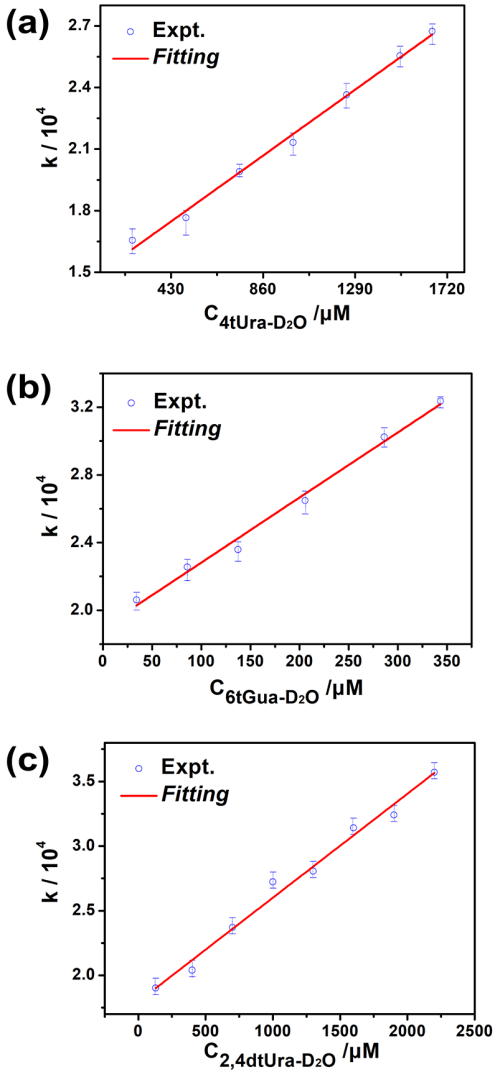

FIG. 5 Stern-Volmer plot obtained from the decay lifetime (τ) of singlet oxygen luminescence. The rate of $^1O_2$ decay displays a linear increase with the concentration of (a) 4tUra, (b) 6tGua, and (c) 2,4dtUra.

(0.1 kcal/mol) is smaller than that for 4tUra with $^1O_2$ (1.9 kcal/mol). In addition, it is worth mentioning that the rate constant of guanine (Gua) with $^1O_2$ (1.36×10^5 $(\text{mol/L})^{-1}\text{s}^{-1}$) [33] is much smaller than that of 6tGua with $^1O_2$, indicating that thioguanine is more reactive than guanine which is in accordance with the previous sulfur addition results [2–5].

The double-thionated pyrimidine 2,4dtUra was also tested and the results are shown in FIG. 5(c) and Table I. The rate constant of 2,4dtUra with $^1O_2$ in D_2O (7.49×10^6 $(\text{mol/L})^{-1}\text{s}^{-1}$) is similar to that of 4tUra (7.46×10^6 $(\text{mol/L})^{-1}\text{s}^{-1}$) in D_2O as well as in CH_3CN, while the $^1O_2$ quantum yield of 2,4dtUra is larger than that of 4tUra [5]. As shown in FIG. 1, the absorption of 2,4dtUra at 355 nm is stronger than that of 4tUra, which will result in that more 2,4dtUra molecules were

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TABLE I Reaction rate constants of $^1O_2$ with different thiobases in different solvents.

Solvents	$k_q/((\text{mol/L})^{-1}\text{s}^{-1})$		
	6tGua+$^1O_2$	4tUra+$^1O_2$	2,4dtUra+$^1O_2$
D_2O	$(3.85\pm0.18)\times10^7$	$(7.46\pm0.25)\times10^6$	$(7.49\pm0.38)\times10^6$
2:1 D_2O/CH_3CN	$(1.04\pm0.22)\times10^8$	$(1.69\pm0.26)\times10^7$	$(2.89\pm0.16)\times10^6$
1:1 D_2O/CH_3CN	$(1.12\pm0.17)\times10^8$	$(1.25\pm0.30)\times10^7$	$(3.11\pm0.29)\times10^6$
1:2 D_2O/CH_3CN	$(1.73\pm0.20)\times10^8$	$(2.05\pm0.11)\times10^7$	$(2.38\pm0.09)\times10^7$
CH_3CN	$(4.00\pm0.26)\times10^8$	$(2.06\pm0.16)\times10^7$	$(1.25\pm0.24)\times10^7$

populated to triplet state to generate more $^1O_2$ via photosensitization, leading to the higher quantum yield of $^1O_2$ produced by 2,4dtUra. Actually, under current experimental conditions, the concentrations of ground thiobases are much greater than that of $^1O_2$ generated, which implies that one 2,4dtUra molecule can only react with one $^1O_2$ molecule, though 2,4dtUra has two C=S bonds to potentially participate in the reaction with $^1O_2$. The similar rate constants of $^1O_2$ with 2,4dtUra and 4tUra may be due to the same reaction mechanism as follows. According to the previous result [5], the thionation at the C4 position results in faster intersystem crossing than thionation at the C2 position, indicating the C4=S bond may have higher reactivity than C2=S bond. Therefore, when reacting with $^1O_2$, the addition of $^1O_2$ may occur on the C4=S bond, rather than on C2=S bond. However, more detailed reaction mechanisms between thiobases with $^1O_2$ need further investigation.

C. Solvent effect on reaction rate constants

The solvent polarity may influence the reaction rates [34]. Interestingly, as can be seen in Table I, the reaction rate constant of 4tUra with $^1O_2$ is 7.46×10^6 $(\text{mol/L})^{-1}\text{s}^{-1}$ in D_2O, while in less polar acetonitrile, the rate constants are two times larger (2.06×10^7 $(\text{mol/L})^{-1}\text{s}^{-1}$). To further testify the influence of solution polarity on the reaction rate constants of thiobases and singlet oxygen, the mixed solvents of D_2O and CH_3CN in different volume ratio as 2:1, 1:1 and 1:2 were used. The detected rate constants in different polarity solvents are also listed in Table I. As for 6tGua in those five different solvents, the rate constants show an obvious increasing trend with the decreasing of the solvent polarity. Similar trends are observed for the thiobases of 4tUra and 2,4dtUra, which is in accordance with the previous rules of polarity effect on the rate constants for addition reaction [34, 35].

IV. CONCLUSION

By means of laser flash photolysis combined with time-resolved luminescence spectra of $^1O_2$ at 1270 nm after UVA irradiation of thiobases, we measured the kinetics rate constants of several thiobases reacting with singlet oxygen. It is determined that the reaction rate constant of 4tUra with $^1O_2$ is 7.46×10^6 $(\text{mol/L})^{-1}\text{s}^{-1}$ in D_2O and 2.06×10^7 $(\text{mol/L})^{-1}\text{s}^{-1}$ in CH_3CN at room temperature respectively and that of 6tGua with $^1O_2$ is 3.85×10^6 $(\text{mol/L})^{-1}\text{s}^{-1}$ in D_2O and 4.00×10^8 $(\text{mol/L})^{-1}\text{s}^{-1}$ in CH_3CN respectively. Our results illustrate that the reaction rate constants of thiouracils with $^1O_2$ are smaller than that of thioguanine with $^1O_2$, suggesting that thiopyrimidine is more stable than thiopurine, thus should be more suitable to be used as the photosensitive drug in PDT. We also examined the solvent effect on the reactions of singlet oxygen with thiobases and found that the reaction rate constants of thiobases with $^1O_2$ are smaller in water than in less polar solvent. Additionally, the reaction rate constant of thiouracil with $^1O_2$ is roughly equal to that of dithiouracil with $^1O_2$, which indicates that double sulfur substitution does not cause further change for the reactivity. These results can provide some new perspectives on the reaction kinetics of nucleic acid and reactive oxygen species, guiding the selection of photosensitive drug in PDT.

V. ACKNOWLEDGEMENTS

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