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Characteristics of Excited Triplet States of Thiolated Guanosine Derivatives and Singlet Oxygen Generation

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Thioguanine is sensitive to UVA light and generates singlet molecular oxygen ($^1O_2$*) when exposed to UVA. Three thioguanosine derivatives, 2',3',5'–tri–O–acetyl–6–thioguanosine (ta6TGuo), 2',3',5'–tri–O–acetyl–8–thioguanosine (ta8TGuo), and 2',3',5'–tri–O–acetyl–6,8–dithioguanosine (taDTGuo) were explored photophysically and photochemically. The nanosecond transient absorption and time–resolved near–infrared emission measurements were carried out to investigate the characteristics of their excited triplet states in acetonitrile solution. The quantum yield of intersystem crossing ($\emptyset_{ISC}$), the intrinsic decay rate constant (k_0), the quenching rate constant by $^3O_2$ (k_q) and the self–quenching rate constant (k_{SQ}) of their triplet states were all determined. From precise analysis of the quantum yield of $^1O_2$* generation ($\emptyset_\Delta$) against the concentration of dissolved molecular oxygen, the fraction of the triplet states quenched by dissolved oxygen which gives rise to $^1O_2$* formation (S_Δ) was successfully obtained with high accuracy. The $\emptyset_\Delta$ values at low oxygen concentrations reveal these thioguanosines, particularly taDTGuo, can still effectively generate $^1O_2$* under low molecular oxygen concentration like carcinomatous microenvironments. These findings indicate that taDTGuo would perform well as a potential agent for photo–induced cancer therapies.

Introduction

Nucleic acids (DNA and RNA) are one of the most important classes of biological molecules. Chemically they are made of nucleotides, and each nucleotide consists of a base (adenine, guanine, cytosine, thymine or uracil), sugar (ribose or deoxyribose) and phosphate moiety. The bases are the chromophore and strongly absorb light in the UV region (λ_{abs} < 300 nm).[1–4] Excited state dynamics of nucleic acid bases have been intensively studied by various spectroscopic techniques (such as fluorescence, resonant ionization and photoelectron spectroscopies).[5–12] Current studies by femtosecond time–resolved spectroscopy have clarified that nucleic acid bases and nucleosides/nucleotides in an initially excited singlet state, following UV exposure, undergo ultrafast internal conversion to the electronic ground state.[2,13–19] Since such ultrashort lifetimes would not allow any further photoreaction to occur, it was claimed that this ultrafast relaxation of the excited state is the origin of the high photostability of DNA/RNA, and protects our genes from the harmful UV radiation from the sun.[18]

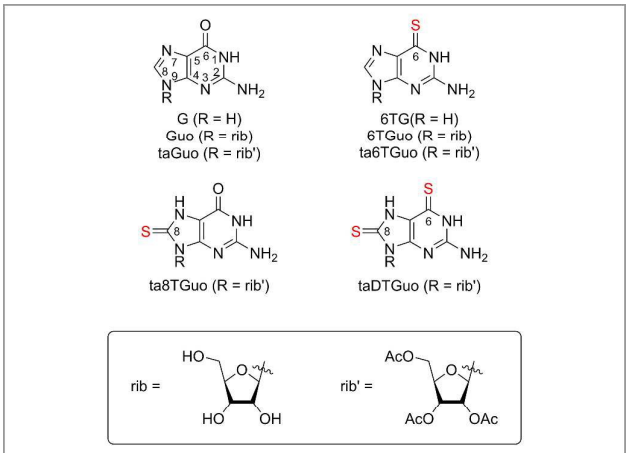

Fig. 1 Structures of guanine, thioguanines, and their nucleosides.

Nucleic acids generally do not contain sulfur atoms in them. However, sulfur–containing analogues of nucleic acid bases (thio–bases) have recently become a focus of attention because of their remarkably different photochemical characteristics from the native (un–thiolated) bases. For instance, 6–thioguanine (6TG) and 4–thiouracil have been used as carcinostatic agents, leukemia drugs, antithyroids and DNA markers among others.[20–35] Thio–bases have a strong absorption in UVA (320–400 nm) region,[36–46] in which native bases are transparent. Thio–bases localizing in proliferating cells have been reported to generate reactive oxygen species (ROS) when exposed to UVA light, thus leading to cell

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deaths.[24,47] These findings indicate that thio–bases (and their nucleosides) have some potential for photochemotherapy by acting as a photoactivatable genotoxic agent and/or a photosensitizer.[23,48,49]

We previously reported that 2–thiothymine and 4–thiothymidine can effectively generate singlet molecular oxygen ($^1O_2$*,$^1\Delta_g$), one type of ROS, by photoexcitation.[37–39] Crespo–Hernández and co–workers also described the generation of $^1O_2$* ($^1\Delta_g$) from other thio–pyrimidines and from 6–thioguanosine (6TGuo).[42–45] This type of $^1O_2$* ($^1\Delta_g$) generation is in a high yield and results from high quantum yield of intersystem crossing (from its excited singlet state to the triplet manifolds), followed by the photosensitization reaction with $^3O_2$. These are some of the unique photophysical/photochemical characteristics of thio–bases and thio–nucleosides.[50,51] In recent reports, 6-thioguanine (6TG) that accumulates in the DNA interacts with UVA to generate ROS and these cause lethal and mutagenic DNA damage.[27] Conversely, ROS generation plays a minor role in the photocytotoxic of thiobases.[52,53] In addition, 6TG oxidation products by the interaction between DNA6TG and UVA have a potential to cause cytotoxic.[40,54] In order to demonstrate mechanisms of breaking cells, it is essential to obtain ultimate kinetic parameters of thiolated nucleic acid bases, the values of the quantum yield of $^1O_2$* ($^1\Delta_g$) generation ($\emptyset_\Delta$), the fraction of the triplet states quenched by dissolved oxygen which gives rise to $^1O_2^*$ formation (S_Δ), and the quantum yield of intersystem crossing ($\emptyset_{ISC}$).

As part of our on–going effort to understand the phototherapeutic ability of thio–bases and thio–nucleosides, recently we synthesized and explored tri–acetyl–protected thioguanosine derivatives: namely 2',3',5'–tri–*O*–acetyl–6–thioguanosine (ta6TGuo), 2',3',5'–tri–*O*–acetyl–8–thioguanosine (ta8TGuo) and 2',3',5'–tri–*O*–acetyl–6,8–dithioguanosine (taDTGuo) (for chemical structures, see Fig. 1).[55] The presence of these acetyl groups on the thioguanosines greatly increases their solubilities in dehydrated organic solvents. However, these acetyl groups are removable due to their proneness to hydrolysis under a very mild condition such as by biological agents (e.g. deacylase) or chemical environments (e.g. a changed pH). Furthermore, these acetylated thioguanosines retain strong absorption maxima at UVA region (see Fig.S1 in the ESI) and generate $^1O_2$* ($^1\Delta_g$). The doubly thiolated analogue (taDTGuo) is of particular interest since it absorbs UV light most strongly and at the longest wavelength among all thio–nucleobases and thio–nucleosides reported,[36–46] revealing that taDTGuo would be much more sensitive to the light penetrating into the human skin. Therefore these acetylated thio–guanosines, especially taDTGuo, are well worth further investigating as potential agents for light–induced therapies.

In general, the triplet formation of the thioguanosines is considered to be essential to the $^1O_2$* ($^1\Delta_g$) generation, however, direct evidence of the triplet formation and photophysical characteristics such as lifetime and quantum yield of the triplet states are still lacking. Although the quantum yield of $^1O_2$* ($^1\Delta_g$) generation ($\emptyset_\Delta$) has just been determined under the atmospheric oxygen condition,[55] further detailed information on the $\emptyset_\Delta$ values under cellular conditions are still required and would be much more therapeutically relevant because the oxygen concentration in the living cells is known to be much smaller (~100 μM) than the atmosphere.[51] In this article, we clarify the characteristics of excited triplet states of these thioguanosines and report their $\emptyset_\Delta$ values are dependent on concentration of dissolved molecular oxygen. These findings should contribute to a better understanding of these thioguanosines as effective agents for photochemotherapies.

Experimental

Materials.

Tri–acetyl–protected guanosine and thioguanosines, taGuo, ta6TGuo, ta8TGuo and taDTGuo, were prepared as described in the previous article.[55] Their structures were characterized by 1H NMR and their purities estimated to be above 97% with a minor amount of impurity being H_2O.

Apparatus.

Nanosecond transient absorption measurements were carried out with a XeCl excimer laser (Lambda Physik, COMPex 102; 308 nm, 120 mJ/pulse, 20 ns pulse duration, repetition rate 2 Hz) as an excitation light source. White continuum light from a Xe flash lamp (Ushio UXL–300D; 300 W) was used as a probe light to monitor the transient absorption change. The monitoring light, passing through a flow cell (Tosoh Quartz T514M–ES–10; 10 mm optical pass length), was dispersed by a monochromator (Nikon, P–250) and then detected by a photomultiplier tube (Hamamatsu Photonics, R928). The output signal was visualized by a digital oscilloscope (Sony Tektronix, TDS380P; 400 MHz, 2GS/s) and analyzed by a personal computer. Signals were averaged over 50 shots. The incident laser power was controlled to avoid triplet–triplet annihilation or any multiphotonic events. The ground state absorbance of thioguanosine solutions was prepared to be typically *ca*. 0.8 at excitation wavelength (308 nm) in transient absorption spectrum measurements. The linearity of the signal intensity against the incident laser power and the ground state concentration was confirmed on each experiment.

Time–resolved near–infrared emission measurements were carried out with a thermoelectric cooled near–infrared photomultiplier tube (Hamamatsu Photonics, H10330–45; InP/InGaAsP, spectral response 950 to 1400 nm) combined with a longpass filter (Thorlabs, FEL1250; cut–on wavelength 1250 nm) and a bandpass filter (Edmund, Hard–coated bandpass filter; 1275 ± 50 nm). The XeCl excimer laser was used as an excitation light source. The sample solution flowed in the cell to avoid the contamination of photoproducts.

All measurements were carried out in acetonitrile solution at room temperature. Each of the thioguanosines was dissolved in acetonitrile and the solution was then bubbled over 15 minutes with Ar or strictly adjusted partial pressure of N_2/O_2

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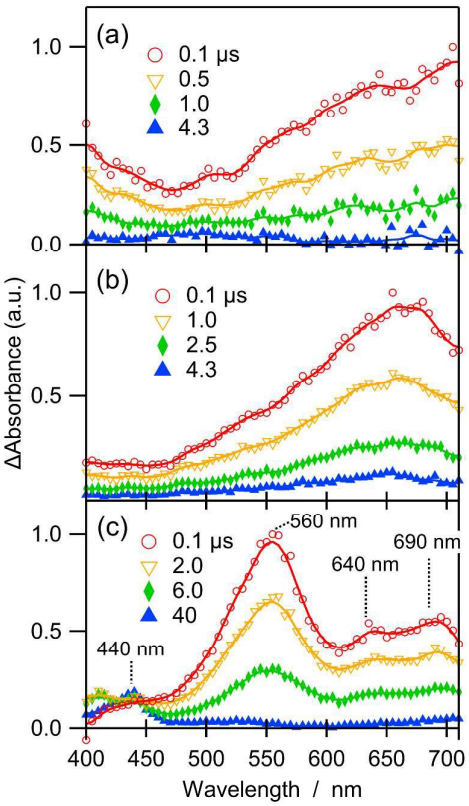

Fig. 2 Transient absorption spectra of (a) ta6TGuo, (b) ta8TGuo, and (c) taDTGuo in Ar–saturated acetonitrile solution at several delay times after the laser.

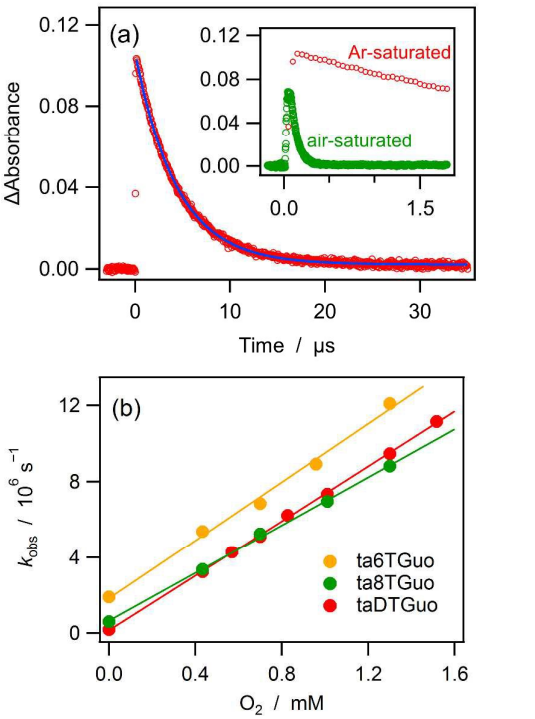

Fig. 3 (a) Time profile of transient absorption of taDTGuo (65.7 μM) monitored at 560 nm in Ar–saturated acetonitrile solution. Time profile of transient absorption in air–saturated solution is also shown in the inset. (b) Plots of the decay rate constant (k_{obs}) against the concentration of dissolved oxygen.

mixture gas under atmosphere pressure to control the dissolved oxygen concentration in the solution.

Results and Discussion

Transient Absorption Measurement.

Transient absorption spectra of ta6TGuo in Ar–saturated acetonitrile are shown in Fig. 2a. Immediately after the laser, a broad spectrum extending over 400–710 nm was observed. The absorption decayed to almost zero within 5 μs. Furthermore the decay of the absorption intensity became faster with the increase in the concentration of dissolved oxygen (see Fig. S2 in the ESI). These spectral features (presented in Fig. 2a) well agree with the reported spectrum of triplet–triplet absorption of 6TGuo.[41] Since the acetyl groups are not conjugated with the thiobase and make no interference or contribution, thus the spectrum presented in Fig. 2a can be safely assigned to the triplet–triplet absorption of ta6TGuo.

Transient absorption spectra of ta8TGuo in Ar–saturated acetonitrile are shown in Fig. 2b. A broad spectrum with absorption maximum at around 650 nm was observed. The absorption decayed to almost zero within 5 μs, and furthermore the decay of the absorption intensity also became faster with increased concentrations of the dissolved oxygen (see Fig. S3 in the ESI). For the same reasons discussed for ta6TGuo (above), the absorption spectrum should be assigned to the triplet–triplet absorption of ta8TGuo. For taGuo, no transient signals were detected, indicating that the excited state of taGuo will decay through the undetectable ultrafast internal conversion.

Transient absorption spectra of taDTGuo in Ar–saturated acetonitrile shown in Fig. 2c. Three absorption maxima at 560, 640 and 690 nm were observed immediately after the laser. Fig. 3a shows a typical time profile of the transient absorption at 560 nm of taDTGuo in Ar–saturated acetonitrile. The absorption intensity decreased single–exponentially, and this transient species was efficiently quenched by dissolved oxygen molecules (see Fig. 3a inset). We previously reported that thiolated guanosines, including taDTGuo, exhibit a phosphorescence from the T_1 state in glassy ethanol matrix and have a high quantum yield of singlet oxygen generation ($Ø_\Delta$) in O_2–saturated acetonitrile solution.[55] Thus, this transient species should be assigned to the lowest excited triplet taDTGuo. A slowly rising component observed at around 440 nm in the spectrum of taDTGuo (Fig. 2c) disappeared with increased oxygen concentrations. This band is likely to result from a photoproduct, generated by a reaction between triplet taDTGuo and ground–state of taDTGuo. Such a photoproduct was also shown in 6-aza-2-thiothymine,[39] but these products are not observed in the absorption spectra after irradiation,

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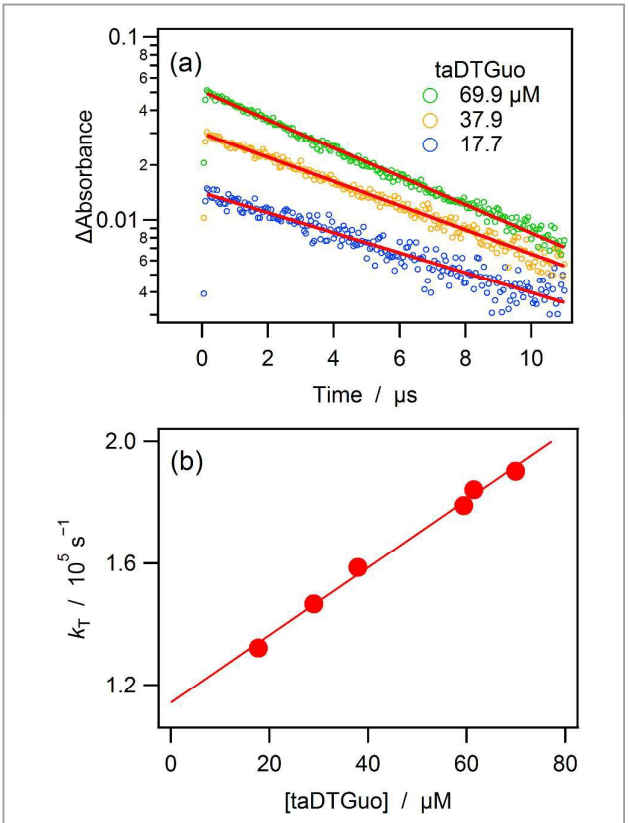

Fig. 4 (a) Time profiles of transient absorption monitored at 560 nm for different concentrations of taDTGuo in Ar–saturated acetonitrile, and (b) plot of the triplet decay rate constant (k_T) of taDTGuo against the ground–state taDTGuo concentration.

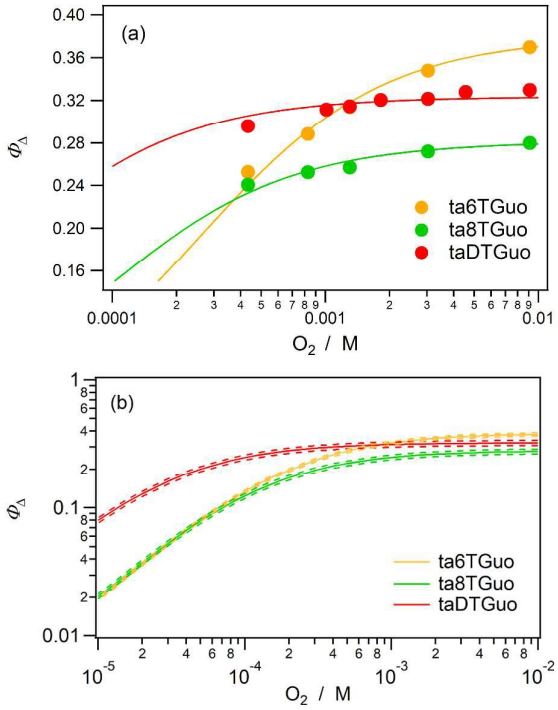

Fig. 5 (a) Plots of the Φ_Δ values of ta6TGuo (yellow), ta8TGuo (green), and taDTGuo (red) against the dissolved oxygen concentration. (b) Extrapolation of the Φ_Δ values under infinitely small oxygen concentration using the kinetics parameters estimated in this study. The concentration of the thioguanosines was set to 100 μM in this simulation. The broken curves show the simulated ones including ±0.02 errors of the Φ_{ISC} values.

thus they will be transients having long lifetime (≥ 1 ms), not final products.

To investigate the effects of dissolved oxygen on the excited triplet states of these thioguanosines, the time profile of the transient absorption was measured with varied oxygen concentrations. Fig. 3b shows the plots of the decay rate constant (k_{obs}), obtained by the fitting analysis with the single exponential equation, against the concentration of oxygen dissolved in the solution. These plots show good linear relationships. These kinetics of the decay process can be described using the following equation,

$$k_{obs} = k_T + k_q [{}^3O_2] \quad (1)$$

where k_T and k_q denote the decay rate constant of the triplet–state itself and the quenching rate constant of the triplet thioguanosines by molecular oxygen, respectively. The k_q value was obtained from the plots of the triplet decay rate constant against dissolved molecular oxygen concentration as shown in Fig. 3b. The linear regression analysis of the plots by using eq. 1 gives the quenching rate constant, (7.6 ± 0.1)×10^9 M^{-1} s^{-1} for ta6TGuo, (6.3 ± 0.1)×10^9 M^{-1} s^{-1} for ta8TGuo, and (7.2 ± 0.1)×10^9 M^{-1} s^{-1} for taDTGuo. These values are close to the k_q values reported for other thio–bases and thio–nucleosides,[37,39,44] showing the triplet quenching will occur almost at the diffusion–controlled rate. Under the high $^3O_2$ concentration condition, the dominant relaxation process from the T_1 state of thioguanosines will be the quenching by molecular oxygen, and the excited triplet state of these thioguanosines as well as other thio–bases and thio–nucleosides can generate $^1O_2$* through the photosensitization reaction.[55]

Fig. 4a shows the time profile of the transient absorption monitored at 560 nm for different concentrations of taDTGuo in Ar–saturated solution. The decay rate constant of the excited triplet state was accelerated as the concentration of taDTGuo increased, suggesting the existence of self–quenching process by ground state taDTGuo. Fig. 4b shows the plot of the decay rate constant of the excited triplet state (k_T) against the concentration of taDTGuo in Ar–saturated solution. The plot gives a good linear relationship between the constant and the concentration. The triplet self–quenching kinetics can be written as a Stern–Volmer relationship,

$$k_T = k_0 + k_{SQ} [\text{taDTGuo}] \quad (2)$$

where k_0 and k_{SQ} denote the intrinsic decay rate constant of triplet state and the self–quenching rate constant,

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Table 1. Photophysical Characteristics of Acetylated Thioguanosines

	k_0 [a] / s^{-1} [τ_0 [b] / μs]	k_{SQ} [c] / M^{-1} s^{-1}	k_q [d] / M^{-1} s^{-1}	Φ_Δ [e]	Φ_{ISC} [f]	S_Δ [g]
ta6TGuo	(1.1±0.1)×10^6 [0.95±0.02]	(3.4±0.1)×10^9	(7.6±0.1)×10^9	0.37±0.01	0.65±0.02	0.57±0.02
ta8TGuo	(4.4±0.1)×10^5 [2.3±0.1]	(3.7±0.2)×10^9	(6.3±0.1)×10^9	0.28±0.01	0.46±0.02	0.59±0.03
taDTGuo	(1.1±0.1)×10^5 [8.8±0.1]	(1.1±0.1)×10^9	(7.2±0.1)×10^9	0.33±0.01	0.43±0.02	0.74±0.04

[a] Intrinsic rate constant of triplet–state decay. [b] Intrinsic lifetime of triplet–state. [c] Self–quenching rate constant. [d] Bimolecular quenching rate constant of the triplet state by molecular oxygen. [e] Quantum yield of singlet oxygen generation under $^3O_2$–saturated condition determined in the previous study.[50] [f] Quantum yield of intersystem crossing. [g] Fraction of the triplet states quenched by dissolved oxygen which gives rise to $^1O_2{}^*$ formation.

respectively. By the linear regression analysis using eq. 2, the intrinsic decay and self–quenching rate constants of taDTGuo were determined to be (1.1 ± 0.1) ×10^5 s^{-1} and (1.1 ± 0.1) ×10^9 M^{-1} s^{-1}, respectively. Excited triplet states of ta6TGuo and ta8TGuo also exhibited self–quenching process (as shown in Figs. S4–S7 in the ESI), and their rate constants have also been determined (see Table 1). The self–quenching rate constant of the doubly thiolated guanosine (taDTGuo) was about one third of those of the singly thiolated guanosines (i.e. ta6TGuo and ta8TGuo), and intrinsic triplet lifetime of taDTGuo was more than three–fold in comparison with the other thioguanosines. These photophysical characteristic parameters of the acetylated thioguanosine derivatives are summarized in Table 1.

Estimation of Quantum Yield of Singlet Oxygen Generation in Low Oxygen Concentration like Carcinomatous Condition.

Under any high oxygen concentration condition, the dominant relaxation process from the T_1 state of the thioguanosines will be the quenching process by oxygen. However, the concentration of oxygen in the living cells is known to be low. Especially, the concentration in tumor cells was reported to be much lower than that of health cells.[56] Thus, determining Φ_Δ values of the thioguanosines under the condition of such a low oxygen concentration would be therapeutically relevant and useful when optimizing the thioguanosines as potential photochemotherapeutic agents.

The Φ_Δ value under the oxygen saturated condition was reported in our previous paper to be 0.37 ± 0.01 for ta6TGuo, 0.28 ± 0.01 for ta8TGuo, and 0.33 ± 0.01 for taDTGuo.[55] Individual long–lived phosphorescence traces of $^1O_2{}^*$ were also observed at several dissolved oxygen concentrations, and fitted by using a single–exponential function to estimate the emission intensity maxima immediately after laser irradiation (I_S^0). The I_S^0 value was plotted against the laser fluence (I_L) (see Figs. S8–S10 in the ESI), showing good linear relationships between I_S^0 and I_L. The plots were analyzed with the least squares' fitting to obtain a best–fit straight line. By comparing the slope of the line at each dissolved oxygen concentration with that at oxygen–saturated solution, the oxygen concentration dependence of the Φ_Δ value was established, as shown in Fig. 5a. The Φ_Δ values of the thioguanosines, especially ta6TGuo, are found to be largely dependent on the dissolved oxygen concentration.

The oxygen concentration–dependent Φ_Δ value can be written down as

$$\Phi_\Delta = \frac{S_\Delta \cdot \Phi_{ISC} \cdot k_q[^3O_2]}{k_0 + k_{SQ}[TGuo] + k_q[^3O_2]} \quad (3)$$

where S_Δ and Φ_{ISC} denote the fraction of the triplet states quenched by dissolved oxygen which gives rise to $^1O_2{}^*$ formation and triplet quantum yield of the thioguanosines, respectively.[37,39,44] In these measurements, the concentration of thioguanosines, [TGuo], was 247 μM for ta6TGuo, 35.6 μM for ta8TGuo, and 66.7μM for taDTGuo. The Φ_{ISC} value was determined to be as large as 0.65 ± 0.02 for ta6TGuo, 0.46 ± 0.02 for ta8TGuo, and 0.43 ± 0.02 for taDTGuo in a similar fashion in our previous article.[57,58] Briefly, the molar absorption coefficient of triplet–triplet absorption was measured by energy transfer method with a reference substance whose molar absorption coefficient is known. The Φ_{ISC} was determined from the triplet–triplet absorption intensity of thioguanosine solution relative to optically matched benzophenone solution as a reference. The Φ_{ISC} value for ta6TGuo (0.65 ± 0.02) was slightly smaller than that of 6-thioguanosine (0.8 ± 0.2)[41], which may result from the tri-acetylation on the ribose and/or the different solvent. The plots of Φ_Δ against the dissolved oxygen concentration were analyzed by using eq. 3. The best-fitting curves are shown as solid lines in Fig.5a (The expanded figure to the lower oxygen concentration is also shown in Fig. S11). The S_Δ values were successfully estimated to be 0.57 ± 0.02 for ta6TGuo, 0.59 ± 0.03 for ta8TGuo, and 0.74 ± 0.04 for taDTGuo. These S_Δ values were sufficiently high in comparison with the reported values for other thio–bases and thio–nucleosides.[37,39,44] Therefore, these thioguanosines were found to generate $^1O_2{}^*$ effectively by a collision between a triplet thioguanosine molecule and a ground–state molecular oxygen. In general, a triplet state having ππ* character was reported to have a S_Δ value of the range of 0.7–1.0.[59] Since the T_1 state of these thioguanosines was assigned to the ππ* state,[55] the high S_Δ values were reasonable.

To evaluate the thioguanosines as photochemotherapeutic agents, their Φ_Δ values under infinite small $^3O_2$ concentration

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were extrapolated by using the photophysical parameters estimated in the present study. The concentration of the thioguanosines was standardized to 100 μM in this calculation. According to the simulated results (Fig. 5b), the $\emptyset_\Delta$ value of ta6TGuo is the largest at higher oxygen concentrations (larger than 1.3 mM) among the thioguanosines examined. The $\emptyset_\Delta$ value of these thioguanosines at high oxygen concentration depends on the product of $\emptyset_{ISC}$ and S_Δ. On the other hand, at quite low oxygen concentration such as general carcinomatous conditions (at around 18 μM of $^3O_2$),[56] taDTGuo has the largest value of $\emptyset_\Delta$ among these thioguanosines. It is noted that the intrinsic decay rate constant of triplet state (k_0) and the self–quenching rate constant (k_{SQ}) of taDTGuo was small in comparison with those of ta6TGuo and ta8TGuo (see Table 1). Thus, the relatively long intrinsic T_1 lifetime and small influence of self–quenching deactivation of taDTGuo can readily achieve the collision event with molecular oxygen, which enables taDTGuo to generate $^1O_2$* effectively at low oxygen concentrations such as carcinomatous conditions.

In our previous article,[60] taDTGuo in phosphate buffer solution was reported to establish acid dissociation equilibria among neutral form, 1-imide anionic form, and 1,7-di-imide anionic form, and to exhibit pH-dependent $\emptyset_\Delta$ values. According to the equilibrium constants obtained, neutral form of taDTGuo exists dominantly under the low pH condition like carcinoma cells and has a larger $\emptyset_\Delta$ value than anionic species. Taking these results together, taDTGuo would generate $^1O_2$* effectively under low-oxygen and low-pH condition and therefore should offer a good potential as a photochemotherapeutic agent for cancer therapies.

Conclusions

The excited triplet state characteristics of the thioguanosines, ta6TGuo, ta8TGuo and taDTGuo, were studied in detail by the nanosecond transient absorption and time–resolved near–infrared emission measurements. The broad transient absorption spectra of the thioguanosines in acetonitrile were observed over 440–710 nm. These spectra were assigned to the triplet–triplet absorption because of their efficient quenching by the dissolved oxygen. The excited triplet lifetime also depended on the ground state concentration, and the self–quenching rate constant and intrinsic triplet lifetime were determined by the Stern–Volmer plots. By near–infrared emission measurements, the $\emptyset_\Delta$ value of the thioguanosines was found to depend on the concentration of dissolved oxygen. The fitting analysis of the plots of $\emptyset_\Delta$ value against oxygen concentration gave the large S_Δ values, revealing that the excited triplet thioguanosine should collide with a molecular oxygen efficiently to generate $^1O_2$* with high probability (larger than 0.5). The $\emptyset_\Delta$ values under infinite low oxygen concentrations were extrapolated by using the photophysical parameters estimated in this study. The simulations revealed that taDTGuo should generate $^1O_2$* more effectively in comparison with ta6TGuo and ta8TGuo at quite low concentration of oxygen such as carcinomatous conditions. This results from the long intrinsic lifetime and its low self–quenching rate constant of the triplet taDTGuo. In short, taDTGuo would be most effective for photochemotherapies among all examined thioguanosines.

Conflicts of interest

There are no conflicts to declare.

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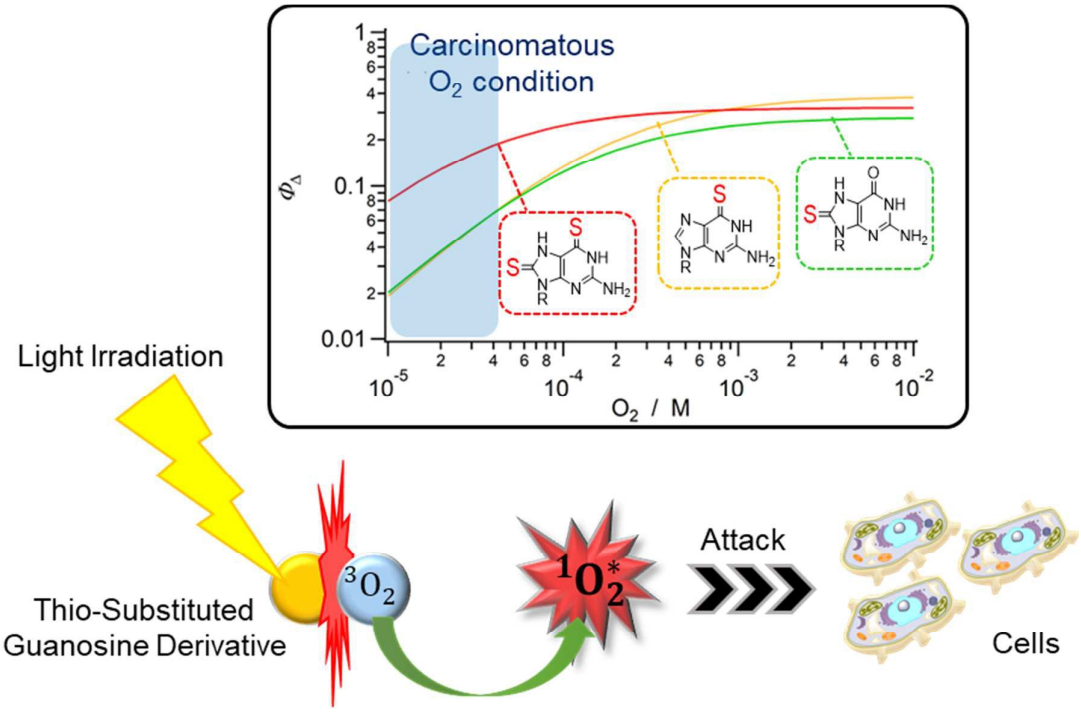

The long intrinsic lifetime and its low self–quenching rate constant of the triplet 6,8-dithioguanosine lead to efficient singlet molecular oxygen generation under carcinomatous oxygen conditions.