

Dual Functionality of 6-Methylthioguanine: Synergistic Effects Enhancing the Photolability of DNA Nucleobases

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nucleobases (thionation and methylation) boosts the photoactivity by introducing more reactive channels. Intriguingly, ${}^{1}n_{N}\pi^{*}$, rather than ${}^{1}n_{S}\pi^{*}$, acts as the doorway state engendering the formation of the long-lived reactive triplet state in me6-TG. The ${}^{1}n_{N}\pi^{*}$ induces a low spin-orbit coupling of 8.3 cm⁻¹, which increases the intersystem crossing (ISC) time (2.91 ± 0.14 ns). Despite the slowed ISC, the triplet quantum yield (Φ_{T}) still accounts for a large fraction (0.6 ± 0.1), consistent with the potential energy surface that favors excited-state bifurcation to ${}^{1}n_{N}\pi^{*}_{min}$ (3.36 ± 0.15 ps) rather than ${}^{1}\pi\pi^{*}_{min}$ (5.05 ± 0.26 ps), such that the subsequent ISC to triplet via ${}^{1}n_{N}\pi^{*}_{min}$ constitutes the main relaxation pathway in me6-TG. Although this Φ_{T} is inferior to its single-substituted predecessor 6-thioguanine (6-TG, 0.8 ± 0.2), the effect of thionation in synergy with methylation opens a unique C–S bond cleavage pathway through crossing to a repulsive ${}^{1}\pi\sigma^{*}$ state, generating thiyl radicals as highly reactive intermediates that may invoke biological damage. This photodissociation channel is extremely difficult for conventional nucleobases. These findings demonstrate the synergistic effects of double functionality substitution in modulating excited-state dynamics and enhancing the photolabile character of DNA nucleobases, providing inspirations for the rational design of advanced photodynamic and photochemotherapy approaches.

KEYWORDS: excited-state dynamics, electronic structure, conical intersection, photoreactivity, double functionality substitution, nucleobase

INTRODUCTION

Enhancing the UV sensitivity of nucleic acid bases is essential for the development of effective sensitizers for photodynamic therapy $(PDT)^{1-3}$ of cancer, which can accelerate DNA damage processes and cell death upon exposure to ionizing radiation or UV photolysis. In general, the canonical nucleobases absorb maximally at around 260 nm and their excited-state lifetimes in solution are known to be in the subpicosecond range.⁴⁻⁶ Such highly efficient nonradiative decay to the electronic ground state (S_0) significantly reduces the possibility of DNA photodamage. Surprisingly, modified nucleobases introduce a longer-wavelength-absorbing chromophore and can be incorporated into native DNA as photosensitizers without affecting the biological function. They exhibit remarkable photoreactivity and can cause selective DNA damage to malignant cells or pathogens, thus offering promising applications in PDT treatment of cancer or viruses.7

A representative example of nucleobase substitution is thionucleobases, which arise from the substitution of the carbonyl oxo group by the thio group for both purines and pyrimidines as 4-thiothymine (4-TT) and 6-thioguanine (6-TG). In contrast to the common nucleic acid bases, their absorption spectra show a significant red shift from the UVC (200-280nm) and UVB (280-320 nm) region to the UVA (320-400nm) region. Furthermore, the sulfur substitution inhibits the ultrafast internal conversion to the ground state and dramatically increases the rate of intersystem crossing (ISC) to the triplet manifold due to the strong heavy-atom effect,

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resulting in near-unity triplet yields.¹¹⁻¹⁴ The long-lived, reactive triplet states with high quantum yields can subsequently generate singlet oxygen $({}^{1}O_{2})$ by triplet-triplet energy transfer,^{15–17} which is the main cytotoxic substance of the type II photochemical reaction in PDT.¹⁻³ Methylsubstituted nucleobases have also been extensively studied, such as 5-methylated cytosine (5mC),^{18,19} 6-methylated adenine (6mA),²⁰ and 6-methylated guanine (6mG).^{21,22} The singlet excited-state decay processes for methyl derivatives are all remarkably slower than those of their parent compounds due to a significant energy barrier on the main nonradiative decay pathway (5mC),¹⁹ a long-lived intramolecular charge transfer state (6mA),²⁰ or the change in topology of the electronic surfaces (6mG).²¹ The existence of such a longer singlet excited-state lifetime may reduce the photostability, increasing the likelihood of photodamage within cellular DNA. However, the single sulfur or single methyl substitution only brings limited phototoxicity to DNA, presumably because their photoactivity is only associated with the generation of longerlived excited states.

There is an intriguing double functionality substitution (thionation and methylation) of the nucleic acid base, 6-methylthioguanine (me6-TG). It is generated spontaneously by the enzymatic metabolism of 6-TG in the presence of *S*-adenosyl-L-methionine (S-AdoMet) and thiopurine methyl-transferase (TPMT),^{23–26} as shown in Scheme 1. Due to its

Scheme 1. Structures of 6-TG and me6-TG and Metabolism Pathways



high miscoding potential, me6-TG may induce cell death by triggering the postreplicative mismatch repair pathway, thereby exerting its cytotoxic and mutagenic effects in vivo.^{26,27} More importantly, it was reported that me6-TG also induced the DNA damage under UV irradiation,²³ despite a low photosensitized ¹O₂ production similar to 6-TG of 0.21 ± 0.02 .^{15,16} However, the underlying precise molecular mechanism for the origin of its phototoxicity has not been elucidated. In this context, we are intrigued to explore whether the double substitution can introduce an unusual effect that modulates the photoactivity and excited-state dynamics of DNA nucleobases.

Herein, we integrate the state-of-the-art time-resolved spectroscopy methods to follow the excited-state reaction dynamics of me6-TG upon UVA photoexcitation, allowing the capture of transient intermediates in detail. The time constants and branching ratios of excited-state decay pathways have been determined. In conjunction with high-level theoretical ab initio calculations, we decipher the effects of double-substitution in shaping the excited-state potential energy surface and enhancing the photolability of DNA nucleobases. The striking feature of dual fluorescence decay dynamics due to emission of ${}^{1}\pi\pi^{*}$ and ${}^{1}n_{N}\pi^{*}$ has been observed, confirming the calculated double potential well character. Interestingly, we discover that the methyl substitution of the C=S bond in me6-TG tunes the active orbitals participating low-lying excited states and the "dark" state becomes the ${}^{1}n_{N}\pi^{*}$ character instead of ${}^{1}n_{S}\pi^{*}$, allowing the ${}^{1}n_{N}\pi^{*}$ state to act as an important gateway to

form the long-lived triplet state. The ${}^{1}n_{N}\pi^{*}$ state results in much smaller SOC and slower intersystem crossing but still with a triplet quantum yield $\Phi_{\rm T}$ of 0.6 \pm 0.1 owing to the initially preferential bifurcation to ${}^{1}n_{N}\pi^{*}_{min}$ (3.36 ± 0.15 ps) rather than ${}^{1}\pi\pi^{*}_{min}$ (5.05 ± 0.26 ps) in the potential energy surfaces (PESs). Although this Φ_{T} is inferior to its singlesubstituted predecessor 6-thioguanine (6-TG, 0.8 ± 0.2),¹² the effect of thionation in synergy with methylation opens a unique C-S bond cleavage pathway through a repulsive ${}^{1}\pi\sigma^{*}$ state, generating thiyl radicals as highly reactive intermediates that may invoke successive biological damage. This photodissociation channel is extremely difficult for conventional nucleobases but has been successfully achieved here by doublefunctionality substitution. These results denote clearly a detailed and comprehensive picture for understanding the prosperous photoreactivity brought by the double substitution of sulfur and methyl, which is unique to me6-TG.

MATERIALS AND METHODS

Materials

6-Methylthioguanine (me6-TG, 99.7%, Sigma-Aldrich), 6-thioguanine (6-TG, 98%, TCI), 1,1'-binaphtyl (BN, Damas-beta), and benzophenone (BP, TCI) were used as purchased. High purity water and acetonitrile (99.9%) were used as solvents.

Steady-State UV Absorption Spectra

The steady-state UV absorption spectra were measured using a double-beam spectrometer (model U-3010, Hitachi). Each measurement was collected from 400 to 200 nm at a scan rate of 600 nm/min. The spectrum of a blank sample containing only ultrapure water was used as the background, which was subtracted from the averaged spectra. All measurements were performed in 1 cm quartz cuvettes at ambient temperature.

Steady-State Emission Spectra

The steady-state emission spectra were recorded using a spectrofluorometer (F4500, HITACHI) in 1 cm path length quartz cuvettes at ambient temperature.

Time-Resolved Laser Flash Photolysis

Nanosecond transient absorption spectra were measured using a flash photolysis setup, an Edinburgh LP980 spectrometer (Edinburgh Instruments Ltd.) combined with a Nd:YAG laser (Spectra-Physics Lab 170, Newport Corp.). Each measurement was performed in a 1 cm path length quartz cuvette at room temperature. The sample was excited by a 325, 320, or 310 nm laser pulse (1 Hz, 10 mJ/pulse/cm², fwhm \approx 7 ns). The analyzing light came from a 150 W pulsed xenon lamp. A monochromator equipped with a photomultiplier to collect the spectral range from 300 to 800 nm was used to analyze the transient absorption spectra. The signals from the photomultiplier were displayed and recorded as a function of time on a 100 MHz (1.25 Gs/s sampling rate) oscilloscope (Tektronix, TDS 3012C), and the data were transferred to a personal computer. The data were analyzed using the online software of the LP980 spectrophotometer. The fitting quality was judged by weighted residuals and the reduced χ^2 value.

Femtosecond Transient Absorption Spectra

The femtosecond transient absorption spectra were measured using a femtosecond transient absorption spectrometer (Harpia-TA, Light Conversion). The fundamental pulses (800 nm, 40 fs, 200 nJ, 1 kHz) were generated with a Ti:Sapphire laser system (Coherent Astrella) and split by a beam splitter to generate the pump and probe beams. A small fraction of the fundamental pulses was sent to a computercontrolled optical delay line and then focused onto a CaF_2 plate to generate the white light continuum probe beam. The rest of the fundamental pulses were sent to the optical parametric amplifier (TOPAS-C, Coherent Inc.) to generate the 330 nm pump beam,



Figure 1. (a) UV–vis absorption spectra of the me6-TG (40 μ M, black) and 6-TG (40 μ M, red) in H₂O. (b) Fluorescence spectra of me6-TG and 6-TG in H₂O (λ_{ex} at the maximum absorption wavelength). Inset: photographs taken under UVA light excitation. (c) Fluorescence spectra of me6-TG in H₂O at different excitation wavelengths (270–345 nm).

which was chopped at 500 Hz. To avoid anisotropic signals, the polarizations of pump and probe beams were set to the magic angle (54.7°) in all measurements. The focused signal and pump beams were overlapped into a fused silica cuvette with a 1 mm beam path length. The frequent movement of the electrically driven sample holder ensures that the sample in the probed volume was continuously renewed to avoid re-excitation of the excited volume by successive laser pulses, similar to the function of a magnetic stirrer. Moreover, considering the relatively low laser frequency of 1 kHz operated in our experiments, there is negligible thermal effect, especially for solutions. The absorption spectra and fluorescence spectra of the solutions before and after the laser experiments were consistent with each other (Figure S1). The OD at 330 nm of the sample solution was maintained at ~ 0.5 . All experiments were carried out at room temperature. The differential absorbance ΔA (t, λ) obtained from the femtosecond transient absorption spectra as a function of wavelength and time delay was analyzed using the Glotaran program.²⁸

Time-Resolved Fluorescence Spectra

The time-resolved fluorescence spectra were measured using a highresolution streak camera system: Universal Streak Camera (C10910-05, Hamamatsu) combined with a CMOS camera (C13440-20CU) and a spectrometer (HRS-300-S). The camera had two time sweep regimes: fast (minimum duration, 70 ps) and slow (minimum duration, 1 ns). The maximum temporal resolution of the streak camera in the single-pulse regime was 1.37 ps. In our experiments, the time-resolved fluorescence spectra and dynamics on the 20 ns time scales were collected through the slow sweep regimes. The fs 330 nm pump beam was generated from the optical parametric amplifier (TOPAS-C, Coherent Inc.). The fundamental pulse (800 nm, 40 fs, and 1 kHz repetition rate) was generated with a Ti:Sapphire laser system (Coherent Astrella). The sample solutions were in a 1 mm fused silica cuvette, and the sample cell was continuously moved to avoid photodamage. The absorption and fluorescence spectra of the solutions before and after the laser experiments were consistent with each other (Figure S1).

HPLC-ESI-Q-TOF-MS

The samples (500 μ M) were analyzed by HPLC coupled to TIMS-QTOF MS (Bruker Daltonics Inc., Billerica, MA). The separation column was a Hypersil GOLD column (100 × 2.1 mm, 1.9 μ m) from Thermo. Optimum separation was achieved with a binary mobile phase (50 mM acetic acid:acetonitrile = 80:20 in volume) with a flow rate of 0.2 mL/min. Typical ESI operating conditions were 4500 V capillary voltage, 500 V end plate offset, 1.8 bar nebulizer pressure, 8.0 L/min dry gas, and 220 °C dry temperature. The pressure in the TOF region was less than 1.0 × 10⁻⁷ mbar. Calibration of the mass spectrometer was performed using an ESI-L Low Concentration Tuning Mix (G1969-85000).

Theoretical Calculations

The stationary geometries and intersections of 6-TG and me6-TG were optimized in the five lowest electronic states (S_0, S_1, S_2, T_1) and

 T_2) and six lowest electronic states (S₀, S₁, S₂, T₁, T₂, and T₃), respectively, by using of the state-averaged complete active space selfconsistent field (SA-CASSCF) method,^{29–31} with the active spaces of 12 electrons in 9 orbitals and 14 electrons in 11 orbitals for 6-TG and me6-TG, respectively. The graphical representations of the active orbitals are shown in Figure S10 and Figure S12. Frequency calculations were performed at the same theory level to confirm the nature of the stationary points. Based on the above CASSCF optimized structure of ${}^{1}n_{N}\pi^{*}_{min}$, $\pi\pi^{*}_{min}$, and ${}^{3}\pi\pi^{*}$ of me6-TG, the corresponding transient absorption spectra for these states are calculated at the level of TD-B3LYP/PCM/6-311G** for 30 lowest excited singlet states broadened by Gaussian line shape with 0.25 eV full-width at half-maximum. The polarized continuum model (PCM) is used to simulate the solvation effects (in water) in TD-DFT calculations.

To account for dynamic electron correlation and solvent effects, the single-point energies of all CASSCF optimized structures were obtained at the multistate second-order perturbation theory (MS-CASPT2)^{32,33} level by using the polarizable continuum model (PCM) on the basis of the CASSCF wave functions, referred to as MS-SA-CASPT2//CASSCF//PCM hereafter, and the relative energies of involved states for all stationary and internal/intersystem conversion funnels are shown in Tables S2 and S4. An imaginary shift of 0.2 au was applied to avoid intruder state issues, and the ionization potential electron affinity (IPEA) shift was set to be zero. The basis sets of 6-311G** and cc-pvtz are applied in the CASSCF and MS-CASPT2 calculations, respectively. The energy gaps between the electronic states involved in conical intersections and intersystem crossings are less than 0.1 eV at the MS-CASPT2//CASSCF//PCM level, except that S_1/S_0 for 6-TG is around 0.3 eV and T_2/T_1 for me6-TG is around 0.2 eV, which is acceptable, as shown in Table S2 and Table S4.

To obtain the photodissociation (radical production) potential energy profile, we performed a relaxed scan on the adiabatic S₁ state (constrained optimization of all other coordinates while keeping the C–S distance fixed) at the SA3-CAS(14,11)/6-311G** level. The scan starts from the FC point with a certain step size. The energies of each optimized geometry are further calculated at the MS-SA3-CASPT2(14,11)/cc-pvtz//PCM (in water) on the basis of the SA3-CAS(14,11) wave functions to include the dynamic electronic correlation energy and solvent effect.

All of the optimization calculations at the CASSCF level were performed by using of the MOLPRO 2012.1 package,³⁴ while the single-point energy calculations at the MS-CASPT2 level with the PCM model were carried out by the MOLCAS 8.0 package.³⁵ Besides, the spin–orbit coupling (SOC)^{36–38} between the singlet and triplet states for 6-TG and me6-TG was calculated at the MS-CASPT2//CASSCF//PCM levels in MOLCAS 8.0. MEPs were calculated using mass weighted coordinates and a hypersphere radius of 0.1 atomic units as implemented in the MOLCAS 8.0 package based on the CASSCF//PCM level. The definition of the key dihedral angles of 6-TG and me6-TG is shown in Scheme S1.

Table 1. Photophysical Properties of me6-TG and 6-TG

	$\lambda_{ m abs}/ m nm$		$\lambda_{ m FL}/ m nm$		$\Phi_{ extsf{FL}}{}^a$	
solvent	H ₂ O	CH ₃ CN	H ₂ O	CH ₃ CN	H ₂ O	CH ₃ CN
me6-TG	310	307	404 ^b	398 ^b	0.071 ± 0.014^{b}	0.073 ± 0.013^{b}
6-TG	341	346	с	с	с	с

^aFluorescence quantum yield estimated with quinine bisulfate⁴⁰ as the standard ($\Phi_{FL} = 0.540$ in 0.050 M sulfuric acid solution). ^b λ_{ex} is 310 nm. ^cNot observed.



Figure 2. (a) Potential energy profile for the excited-state relaxation of 6-TG calculated at the MS-SA5-CASPT2(12,9)/PCM/cc-pvtz//SA5-CASSCF (12,9)/6-311G** level. (b) Left panel: Potential energy profile for the excited-state relaxation of me6-TG, calculated at the MS-SA6-CASPT2(14,11)/PCM/cc-pvtz//SA6-CASSCF (14,11)/6-311G** level. Right panel: photodissociation potential energy profile obtained by a relaxed scan along C–S bond elongation on the adiabatic S₁ state, where the change of electronic excitation nature is depicted with different colors ($\pi \rightarrow \pi^*$ in red, $\pi \rightarrow \pi^*/\sigma^*$ in pink, and $\pi \rightarrow \sigma^*$ in orange), at the level of SA3-CASPT2(14,11)/cc-pvtz//PCM//SA3-CAS(14,11)/6-311G**. The relative energies are given in eV. The reaction coordinates of φ_1 and φ_2 correspond to the dihedral angle involving the exocyclic S-CH₃ rotation and the dihedral angle involving ring bending as shown in Scheme S1, respectively.

The geometry of the photodissociation product (6-TG[•]) was calculated by B3LYP/PCM/6-311++g (d, p), and its absorption spectrum was calculated by TD-B3LYP/PCM/6-311++g (d, p). All of the DFT and TD-DFT calculations were performed with the Gaussian 09 program package.³⁹

RESULTS AND DISCUSSION

Steady-State Spectral Properties and Excited-State Electronic Structure Calculations

The steady-state spectra were measured for me6-TG in comparison to those for 6-TG (Figure 1 and Figure S2) for understanding the optical properties. The characteristic spectral parameters are summarized in Table 1. The lowestenergy absorption band of 6-TG has a maximum at 341 nm and an extinction coefficient of (2.3×10^4) M⁻¹·cm⁻¹ at the same wavelength in aqueous solution, whereas me6-TG exhibits an absorption maximum at 310 nm with an extinction coefficient $(1.2 \times 10^4) \text{ M}^{-1} \cdot \text{cm}^{-1}$.²³ Obviously, the methyl substitution of the S6 site in 6-TG results in absorption hypsochromic shifts. In the emission spectra, no fluorescence of 6-TG is observed with excitation at 340 nm, indicating that the quantum yield of fluorescence ($\Phi_{\rm FI}$) of 6-TG is quite low, in agreement with a previous study.¹² In sharp contrast, me6-TG emits bright fluorescence emission at 404 nm in H₂O upon 310 nm excitation (Figure 1b), and the Φ_{FL} is determined to be 0.071 ± 0.014 using quinine bisulfate⁴⁰ as a reference standard (Table 1, Table S1). The emission is further confirmed as fluorescence instead of phosphorescence by a

deoxygenation experiment (Figure S3a). The excitation spectra collected at the 404, 450, and 500 nm emission wavelengths all satisfactorily match the absorption spectrum of me6-TG (Figure S3b). This provides evidence that the fluorescence emission is an intrinsic property of me6-TG. Notably, the fluorescence band of me6-TG shows a long-tail covering the entire visible region, hinting the possibility of dual fluorescence. Figure 1c presents the dependence of the fluorescence emission spectrum of me6-TG in H₂O on the excitation wavelength. As the excitation wavelength is increased from 270 to 345 nm, the fluorescence maximum is gradually red-shifted from 396 to 406 nm. Such a progressive evolution implies that the fluorescence emission may be contributed by two luminescence states. The above spectral differences between me6-TG and 6-TG suggest that the photophysical pathways in the excited state have been altered by S6-methylation.

To aid the interpretation of the above steady-state and the following time-resolved spectra, we mapped out the low-lying excited-state potential energy surfaces (PESs) by high-level *ab initio* calculations (see the Supporting Information for computational details) for me6-TG. For comparison, calculations were also performed for 6-TG. All optimized structures and calculated energies of the minima and intersections for low-lying states are shown in Figures S9–15 and Table S2–S6, and the PESs are shown in Figure 2.

For 6-TG, the obtained results are basically consistent with previous reports,^{13,14} although we used different active spaces



Figure 3. (a) Two-dimensional spectra of me6-TG ($40 \mu M$) at 330 nm excitation in pH 7.0 phosphate buffer. (b) Fluorescence decays of me6-TG ($40 \mu M$) at different wavelengths after excitation at 330 nm in buffer solutions. (c) Fluorescence decays of me6-TG ($40 \mu M$) at 404 nm in buffer after excitation at 330 nm (red) and 310 nm (black).

and basis sets. The calculations show that 6-TG exists a "bright" S₂ state with a $\pi\pi^*$ character at the Franck–Condon point $({}^{1}\pi\pi^{*}_{FC})$, having a considerable oscillator strength value (f) of 0.85. The vertical excitation energy from the S_0 state to the $S_2({}^{1}\pi\pi^*_{FC})$ is predicted to be 3.92 eV, which is in good agreement with the experimentally observed absorption maxima of 340 nm (3.65 eV, Figure 1a). Two different deactivation pathways from the $S_2({}^1\pi\pi^*_{FC})$ state can be predicted, which have in common the initial barrierless deactivation via the conical intersection (CI) S₂ $({}^{1}\pi\pi^{*})/$ $S_1(n\pi^*)$ (Figure 2a). This CI distributes the population into two different minima of the S₁ potential, with ${}^{1}\pi\pi^{*}$ and ${}^{1}n_{S}\pi^{*}$ characters (the left and right panel of Figure 2a). Along the ${}^{1}\pi\pi^{*}$ state, the CI S₁(${}^{1}\pi\pi^{*}$)/S₀ is close to the ${}^{1}\pi\pi^{*}$ minimum $({}^{1}\pi\pi^{*}_{min})$ structurally and energetically (Figure S9, Table S2), which makes the primary relaxation pathway of ${}^{1}\pi\pi^{*}_{min}$ to be the nonradiative decay to the ground state, rationalizing the absence of fluorescence of 6-TG. Along the ${}^{1}n_{s}\pi^{*}$ state, there is an energetically favorable ${}^{1}\mathrm{n}_{\mathrm{S}}\pi^{*}/{}^{3}\pi\pi^{*}$ crossing point, which is only 0.03 eV higher than that of the ${}^{1}n_{s}\pi^{*}{}_{min}$. Moreover, the large spin-orbit coupling (SOC) value of 95.0 cm⁻¹ for ${}^{1}n_{s}\pi^{*}/{}^{3}\pi\pi^{*}$ should result in favorable ISC from singlet to triplet manifold. It should be pointed that the ${}^{1}n_{s}\pi^{*}$ state of 6-TG mainly involves an excitation from the lone pair on the S6 atom to a π^* orbital (thus labeled as ${}^{1}n_{s}\pi^*$), which is a dark state with an extremely small oscillator strength (absorption fof 0.00012 and emission f of 0.000039). Compared to the S₀ structure, the C-S bond length is remarkably elongated in the ${}^{1}n_{S}\pi^{*}$ states (0.15 Å, see Figure S9). The ${}^{1}n_{S}\pi_{min}^{*}$ energy is lowered to be 2.84 eV, which is 0.74 eV more stable than the ${}^{1}\pi\pi^{*}_{min}$ state (3.58 eV). Therefore, excited-state depopulation to ${}^{1}n_{S}\pi^{*}{}_{min}$ and subsequent ISC to the triplet state ${}^{3}\pi\pi^{*}$ is the main relaxation pathway of 6-TG, which is more favorable than the internal conversion from ${}^{1}\pi\pi^{*}_{min}$ to the ground state. The ISC time constant of 6-TG was reported to be \sim 0.3 ps with a triplet quantum yield of ~ 0.8 .¹⁷

For me6-TG, the potential energy profiles are shown in the left panels of Figure 2b. The calculations reveal that the vertical excitation energy of S₀ to S₁ is 4.16 eV with f = 0.11, while that of S₀ to S₂ is 4.39 eV with f = 0.013 (Table S4). Apparently, the excitation of me6-TG populates the S₁ (${}^{1}\pi\pi^{*}$) state in the FC region (${}^{1}\pi\pi^{*}_{FC}$) directly, rather than the S₂ state as in 6-TG. Indeed, me6-TG exhibits an absorption band with a maximum at 310 nm (4.00 eV) in the UV–vis absorption spectrum (Figure 1a), which coincides with the calculated S₁ vertical excitation energy (4.16 eV). This energy is higher than the bright state S₂(${}^{1}\pi\pi^{*}_{FC}$) of 6-TG (3.92 eV), explaining the blue shift of absorption maxima of me6-TG relative to 6-TG.

For me6-TG after the initial population in the Franck-Condon point ${}^{1}\pi\pi^{*}_{FC}$, two local state minima of ${}^{1}\pi\pi^{*}_{min}$ (3.68) eV) and ${}^{1}n_{N}\pi^{*}_{min}$ (3.76 eV) as well as the conical intersection between these two states ${}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*}$ (3.90 eV) have been located. Two decay pathways can be branched through the CI ${}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*}$, depopulating me6-TG to ${}^{1}\pi\pi^{*}_{min}$ and ${}^{1}n_{N}\pi^{*}_{min}$ and showing the characteristic of a double potential well. It is interesting that there is no amenable S_1/S_0 intersections found for both the ${}^{1}\pi\pi^{*}$ and ${}^{1}n_{N}\pi^{*}$ states of me6-TG as that for 6-TG; therefore, the chances of fluorescence emission are greatly increased for me6-TG. This explains the observation of bright fluorescence for me6-TG. In fact, the emission wavelength of $\pi \pi \pi^*_{\rm min}$ is calculated to be 391.0 nm (3.17 eV), which is close to the experimental fluorescence peak wavelength of 404 nm (3.07 eV). Moreover, the calculated emission oscillator strength values (f) are 0.10 and 0.0071 for ${}^{1}\pi\pi^{*}_{min}$ and ${}^{1}n_{N}\pi^{*}_{min}$, respectively (Table S4). Unlike 6-TG (f = 0.000039, Table S2), the ${}^{1}n_{N}\pi^{*}$ state of me6-TG has a substantial emission oscillator strength (0.0071). This feature may render the ${}^{1}n_{N}\pi^{*}$ state of me6-TG a weak fluorescence emitter. The calculated emission wavelength of ${}^{1}n_{N}\pi^{*}_{min}$ is 435 nm (2.85 eV).

After depopulating to ${}^{1}n_{N}\pi^{*}_{min}$, there may exist another competing pathway of the relaxation to the triplet manifold. According to the calculated energies, the energy gaps of the ${}^{1}n_{N}\pi^{*}$ state (3.76 eV) with three triplet states T₁ (${}^{3}n\pi^{*}$, 3.45 eV), T₂ (${}^{3}\pi\pi^{*}$, 3.93 eV), and T₃ (${}^{3}\pi\pi^{*}$, 4.51 eV) states are 0.31, 0.17, and 0.75 eV, respectively. The ${}^{1}n_{N}\pi^{*}-T_{3}$ ISC process is less favorable due to the large energy gap. There is a singlet/triplet crossing S₁(${}^{1}n_{N}\pi^{*}$)/T₂ (${}^{3}\pi\pi^{*}$) with an SOC value of 8.3 cm⁻¹, which may result in ISC of S₁ \rightarrow T₂. The T₂ (${}^{3}\pi\pi^{*}$) state should then decay to the T₁ (${}^{3}n\pi^{*}$) state via internal conversion. According to the El-Sayed propensity rules, direct ISC from the S₁(${}^{1}n_{N}\pi^{*}$) state to the T₁ (${}^{3}n\pi^{*}$) state is less likely.

Time-Resolved Fluorescence Spectroscopy Monitoring the Dual Fluorescence Dynamics of me6-TG

After predicting the possible excited-state relaxation pathways on the basis of theoretical calculation, we perform systematic ultrafast time-resolved spectroscopy to examine the evolving photophysical and photochemical dynamics of me6-TG as below. First, the dual emission of me6-TG can be clearly discerned in the time-resolved fluorescence spectra (Figure 3). The longer wavelength UVA excitation of 330 nm was used to prevent possible photochemical pathways under shorter wavelength. In the two-dimensional fluorescence spectra (Figure 3a), while the fluorescence intensity decays with time, a spectral change of gradual red shift from 404 to 420 nm is observed, indicating that the fluorescence emission may be contributed by two luminescence states with different emission wavelengths. The kinetic traces for representative fluorescence wavelength are illustrated in Figure 3b. Indeed, the kinetic analysis of the decay profiles shows complex dynamics requiring biexponential fitting (Figure S4), containing two components with time constants in the hundreds of picoseconds ($\tau_1 = 397 \pm 21$ ps) and a few nanoseconds ($\tau_2 = 3.04 \pm 0.20$ ns) (Table 2). It is also clear that the relative amplitude

 Table 2. Dynamic Parameters Obtained by the Global

 Fitting of Time-Resolved Fluorescence Spectra of me6TG

λ_{ex} (nm)	$\lambda_{\mathrm{flu}} \; (\mathrm{nm})$	$\tau_1 \ (ps)$	A_1 (%)	τ_2 (ns)	A_2 (%)
330	375	397 ± 21	83.4	3.04 ± 0.20	16.6
	404		83.1		16.9
	475		80.3		19.7
310	404	382 ± 20	91.1	2.94 ± 0.19	8.9

of τ_1 decreases while that of τ_2 increases as the fluorescence wavelength or excitation wavelength shifts to the red (Table 2 and Figure 3b,c), indicating that τ_2 should originate from a lower-energy excited state with a longer wavelength emission. Our calculations above indicate that in addition to the bright state emission of ${}^{1}\pi\pi^{*}_{min}$, the ${}^{1}n_{N}\pi^{*}_{min}$ could emit weak fluorescence. Moreover, the calculated emission wavelengths of 391 nm (3.17 eV) for ${}^{1}\pi\pi^{*}_{min}$ and 435 nm (2.85 eV) for ${}^{1}n_{N}\pi^{*}{}_{min}$ agree well with the time-resolved emission spectra (red shift from 404 to 420 nm along with the fluorescence decay). Therefore, these two lifetime emissive states can be assigned to ${}^{1}\pi\pi^{*}$ (τ_{1}) and ${}^{1}n_{N}\pi^{*}$ (τ_{2}). Owing to the larger oscillation strength, the ${}^{1}\pi\pi^{*}_{min}$ state (f = 0.10) is a more efficient radiative state than the ${}^{1}n_{N}\pi^{*}_{min}$ state (f = 0.0071), as indicated by the larger amplitude of τ_1 than τ_2 (Table 2). The possibility of the weak emissive state attributed to the intramolecular charge transfer (ICT) state as that for 6 mA¹⁸ has been ruled out since our high-level ab initio calculations did

not locate any structure with an ICT character. Similar emission behavior due to the ${}^{1}n\pi^{*}$ states has also been reported for 9-methylpurine⁴¹ and 8-azaadenine.⁴² Likewise, the ${}^{1}n_{N}\pi^{*}$ state of me6-TG here cannot be considered as a traditional "dark" state.

Femtosecond Transient Absorption Spectroscopy Monitoring the Ultrafast Excited State Relaxation Dynamics of me6-TG

The above time-resolved fluorescence spectroscopy only provides information related to two emissive states. To elucidate the holistic excited-state picture, fs transient absorption (fs-TA) spectroscopy of me6-TG was performed further. Figure 4a-d shows the fs-TA contour plots and transient absorption spectra at typical time delays of me6-TG in phosphate buffer after 330 nm photoexcitation. Immediately after the pump pulse, two broad excited-state absorption (ESA) bands are developed at 410 and 510 nm (Figure 4b), both peaking within the instrumental response time (~ 200 fs). These positive bands in the initial time 0.16 ps should correspond to the ESA of the initially populated ${}^{1}\pi\pi^{*}_{FC}$ state. Then, the ${}^{1}\pi\pi^{*}_{FC}$ features decay in the following 10 ps, accompanied by a gradual red-shift from 510 to 550 nm, corresponding to the appearance of new excited species 2 (Figure 4b). Thereafter, from 10 to 800 ps, these two bands at 410 and 550 nm continue to decay, with the relative strength of the 410 nm band becoming greater than that of 550 nm (Figure 4c). This suggests that the excited species 2 are transformed into another species 3. After 800 ps, excited species 3 further decay to form the eventual long-lived species with an ESA band peaking at 410 nm and a flat absorption from 460 to 700 nm (Figure 4d). These eventual ESA bands at 7 ns (shape and peak position) closely match the triplettriplet absorption bands of the T₁ state of me6-TG measured by the nanosecond laser flash photolysis (Figure S5), indicating their assignment to the T₁ state.



Figure 4. (a) 2D broadband and (b–d) fs-transient absorption spectra of me6-TG (400 μ M, path length: 1 mm) at 330 nm excitation at typical delay times in pH 7.0 phosphate buffer. (e) Time traces at typical wavelengths (circles) and fits (lines) from global analysis of the fs-transient absorption spectra of me6-TG. (f) SADSs of the corresponding kinetic components obtained from global analysis of the ultrafast transient absorption spectra for me6-TG. Concentration of transients as a function of delay time plotted in Figure S6.

On the basis of the calculated excited-state potential energy profiles (Figure 2b), the ESA bands for intermediate species 2 and 3 in the fs-TA spectra can be assigned. After initially populating in the ${}^{1}\pi\pi^{*}_{FC}$ state, me6-TG passes through the conical intersection (CI) ${}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*}$, leading to two competing decay processes, populating at ${}^{1}\pi\pi^{*}_{min}$ and ${}^{1}n_{N}\pi^{*}_{min}$. Therefore, the ESA bands at 410 and 550 nm (10 ps) for species 2 may correspond to the features of the ${}^{1}\pi\pi^{*}_{min}$ state overlapping with the ${}^{1}n_{N}\pi^{*}_{min}$ state whose absorptions are in the same region. Subsequently, the ${}^{1}\pi\pi^{*}_{min}$ state should decay to the ground state with a lifetime of \sim 397 ± 21 ps as measured by time-resolved fluorescence spectroscopy. During hundreds of picoseconds, the ${}^{1}n_{N}\pi^{*}{}_{min}$ state (with a lifetime \sim 3.04 ± 0.20 ns as determined by time-resolved fluorescence spectroscopy) is not fully relaxed, so the ESA bands for the species 3 at 800 ps should belong mainly to the ${}^{1}n_{N}\pi^{*}_{min}$ state. Then, the ${}^{1}n_{N}\pi^{*}{}_{min}$ state should serve as a gateway leading to ISC to triplet manifold through ${}^{1}n_{N}\pi^{*}/{}^{3}\pi\pi^{*}$ surface intersections as discussed in the calculation. The radiative decay to ground state contributing to the ${}^{1}n_{N}\pi^{*}$ decay is minor due to the small f(0.0071). So, the spectral evolution from 800 ps to 7 ns mainly corresponds to the triplet state formation process.

As can be seen in the potential energy profiles (Figure 2b left panel), the photophysical relaxation from ${}^{1}\pi\pi^{*}{}_{FC}$ state involves bifurcation to ${}^{1}\pi\pi^{*}_{min}$ and ${}^{1}n_{N}\pi^{*}_{min}$ and there are competing pathways from one initial state. Therefore, a target model involving four time constants $(\tau_1 - \tau_4)$ as plotted in Scheme S2 is used to perform global analysis of the fs-TA spectra, yielding species associated difference spectra (SADS) as shown in Figure 4f. The representative kinetic traces with fittings are shown in Figure 4e, showing the satisfactory fitting to experimental data. The first time constant τ_1 (3.36 ± 0.15 ps) corresponds to the decay of the ${}^{1}\pi\pi^{*}_{FC}$ state to the ${}^{1}n_{N}\pi^{*}_{min}$ state. The second time constant τ_{2} (5.05 \pm 0.26 ps) represents the decay of the ${}^{1}\pi\pi^{*}_{FC}$ state to the ${}^{1}\pi\pi^{*}_{min}$ state. The third time constant $(372 \pm 17 \text{ ps})$ corresponds to the decay of the ${}^{1}\pi\pi^{*}_{min}$ state to the ground state. The last time constant τ_4 (2.91 \pm 0.14 ns) is attributed to the ISC decay of the ${}^{1}n_{N}\pi^{*}{}_{min}$ state to triplet state ${}^{3}\pi\pi^{*}$. It is noticeable that the derived decay lifetimes of ${}^{1}\pi\pi^{*}_{min}$ (372 ± 17 ps) and ${}^{1}n_{N}\pi^{*}_{min}$ $(2.91 \pm 0.14 \text{ ns})$ states are both consistent with those determined by fluorescence measurements above $({}^{1}\pi\pi^{*}{}_{\min})$ state: 397 \pm 21 ps; ${}^{1}n_{N}\pi^{*}_{min}$: 3.04 \pm 0.20 ns), which in turn demonstrates the reliability of the global fitting model.

To further support the assignments of the transient absorption species and relaxation mechanism, the absorption spectra of the ${}^{1}n_{N}\pi^{*}{}_{min}$, ${}^{1}\pi\pi^{*}{}_{min}$, and ${}^{3}\pi\pi^{*}$ excited states of me6-TG were calculated using the TD-B3LYP/PCM/6-311G** method (Figure S13). In the energy range between 1.77 and 3.54 eV (350 and 700 nm), the theoretical spectrum shows two absorption bands, with maxima centered around 420 and 510 nm. The major difference for the calculated spectra of the ${}^{1}\pi\pi^{*}_{min}$ state and ${}^{1}n_{N}\pi^{*}_{min}$ state is that the former state has stronger absorption intensity at 510 nm than that at 420 nm and the latter state is the opposite. This feature reproduces well the SADS spectra derived from the global fit of fs-TA experiments (Figure 4f). Moreover, the calculated spectra for the ${}^{3}\pi\pi^{*}$ state agree with experiments, exhibiting only one absorption band centered around 420 nm and a flat absorption from 500 to 700 nm.

The time constants derived from global fit indicate that the excited-state bifurcation to the ${}^{1}n_{N}\pi^{*}_{min}$ state (3.36 ± 0.15 ps)

is predominant over that to the ${}^{1}\pi\pi^{*}_{min}$ state (5.05 ± 0.26 ps) kinetically. Therefore, the subsequent formation of triplet states via the ${}^{1}n_{N}\pi^{*}$ state should still play important roles, although the ISC time constant (\sim 2.91 ± 0.14 ns) is much slower than that of 6-TG (\sim 0.3 ps¹²). The lowest triplet state is thought to act as a long-lived reactive precursor, which may incur severe consequences for the DNA photochemistry by participating in reactions with canonical nucleic acids or amino acids and triplet-energy transfer to generate singlet oxygen $({}^{1}O_{2})$. Therefore, the quantum yields of this long-lived triplet state $(\Phi_{\rm T})$ of me6-TG in comparison to 6-TG are determined further. Details can be found in the SI (Figure S7). Indeed, the $\Phi_{\rm T}$ of me6-TG (0.6 \pm 0.1) is only slightly smaller than that of 6-TG (0.8 \pm 0.2) but still high. Furthermore, it should be noted that the quantification of the Φ_{T} is one of the key parameters to evaluate the efficacy of a potential photo-sensitizer for PDT applications.⁴³ To the best of our knowledge, this is also the first work to report the triplet quantum yield of me6-TG following UVA excitation.

The above measured time constants and branching ratios of excited-state decay pathways can be rationalized further by theoretical calculations. The optimization in S₁ PES starting from FC geometry with the aid of the steepest decent method leads to $CI(S_2/S_1)$ finally, which implies that decaying to the $CI({}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*})$ region from FC is barrierless. Moreover, the minimum energy path (MEP) search is employed to compute the steepest descendant minimum energy reaction path starting from $CI({}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*})$ on S₁ PES. As shown in Figure S14, it finally leads to $S_1({}^{1}n_N\pi^*)$ minimum, which suggests that forming $S_1({}^{1}n_N\pi^*)$ minimum is the most straightforward and efficient. As shown in Figure 2b, the decay from (CI) ${}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*}$ to ${}^{1}n_{N}\pi^{*}{}_{min}$ takes place along mainly the reaction coordinate φ_1 , corresponding to a structural change of φ_1 from -110.9 to -78.8° and φ_2 nearly unchanged (Figure S11). In comparison, the decay from (CI) ${}^{1}\pi\pi^{*}/{}^{1}n_{N}\pi^{*}$ to ${}^{1}\pi\pi^{*}_{min}$ involves both the reaction coordinates φ_1 and φ_2 , corresponding to a structural change of φ_1 from -110.9 to -94.2° and φ_2 from 0.3 to 7.7° (Figure S11). Structurally, the exocyclic S-CH₃ single bond rotation (change of φ_1) should be easier than the ring bending deformation (change of φ_2). Thus, the structural change from CI to ${}^{1}\pi\pi^{*}_{min}$ is more difficult than that from CI to ${}^{1}n_{N}\pi^{*}{}_{min.}$ Moreover, kinetically speaking, the initial momentum of φ_1 change carried from FC to CI is easily passed to the decay path from CI to ${}^{1}n_{N}\pi^{*}{}_{min}$ that is still along φ_{1} , whereas this momentum is to be partitioned to φ_2 in addition to φ_1 for the decay of CI to ${}^1\pi\pi^*_{\min}$. These calculation results explain why the decay of ${}^{1}\pi\pi^{*}_{FC}$ to ${}^{1}n_{N}\pi^{*}_{min}$ (3.36 ± 0.15 ps) is faster than that to ${}^{1}\pi\pi^{*}_{min}$ (5.05 ± 0.26 ps). Although these decay processes from ${}^{1}\pi\pi^{*}{}_{\rm FC}$ are barrierless, the formation time of these two state minima is still on the picosecond time scale, which is likely due to the shallow gradient from $CI(\pi\pi\pi^*/n_N\pi^*)$ to these state minima on PESs as shown in Figure 2b (small energy change). This is in contrast to the case of 6-TG (Figure 2a), where a deep gradient from CI- $({}^{1}\pi\pi^{*}/{}^{1}n_{S}\pi^{*})$ to ${}^{1}n_{S}\pi_{min}^{*}$ is involved to ensure extremely fast internal conversion (too fast to be discernible with the \sim 0.3 ps ISC).¹²

According to the active space (Figure S12), the ${}^{1}n\pi^{*}_{min}$ state of me6-TG mainly involves excitation of electrons from the n orbital of the N1 atom of the C6=N1 moiety (Scheme 1) to the π^{*} orbital on the purine ring; thus, this state is of the ${}^{1}n_{N}\pi^{*}$ character. Knowing that the presence of low atomic number atoms can reduce the transitions of different multiplicities,⁴⁴



Figure 5. Transient absorption spectrum of me6-TG (40 μ M) after laser excitation at 325 nm (a), 320 nm (c) and 310 nm (d) in deaerated water. (b) Transient absorption decays of me6-TG (40 μ M) at 410 (black) and 650 nm (red) after the 325 nm laser excitation. (e) Transient absorption decays of me6-TG (40 μ M) at 350 (black), 420 (red) and 650 nm (blue) after the 310 nm laser excitation. (f) Transient absorption spectrum of me6-TG (40 μ M) recorded at 150 μ s (blue) after 310 nm excitation. The bars show the vertical optical transitions for 6-TG[•] (black) in aqueous solution by the TD-DFT/B3LYP/PCM/6-311++g (d, p) method. (g) HPLC-MS isolation and characterization of me6-TG (top) and the final photoproducts of 6-TG after 310 photoexcitation (bottom). The sample solution was injected into an HPLC instrument and eluted with acetonitrile/acetic acid. (h) Steady-state UV-vis difference spectrum of me6-TG in water after 310 nm laser irradiation.

¹ $n_N \pi^*$ should incur a small spin-orbital coupling. In fact, the calculated SOC value for $S_1({}^1n_N\pi^*)/T_2({}^3\pi\pi^*)$ is quite small (8.3 cm⁻¹), which is much smaller than the SOC value of 6-TG (95.0 cm⁻¹) that involves the ${}^1n_S\pi^*$ state as the gateway to the triplet state. Meanwhile, for me6-TG, the singlet/triplet surface intersection $S_1({}^1n_N\pi^*)/T_2({}^3\pi\pi^*)$ lies ~0.39 eV above the ${}^1n_N\pi^*$ minimum. Thus, a substantial energy barrier is required to be surmount for the occurrence of ISC. This is in contrast to the nearly barrierless ISC of 6-TG (Figure 2a), where the ${}^1n_S\pi^*/{}^3\pi\pi^*$ crossing point is only 0.03 eV higher than that of the ${}^1n_S\pi^*_{min}$. The calculated SOC and energy barrier of singlet/triplet crossings satisfactorily explain why the measured ISC time constant of me6-TG (~3 ns) is slower than that of 6-TG (~0.3 ps¹²). On the other hand, it is because of such a slow ISC that there are some chances of emitting

fluorescence left for the ${}^{1}n_{N}\pi^{*}_{min}$ state of me6-TG, although it is in principle a "dark" state.

Altogether, owing to the preferred initial bifurcation to the ${}^{1}n_{N}\pi^{*}{}_{\min}$ potential well as discussed above, most excited-state population of me6-TG would follow the decay path along ${}^{1}n_{N}\pi^{*}$ state. While there is no amenable surface intersection found between ${}^{1}n_{N}\pi^{*}$ state and ground state and the radiative decay to the ground state by fluorescence is the minor channel due to small f (0.0071), the ISC from ${}^{1}n_{N}\pi^{*}$ to the triplet manifold becomes dominant, accounting for a quite large fractional yield of 0.6 \pm 0.1.

Nanosecond Transient Absorption Spectroscopy Monitoring C–S Bond Photodissociation Dynamics to Produce Reactive Radicals

The above experiments demonstrated that under 330 nm excitation, the double substitution of sulfur and methyl in me6-TG leads to the fluorescence emission and long-lived triplet state. In addition to these changes in photophysical properties, it is interesting to know whether there is a direct photochemical channel upon a higher photon energy excitation to break the C-S bond, forming a highly reactive radical. To investigate the possible C-S bond cleavage channel, we performed nanosecond transient absorption (ns-TA) spectroscopy at three additional shorter excitation wavelengths of 325, 320, and 310 nm. Moreover, since the lifetime of the radical generated by the photodissociation pathway is typically longlived on ns-µs time scales, ns-TA spectra measurements are therefore ideal for probing photodissociation channels, avoiding the complex spectral interference of multiple excited states on *fs-ps* time scales.

Figure 5a shows the *ns*-TA spectra of me6-TG in deaerated aqueous solution excited at 325 nm. A negative absorption band is observed at 310 nm, which is attributed to the ground-state bleaching of me6-TG in agreement with the steady-state absorption spectrum (Figure 1a). The spectrum shows positive absorptions peaking at 410 nm and a flat band from 460 to 800 nm. It is in accordance with the 7 ns *fs*-TA measurement (Figure 4d), which is assigned to the absorptions of the T_1 state. Further assignment evidence was obtained by monitoring the decay of the transient absorption spectra in N₂- and O₂-saturated conditions (Figure S8a). Through monoexponential fitting, the triplet state lifetime is determined to be 7.8 ± 0.4 μ s (Figure 5b, Table 3). No photodissociation occurs under 325

Table 3. Fitting Parameters of the ns-Transient Absorption Bands of me6-TG under 325 or 310 nm Laser Excitation

$\lambda_{\mathrm{excitation}}$ (nm)	$\lambda_{\mathrm{probe}} \ (\mathrm{nm})$	$ au_1 \ (\mu s)$	A_1 (%)	$ au_2$ (μ s)	A_2 (%)
325	410	7.8 ± 0.4	100		
	650				
310	350	7.7 ± 0.4	40.6	140 ± 4.7	59.4
	420		55.4		45.8
	650	8.0 ± 0.4	100		

nm excitation, as evidenced by the complete recovery of the ground-state bleach band (Figure 5a). Prominently, when the excitation wavelength was tuned to 320 and 310 nm, the spectra display distinct features (Figure 5c,d), especially at 310 nm excitation. Upon 310 nm excitation, the ground-state bleach only shows a partial recovery over time (Figure 5d), indicating a permanent consumption of me6-TG due to the photochemical reaction. In addition to the characteristic absorption bands of the T_1 state (a peak at 410 nm and a flat absorption from 460 to 800 nm), two narrower bands at 350 and 420 nm are observed clearly after the decay of T_1 is

Scheme 2. Photodissociation Pathway of me6-TG

complete at \sim 30 μ s. Their kinetics are concomitant and decay very slowly ($\tau \sim 140 \pm 4.7 \ \mu s$) into the millisecond time range (Figure S8b, Table 3), which is quite different from that of the triplet state at 650 nm (Figure 5e). Thus, the new transients at 350 and 420 nm are assigned to long-lived radicals that might be generated from photodissociation channels. For me6-TG, most likely, the C-S bond peripheral to the purine ring is breakable. The emergence of radical species in transient spectra as a function of excitation wavelength also indicates that the 320 nm photon energy has reached the energy threshold of me6-TG for the photodissociation. The fluorescence quantum yield of me6-TG under variable excitation wavelengths (Table S1) corroborates this energy threshold, as reflected by the two folds of higher fluorescence quantum yield obtained at 330 nm excitation than those at 290, 310, or 320 nm excitation when the C-S bond dissociation channel can compete and reduce the fluorescence.

However, there are two C–S bonds (labeled in Scheme 2) in me6-TG. Which C-S bond is more likely to be cleaved? Our calculations (Figure S11) show that the $C-S^a$ bond is ~1.80 Å in the S₀ state and decreased to ~1.75 Å in the S₁ state, and the other one $C-S^b$ is ~1.85 Å in both ground and excited states. Apparently, the C-S^a bond is stronger due to the p- π interaction, while the C-S^b bond is weaker and thus is easier to dissociate. In fact, the $C-S^a$ bond fission generates a C-centered guanine radical and CH₃-S[•] radical, whereas the C-S^b bond cleavage forms the S-centered radical (thiyl) 6-TG[•] and CH₃[•] radical. The CH₃-S[•] and CH₃[•] radicals lack absorptions above 300 nm, so the long-lived species observed in the spectrum should be either C-centered G[•] radicals or Scentered 6-TG[•] radicals. As is well-known, C-centered radicals can be quenched rapidly by O₂, with rate constants typically exceeding $10^9 \text{ M}^{-1} \text{ s}^{-1}$ in liquid solution, $^{45-48}$ whereas Scentered radicals are expected to react with O2 only very slowly or not at all.^{45,46} Hence, we performed transient experiments of me6-TG in O2-saturated aqueous solution. The transient absorption bands are also observed at 350 and 420 nm after 30 μ s (Figure S8c), which are the same as those in N₂-saturated aqueous solution, showing that oxygen cannot scavenge these radicals, thus excluding the C-centered G[•] radicals as the contributor. It also indicates that the photodissociation of me6-TG is unlikely to be related to the triplet state. Furthermore, the optical spectra of the S-centered radical (thiyl radical, 6-TG $^{\circ}$) calculated by the TD-DFT/B3LYP/PCM/6-311++g (d, p) method are in agreement with the experimental spectrum (Figure 5f). Therefore, the photodissociation pathway of me6-TG can be ascertained to break the $C-S^b$ bond, as shown in Scheme 2, and it can be concluded that the absorption bands at 350 and 420 nm are attributed to 6-TG[•].

To isolate and characterize the final stable photodissociation product, we further performed high-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-Q-TOF-MS) measurements. As shown in Figure 5g, in the reference



experiments for the pure reactant without laser irradiation, the HPLC/MS spectrum displays the fractions at 2.63 min (m/z =182), corresponding to me6-TG. After laser irradiation at 310 nm, in addition to the peak of me6-TG, a new peak at 2.05 min (m/z = 168) is observed, which is attributed to the formation of the photodissociation product (6-TG) according to its m/zmass (Scheme 2). Furthermore, the absorption band centered at 340 nm is also observed in the steady-state UV-vis difference spectrum (Figure 5h), which is the characteristic absorption feature of 6-TG. This suggests that the final photodissociation product of me6-TG is 6-TG, which should originate from 6-TG[•] by abstraction of the hydrogen atom from the parent compound to form the enol 6-TG followed by tautomerization to the more stable ketone 6-TG, as shown in Scheme 2. Furthermore, the yield of the photodissociation product can be estimated from the *ns*-TA spectra (Figure 5d). For the negative band of ground-state depletion at 310 nm, there is a prominent residual bleach sustained at long delay times, corresponding to the photochemical consumption. Dividing the amplitude of the residual bleach by that of the maximum bleach at 310 nm, the yield of the photodissociation product is estimated to be ~ 0.2 . The occurrence of photodissociation of me6-TG is in contrast to 6-TG, which undergoes mainly the photophysical relaxation pathway of ISC to the triplet state.¹² It appears that the double substitution of surfur and methyl in me6-TG opens up the photodissociation reaction process, through which the photochemical reactivity is enhanced.

Moreover, the behavior of the C-S bond cleavage can be rationalized by the calculations. To obtain the potential energy profiles of C-S dissociation of me6-TG, we performed a relaxed scan on the adiabatic S1 state (constrained optimization of all other coordinates while keeping the C-S distance fixed). The scan starts from the FC point with a step size of 0.1 Å, and smaller steps are adapted when mapping the dissociation energy threshold. As shown in the right panel of Figure 2b, the initial $S_1(^1\pi\pi^*)$ character is changed upon C–S bond elongation and thus the associated repulsive state can be involved and mapped. Along the dissociation path, the S1 energy decreases from FC to the lowest point 3.72 eV (this region with a ${}^{1}\pi\pi^{*}$ character) and then overcomes a barrier of 0.40 eV (from 3.72 to 4.12 eV with an excitation character of π $\rightarrow \pi^*/\sigma^*$), eventually leading to the C–S bond dissociation along the repulsive ${}^{1}\pi\sigma^{*}$ state. It is suspected that a conical intersection of $({}^{1}\pi\pi^{*}/{}^{1}\pi\sigma^{*})$ exists in the $\pi \to \pi^{*}/\sigma^{*}$ region. However, all efforts to pinpoint the minimum energy conical intersection of ${}^{1}\pi\pi^{*}/{}^{1}\pi\sigma^{*}$ have been unsuccessful, and a schematic CI(${}^{1}\pi\pi^{*}/{}^{1}\pi\sigma^{*}$) is plotted in Figure 2b. Here, the minimum energy structure of 3.72 eV is just the lowest point of the adiabatic S₁ state obtained along the performed relaxed scan, before crossing with the $\pi\sigma^*$ state. A full geometry optimization for this structure would finally go to the true minimum structure, labeled as 3.68 eV, $S_1(^1\pi\pi^*)$ in the left panel of Figure 2. The details of the C-S bond dissociation energy profile are plotted in Figure S15, showing that the ${}^{1}\pi\sigma^{*}$ state and the S_0 states both converge to the same dissociation limit, meaning that they have the same products, the $(6-TG^{\bullet})$ radical. Overall, an excitation energy of ~4.12 eV is required to surmount the dissociation barrier. In our experiment, the photodissociation energy threshold is determined to be roughly 320 nm (~0.13 eV lower than the peak absorption of 310 nm), which is in excellent agreement with the calculated energy for photodissociation (~0.04 eV lower than the FC

point). Based on the above, a photodissociation mechanism can be established for me6-TG, where the singlet C–S bond cleavage is via the surface crossing from ${}^{1}\pi\pi^{*}$ to ${}^{1}\pi\sigma^{*}$.

Mechanistic Implications and Significances

The *fs* and *ns* time-resolved spectroscopy results in conjunction with high-level *ab initio* calculations lead to insightful mechanistic implications as follows:

(i) In me6-TG, it is worth noting that the methyl substitution on the sulfur atom changes the C=S bond to a C-S bond and the neighboring C6-N1 bond to a C=N bond, which changes the dark state from ${}^{1}n_{S}\pi^{*}$ state to ${}^{1}n_{N}\pi^{*}$ character. For 6-TG, the orbital hybridization of the S atom is sp^2 and the n_s orbital is in the same plane of the purine ring (Figure S10). In contrast, the N1 atom adopts equal- sp^2 hybridization and one pair of electrons in the p_z orbital of the N1 atom conjugated with π orbitals renders a π_{N1} orbital. There is no n_{N1} orbital existing, and the other n_N orbital energies are too low to be involved (Figure S10). Thus, the n_s orbital is involved as an active orbital contributing to the transitions of the lowest five excited states (${}^{1}n_{s}\pi^{*}$ included), as shown in Table S3. From 6-TG to me6-TG, methyl substitution of the C=S bond changes the orbital hybridization of the S atom from sp^2 to sp^3 , where one pair of electrons involved in p- π conjugation forms the π_{s} orbital and the other pair of electrons form the n_s orbital whose energy is too low to be involved (Table S5). Meanwhile, the C6=N1 double bond makes the N1 atom adopt unequal-sp² hybridization, rendering an n_{N1} orbital that is in the same plane of the purine ring (Figure S12). For these reasons, the ${}^{1}n_{s}\pi^{*}$ character is diminished in me6-TG, while the $n_{\rm N1}$ orbital is involved and ${}^{1}n_{N}\pi^{*}$ state becomes the "dark" state among the low-lying excited states instead (Table S5).

Interestingly, this change of ${}^{1}n\pi^{*}$ character results in the change of the subsequent decay dynamics to triplet, as manifested by the greatly slowed ISC time of ~3 ns for me6-TG in comparison to the case of 6-TG (~0.3 ps¹²). For me6-TG, the triplet doorway state ${}^{1}n_{N}\pi^{*}$ incurs a significantly reduced SOC (8.3 cm^{-1}), which makes the transitions of different multiplicities not as facile as that for 6-TG (SOC: 95 cm⁻¹). Moreover, a substantial energy barrier of ~0.39 eV is encountered for the ISC from $S_1({}^1n_N\pi^*)$ to $T_2({}^3\pi\pi^*)$ via the singlet/triplet surface intersection in me6-TG, unlike the barrierless ISC in 6-TG. Although the heavy-atom S is present in both me6-TG and 6-TG, the involvement of the ${}^{1}n_{S}\pi^{*}$ state as the doorway to triplet (as in 6-TG) is the key factor to allow the heavy atom to take effect. Nevertheless, the methyl substitution of the C=S bond in me6-TG tunes the active orbitals participating low-lying excited states and renders the ${}^{1}n_{N}\pi^{*}$ state as a gateway to form the long-lived triplet, despite the slowed ISC rate. These results would deepen our comprehensions for the formation of the triplet state in sulfurand methyl-substituted nucleobase analogues.

(ii) The double substitution of sulfur and methyl in me6-TG greatly increases the photolabile character and simultaneously opens photochemical channels, i.e., C–S bond cleavage, to produce highly reactive radicals. In me6-TG, a weak C–S bond peripheral to the purine ring (the C–S^b bond) is introduced, thus the low energy orbital σ^* of the C–S bond is involved such that the ${}^{1}\pi\sigma^*$ state energy is lowered to intersect with the ${}^{1}\pi\pi^*$ state as shown in the calculations. The ${}^{1}\pi\sigma^*$ state involves mainly the excitation from the π orbital to C–S^b σ^* orbital (Table S5), which incurs C–S^b bond dissociation sponta-

neously. Accordingly, the photodissociation energy threshold is reduced to ~3.88 eV (320 nm) as determined by experiments, satisfying the need for a low energy UVA photon to initiate C– S bond cleavage and radical production. In sharp contrast, the single methylated nucleobase, 6mG, cannot undergo the C–O bond cleavage upon photoirradiation since the excited-state character of 6mG is essentially similar to that of natural guanine nucleobases. The lowest ${}^{1}\pi\pi^{*}$ (4.72 eV) and ${}^{1}n\pi^{*}$ (5.42 eV) states of 6mG are high in energy and mainly undergo efficient radiationless decay to the ground state by internal conversion.²⁰

CONCLUSIONS

In summary, the excited-state reaction dynamics of me6-TG under different UVA excitation wavelengths have been thoroughly examined by femtosecond and nanosecond timeresolved spectroscopy in conjunction with high-level ab initio calculations. Interestingly, the potential energy surface of me6-TG is found to be featured with double potential wells, resulting in multiple competing pathways. The initially excited singlet state $({}^{1}\pi\pi^{*}_{FC})$ can undergo picosecond depopulation to two local state minima of ${}^{1}\pi\pi^{*}_{min}$ and ${}^{1}n_{N}\pi^{*}_{min}$. Since there is no amenable S_1/S_0 intersections located for me6-TG, the chances of emitting fluorescence are increased for ${}^1\pi\pi^*_{\rm min}$ and ${}^{1}n_{N}\pi^{*}{}_{min}$, and indeed, the dual fluorescence behavior of ${}^{1}\pi\pi^{*}{}_{min}$ and ${}^{1}n_{N}\pi^{*}_{min}$ is observed. In competition with fluorescence emission, ISC to the triplet manifold through the gateway ${}^{1}n_{N}\pi^{*}_{min}$ state also occurs, forming the long-lived reactive triplet state within nanoseconds. In addition to these photophysical decay pathways, we also observed the opening of a unique C-S bond cleavage photochemical pathway under a higher photon energy excitation (<325 nm), generating thiyl radicals 6-TG[•]. This extra radical production channel, in addition to the triplet channel, can be involved in photosensitizer applications (type I involving radicals and type II involving the triplet state and ${}^{1}O_{2}$). Furthermore, me6-TG can still be selectively excited by UVA photons since natural nucleobases absorb mainly UVB and UVC light (<300 nm).

Importantly, for the interesting group of modified nucleobases as in me6-TG, our results disclose that the double substitution of sulfur and methyl has drastic effects on the photophysical and photochemical properties and opens rich excited-state deactivation pathways, including dual fluorescence, triplet state, and photodissociation, as compared to its single-substituted predecessor 6-thioguanine that only has a triplet pathway. We show that the methyl substitution of the C=S bond of me6-TG sensitively tunes the active orbitals participating low-lying excited states and ${}^{1}n_{N}\pi^{*}$ state becomes the dark state instead of the ${}^{1}n_{S}\pi^{*}$ state. The ${}^{1}n_{N}\pi^{*}$ state incurs a low spin-orbit coupling of 8.3 cm^{-1} and confronts a substantial energy barrier of ~0.39 eV to cross to triplet $T_2({}^3\pi\pi^*)$, which increases the lifetime for ISC (~3 ns). But, the quantum yield of the triplet state is still quite large (0.6 \pm 0.1), rationalized by the potential energy surface, which favors the FC excited-state bifurcation to ${}^{1}n_{N}\pi^{*}{}_{min}$ (3.36 ± 0.15 ps) rather than ${}^{1}\pi\pi^{*}_{min}$ (5.05 ± 0.26 ps) such that the subsequent ISC to the triplet manifold via ${}^{1}n_{N}\pi^{*}{}_{min}$ constitutes the main relaxation pathway in me6-TG. Interestingly, me6-TG also introduces a weak C-S bond peripheral to the purine ring and thus a low energy ${}^{1}\pi\sigma^{*}$ state that can intersect with the ${}^{1}\pi\pi^{*}$ state is involved, resulting in facile photodissociation. The energy threshold for the C-S bond dissociation via the

dissociative ${}^{1}\pi\sigma^{*}$ state of me6-TG is significantly lowered so that low-energy UVA photons can initiate bond cleavage and generate thiyl radicals. These findings demonstrate the synergistic effects of double functionality substitution in shaping the excited-state potential energy surface and enhancing the photoreactivity of DNA building blocks, and pave the way for utilizing thionation-based double substitution as a rational molecular design strategy in various applications such as photochemotherapy and photodynamic therapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.3c00575.

Key dihedral angles of 6-TG and me6-TG; Jablonski diagram; UV-vis absorption spectra and fluorescence emission spectra; steady-state emission spectra; fluorescence decay traces; nanosecond transient UV-vis absorption; concentrations of transient species for me6-TG as a function of time; Stern–Volmer plots; quantum yield of fluorescence; selected dihedral angles and side images of stationary and intersection structures; graphical representation of active orbitals for the CASSCF and CASPT2 calculations; calculated absorption spectra; MEP along the steepest descendant path; Dissociation potential energy curves for the two lowest singlet states of me6-TG; relative energies, spin-orbit coupling, and the oscillator strength for 6-TG and me6-TG; vertical excitation energies, oscillator strength, and excitation orbital of the six lowest excited states for 6-TG and me6-TG; optimized Cartesian coordinates (PDF)

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Notes

The authors declare no competing financial interest.

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