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2,4-Dithiothymine as a Potent UVA Chemotherapeutic Agent

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Supporting Information

ABSTRACT: Substitution of both oxygen atoms in the exocyclic carbonyl groups of the thymine chromophore by sulfur atoms results in a remarkable redshift of its absorption spectrum from an absorption maximum at 267 nm in thymidine to 363 nm in 2,4-dithiothymine (ΔE = 9905 cm^{-1}). A single sulfur substitution of a carbonyl group in the thymine chromophore at position 2 or 4 results in a significantly smaller redshift in the absorption maximum, which depends sensitively on the position at which the sulfur atom is substituted, varying from 275 nm in 2-thiothymine to 335 nm in 4-thiothymidine. Femtosecond transient absorption spectroscopy reveals that excitation of 2,4-dithiothymine at 335 or 360 nm leads to the ultrafast population of the triplet state, with an intersystem crossing lifetime of 180 ± 40 fs—the shortest intersystem crossing lifetime of any DNA base derivative studied so far in aqueous solution. Surprisingly, the degree and position at which the sulfur atom is substituted have important effects on the magnitude of the intersystem crossing rate constant, showing a 1.2-, 3.2-, and 4.2-fold rate increases for 2-thiothymine, 4-thiothymidine, and 2,4-dithiothymine, respectively, relative to that of thymidine, whereas the triplet yield increases 60-fold to near unity, independent of the site of sulfur atom substitution. While the natural thymine monomers owe their high degree of photostability to ultrafast internal conversion to the ground state and low triplet yields, the near-unity triplet yields in the thiothymine series account for their potent photosensitization properties. Nanosecond time-resolved luminescence spectroscopy shows that 4-thiothymidine and 2,4-dithiothymine are efficient singlet oxygen generators, with singlet oxygen quantum yields of 0.42 ± 0.02 and 0.46 ± 0.02, respectively, in O_2-saturated acetonitrile solution. Taken together, these photophysical measurements strongly suggest that 2,4-dithiothymine can act as a more effective UVA chemotherapeutic agent than the currently used 4-thiothymidine, especially in deeper-tissue chemotherapeutic applications.

In 1988, Elion and Hitchings shared the Nobel Prize in Physiology or Medicine "for their discoveries of important principles for drug treatment", principles that resulted in the development of a new series of nucleic acid derivatives that exhibit excellent chemotherapeutic effects against a variety of diseases.[1] Among others, they developed 6-thioguanine and 6-mercaptopurine for use against leukemia and azathioprine as a drug that prevents rejection of transplanted organs.[2] A key functionalization in these drugs is the single replacement of an oxygen atom in an exocyclic carbonyl group of a DNA base by a sulfur atom, thus forming the family of nucleic acid analogues commonly known as the thiobases. Thiobase derivatives are still widely used in clinical applications and have received much attention because of their prospective use in phototherapeutics.[3−9] It is therefore no wonder that their excited-state dynamics are currently under intense investigation from both experimental[10−21] and computational[22−25] perspectives.

Sulfur substitution has two significant effects on the photophysical properties of the nucleic acid bases: (1) it redshifts the absorption spectrum into the UVA region, and (2) it increases the triplet yield by nearly 2 orders of magnitude to yields that approach unity.[21] Red-shifting of the absorption spectrum is important because it allows for their selective excitation and because lower frequency radiation penetrates deeper into tissues, facilitating a greater depth of treatment in phototherapeutic applications.[26−29] A high triplet yield is equally important. Triplet states are usually long-lived, reactive species that can increase the probability of cell damage by directly reacting with biomolecules or by transferring their energy to molecular oxygen,[30] thus forming highly reactive oxygen species such as singlet oxygen.

In this contribution, we investigate the photophysical properties of the thiobase series 2-thiothymine (2tThy), 2-thiothymidine (2tThd), 4-thiothymidine (4tThd), and 2,4-dithiothymine (2,4dtThy) and compare them to those recently reported for thymidine (Thd),[31] 2tThy/2tThd,[19,20] and 4tThd.[14,16] The primary goals are twofold: (1) to determine how the site and degree of sulfur substitution affect the photosensitization properties of this series (shown in Figure 1a), and (2) to measure quantitative yields of singlet oxygen in order to scrutinize their prospective use as photochemotherapeutic agents.[32]

Figure 1b shows that the site and degree of sulfur substitution have striking effects on the absorption spectra of the thiobases, both within the series and compared to the absorption spectrum of the canonical DNA base. Specifically, sulfur substitution of the C2 carbonyl in thymine results in about a 10 nm redshift in the absorption maximum, whereas substitution at the C4 position results in a redshift of about 70 nm. Remarkably, double-substitution results in about a 100 nm redshift of the lowest-energy absorption band of thymidine (ΔE

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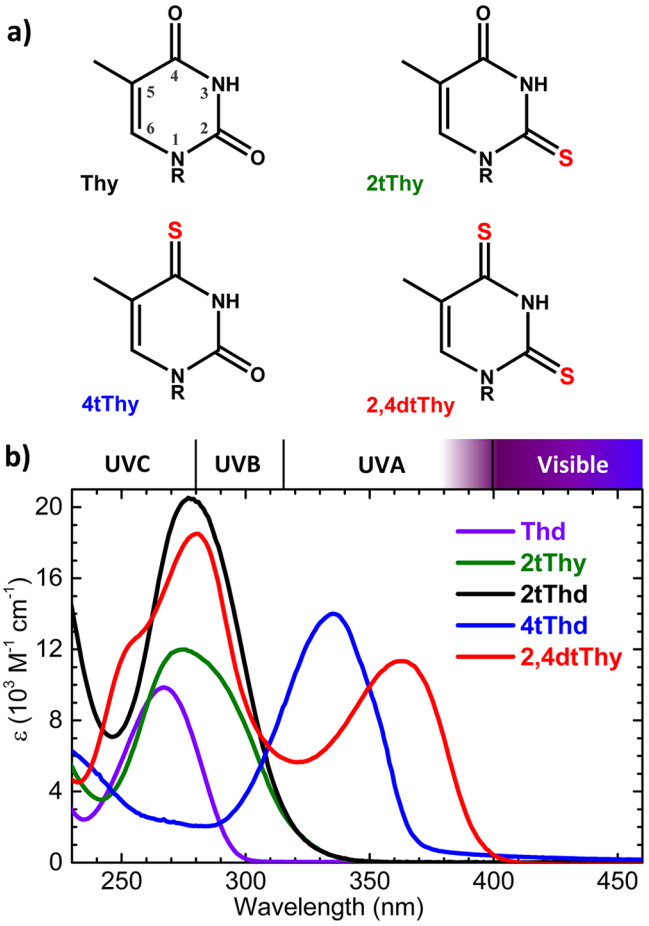

Figure 1. (a) Structures and common ring numbering of thymine (Thy, R = H) and thymidine (Thd, R = 2-deoxyribose) along with their sulfur-substituted analogues: 2-thiothymine (2tThy), 4-thiothymine (4tThy), and 2,4-dithiothymine (2,4dtThy), where R = H, and 2-thiothymidine (2tThd), 4-thiothymidine (4tThd), and 2,4-dithiothymidine (2,4dtThd), where R = 2-deoxyribose. (b) Absorption spectra of the thymine series in phosphate buffer solution at pH 7.4.

= 9905 cm^{-1}), without an appreciable change in the magnitude of the extinction coefficient at the corresponding wavelengths (~1 × 10^4 M^{-1} cm^{-1}). Replacement of an oxygen atom by a sulfur atom in a carbonyl bond is expected to shift the absorption spectrum to the red because the thiocarbonyl is weaker than the carbonyl bond and the excited electronic states in thiocarbonyl compounds are typically found at lower energies.[36] However, the difference in bond strengths between a carbonyl and thiocarbonyl bond cannot solely explain the large spectral shift observed between 2tThy/2tThd and 4tThd. Furthermore, the redshift in the absorption spectrum of 2,4dtThy cannot simply be expressed as a linear combination of the absorption spectra of the 2tThy/2tThd and 4tThd derivatives. In addition, these large spectral shifts do not arise from the presence or absence of a sugar at the N1 position of the thiothymine chromophore. Glycosylation of 2tThy, generating 2tThd, results in a 2 nm redshift in the absorption maximum and about 2-fold increase in the molar absorptivity coefficients (Figure 1b). The same effect has been previously documented in the natural bases and in other thiobases, as reviewed recently.[21] Therefore, glycosylation of 2,4dtThy to form 2,4-dithiothymidine is expected to redshift the absorption spectrum slightly and to increase the molar absorptivity coefficients. Evidently, quantum-chemical calculations are needed to provide a complete understanding of this phenomenon.

Femtosecond broad-band transient absorption spectroscopy was employed to further investigate the effect that varying the degree and position of sulfur substitution has on the electronic properties of these thiothymine derivatives (see Supporting Information for details). Solutions were continuously stirred to replenish the excited-state volume and replaced with fresh solutions as necessary to ensure no contamination of the transient absorption data by photoproducts. The focus of this set of experiments was to measure the intersystem crossing rate and to determine the relative triplet yields for the thiothymine series from back-to-back experiments (Table 1).[37] As observed

Table 1. Triplet-State Properties and Singlet Oxygen Yields of Thymidine and Its Sulfur-Substituted Analogues

	τ_{ISC}[a] (fs)	Φ_T[b]	Φ_Δ[c]
Thd	760[d]	0.014 ± 0.001[e]	0.07 ± 0.01[f]
2tThy	620 ± 60	0.9 ± 0.1[g]	0.36 ± 0.02[h]
2tThd	410 ± 60	0.9 ± 0.1[g]	
4tThd	240 ± 20	0.85 ± 0.15[i]	0.42 ± 0.02
2,4dtThy	180 ± 40	≳0.9	0.46 ± 0.02

[a]Intersystem crossing lifetimes and [b]triplet quantum yields in pH 7.4 phosphate buffer solution. [c]Singlet oxygen quantum yields in O_2-saturated acetonitrile solution. [d]Ref 31. [e]Refs 33 and 34. [f]Ref 35. [g]Refs 19 and 20. [h]Ref 15. [i]Refs 14 and 16.

previously for 2tThy/2tThd[19,20] and 4tThd,[14,16] the excited triplet state of 2,4dtThy can be probed selectively at wavelengths longer than 500 nm (Figure 2a).[37] Representative growth traces are shown in Figure 2b. These traces were taken at 600 nm probe wavelength and show that the triplet state is populated on the femtosecond time scale in all four thiothymine derivatives of this series. Glycosylation of 2tThy shortens the intersystem crossing lifetime from about 620 to 410 fs. Furthermore, Figure 2 and Table 1 show that 2,4dtThy has the shortest intersystem crossing lifetime of the series, which is in fact the fastest rate of intersystem crossing measured for any thiobase derivative to date.[21]

Comparison of the triplet-state dynamics of this series to the dynamics of the parent thymine nucleoside[31] indicates that ultrafast intersystem crossing is an intrinsic property of the thymine monomer, and possibly of pyrimidines in general,[21,38] rather than explicitly the result of sulfur substitution. However, the results presented in Table 1 demonstrate that sulfur substitution does greatly enhance intersystem crossing from being a minor relaxation pathway in thymidine, to being the overwhelmingly primary mode of relaxation in these thiothymine derivatives, independent of the site or the degree of sulfur substitution. Although determination of the absolute triplet yield is beyond the scope of the present work, the transient absorption results support the idea that the triplet yield of 2,4dtThy is equal to or higher than those previously reported for 2tThy/2tThd and 4tThd under similar experimental conditions. This is further supported by the shorter intersystem crossing lifetime of 2,4dtThy, indicating a higher probability for population transfer to the triplet manifold. The sub-200 fs population of the triplet state and its near-unity yield suggest that the spin−orbit and vibronic coupling interactions in 2,4dtThy are close to saturation because of the addition of a second sulfur substituent to the thiothymine base. Hence, we

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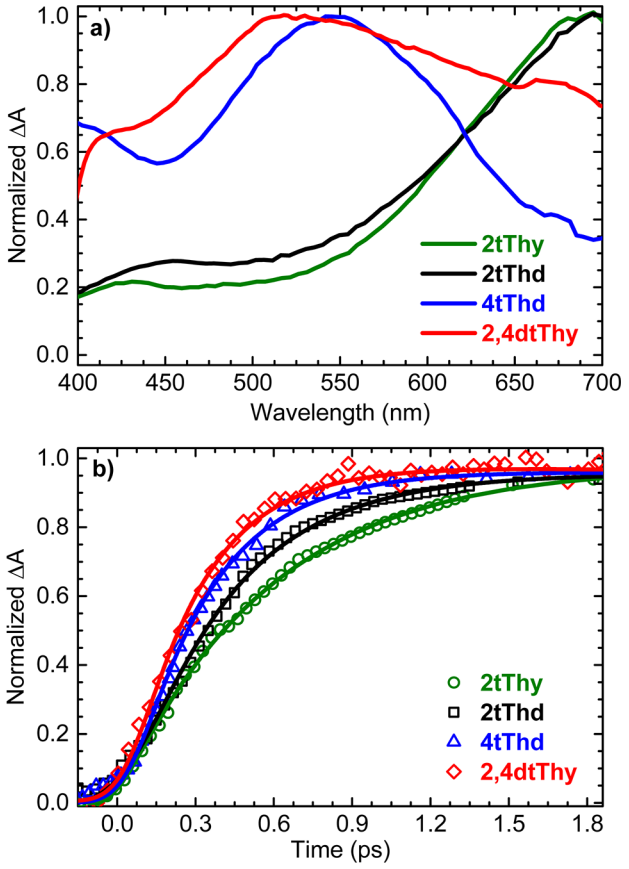

Figure 2. (a) Normalized absorption spectra of the lowest-energy triplet states and (b) representative growth traces of triplet-state population for 2tThy ($\lambda_{exc.}$ = 320 nm), 2tThd ($\lambda_{exc.}$ = 320 nm), 4tThd ($\lambda_{exc.}$ = 320 nm), and 2,4dtThy ($\lambda_{exc.}$ = 335 or 360 nm) at 600 nm probe wavelength in pH 7.4 phosphate buffer solution. Traces are cropped at ~2 ps and normalized to show the relative rates of intersystem crossing. See Supporting Information for details.

propose that intersystem crossing occurs in the strongly non-adiabatic regime between non-equilibrated excited states,[39,40] where the ability of active vibrational modes in the singlet manifold to couple and explore the singlet−triplet crossing regions may control the intersystem crossing rates in this important series of biomolecules.

While ultrafast internal conversion to the ground state and the low triplet yield in thymidine provide the nucleobase with its high degree of photostability, the near-unity triplet yields in the thiothymine series account for their potent photosensitization properties. We have obtained evidence for significant photoreactivity of these triplet states by performing time-resolved energy transfer experiments to molecular oxygen, thus generating singlet oxygen, as monitored by its characteristic phosphorescence at 1270 nm (Figure 3 and Figure S1 in the Supporting Information). The singlet oxygen quantum yields for 4tThd and 2,4dtThy were determined using phenalenone as a standard (Φ_Δ = 0.98)[41] and are summarized in Table 1. It should be remarked that quantification of the singlet oxygen yield is one of the primary methods used to determine the efficacy of a prospective photosensitizer for phototherapeutic applications.[28,42] To our knowledge, this is the first work to report the singlet oxygen yield of 2,4dtThy, whereas the singlet oxygen yield measured herein for 4tThd is in very good agreement with that reported by Harada under

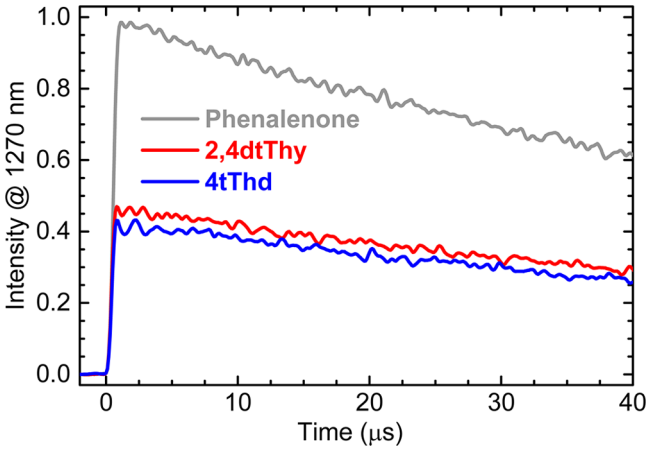

Figure 3. Singlet oxygen phosphorescence decay traces monitored at 1270 nm and generated by pulsed photoexcitation (335 nm, 7 ns pulse length) of 2,4dtThy, 4tThd, and phenalenone in O_2-saturated acetonitrile solutions.

similar conditions.[13] Although, we did not measure the singlet oxygen yield of 2tThy or 2tThd because of the limited UVA absorption of these thiobase derivatives, the singlet oxygen yield of 2tThy was previously measured in acetonitrile under O_2-saturated conditions.[15] Consistent with the spectroscopic analysis reported in this work, the singlet oxygen yield of 2tThy is smaller than those of 4tThd and 2,4dtThy.

Importantly, it has recently been shown that 4tThd in conjunction with a low dose of UVA radiation can effectively kill cancerous cell lines *in vitro*,[5,6,43] treat bladder cancer in animal models,[8] and induce cytotoxic lesions in the lower epidermis of 3D human skin models.[43] However, 4tThd exhibits limited absorption of near-visible UVA radiation, which has raised concerns as to the practicality of this thiobase in clinical applications.[44] We propose that 2,4dtThy has the potential to act as a more effective deep-tissue UVA photosensitizer than 4tThd because of its higher rate of intersystem crossing and increased propensity to generate singlet oxygen, in addition to its ability to absorb UVA radiation more strongly near the visible region of the spectrum, as shown in Figure 1b. We estimate that using 2,4dtThy as photosensitizer could facilitate up to 67% deeper tissue treatment by UVA radiation than 4tThd (see Supporting Information for details).

In summary, we have shown that the site and degree of sulfur substitution have significant effects on the photophysical and photodynamic properties of the 2tThy, 2tThd, 4tThd, and 2,4dtThy series. Surprisingly, the degree and position at which the sulfur atom is substituted play key roles in the magnitude of the intersystem crossing rate constant, showing a 1.2-, 3.2-, and 4.2-fold rate increase for 2tThy, 4tThd, and 2,4dtThy, respectively, relative to that of Thd. It appears that glycosylation also enhances the intersystem crossing rate in this series, at least for the 2tThy/2tThd pair. This paradigm is further highlighted by comparing the photophysical properties of this series to those of the natural thymidine monomer. The lowest-energy absorption band shifts from a maximum at ~267 nm ($\varepsilon \approx 9.9 \times 10^3$ M^{-1} cm^{-1}) in Thd to ~363 nm ($\varepsilon \approx 9.7 \times 10^3$ M^{-1} cm^{-1}) in 2,4dtThy. The triplet and singlet oxygen yields increase from 1.4% and 7% in Thd, respectively, to approximately 90% and 50% in 2,4dtThy. A single sulfur atom

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substitution leads to near-unity triplet yields in this series, as observed in other thiobases.[21]

Of paramount relevance for photochemotherapeutic applications, we have shown that, from this series, 4tThd and 2,4dtThy fulfill three of the most basic requirements of a potent UVA sensitizer: (1) strong absorption cross sections in the UVA spectral region, (2) near-unity triplet yields, and (3) high yields of singlet oxygen generation. On the basis of these photophysical properties, we propose that 2,4dtThy should outperform 4tThd for deeper-tissue UVA chemotherapies.

The thiothymine series investigated in this work represents an important class of biomolecules in which the rate of intersystem crossing outcompetes that of internal conversion to the ground state, exhibiting the fastest rate of intersystem crossing and the highest triplet quantum yields measured for any DNA or RNA nucleobase analogue thus far and challenging conventional wisdom. More generally, this work demonstrates that nanosecond time-resolved luminescence and femtosecond transient absorption spectroscopy are a powerful combination of techniques for quantifying the triplet-state population dynamics and singlet oxygen yields in nucleic acids and their analogues, thus providing key fundamental insights into the relationship between UVA-photosensitization efficacy and the underlying photodynamics.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, data analysis procedures, and supporting results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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