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Increase in the Photoreactivity of Uracil Derivatives by Doubling Thionation†

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The ability of 4-thiouracil to strongly absorb UVA radiation and to populate a reactive triplet state in high yield has enabled its use as a versatile photocrosslinker for nearly 50 years. In this contribution, we present a detailed spectroscopic and photochemical investigation of the 2-thiouracil, 4-thiouracil, and 2,4-dithiouracil series in an effort to further advance this chemistry and to scrutinize the photoreactivity of 2,4-dithiouracil. Our results reveal that excitation of 2,4-dthiouracil leads to intersystem crossing to the triplet manifold in 220 ± 40 fs, which enables the population of the reactive triplet state with nearly unity yield (Φ_T = 0.90 ± 0.15) and ultimately leads to a ca. 50% singlet oxygen generation (Φ_Δ = 0.49 ± 0.02)—one of the highest singlet oxygen yields reported to date for a photoexcited thiobase. In addition, the long-lived triplet state of 2,4-dithiouracil efficiently reacts with nucleic acid bases through a direct, oxygen-independent photocycloaddition mechanism and at a rate that is at least 3-fold faster than that of 4-thiouracil under equal conditions. The new physico-chemical insights reported for these RNA-thiobase derivatives are compared to those of the DNA and RNA bases and the DNA-thiobase analogues. Furthermore, the strong near-visible absorption and increased photoreactivity measured for 2,4-dithiouracil lays a solid foundation for developing RNA-targeted photocrosslinking and phototherapeutic agents that are more effective than those currently available.

Introduction

Thionated RNA derivatives are among the most highly conserved and ubiquitous nucleobases in bacterial tRNAs.[1] Since their discovery in the mid-1900s, these sulfur-substituted analogues of the canonical RNA bases have been employed in structural biology studies[2-5] and investigated for their potential pharmacological properties.[6-10] In particular, the thio-RNA derivative, 4-thiouracil (Scheme 1), has been widely used due to its unique photosensitizing properties. The subtle replacement of a carbonyl oxygen atom by a sulfur atom shifts the absorption spectrum to longer wavelengths and enhances population transfer to the reactive triplet state, as compared to the uracil nucleobase.[11-14] Over the past five decades, 4-thiouracil has been used in photocrosslinking investigations to uncover *in vivo* RNA structures and important RNA-protein interactions.[15-21] Currently, 4-thiouracil is being used in the PAR-CLIP approach (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) to determine the protein binding interactions of specific RNA sequences throughout the entire human transcriptome.[22-24] Furthermore, Photo-SELEX (Photochemical Systematic Evolution of Ligands by Exponential Enrichment) derived aptamers containing 4-thiouracil have been used recently to target and probe the membrane protein interactions of live breast cancer cell cultures.[25]

Although 4-thiouracil has demonstrated ample applicability in these structural-biology studies,[15-26] little work has been done toward further enhancing this chemistry or toward developing phototherapeutics based on thio-RNA derivatives.[18, 27, 28] Conversely, members of the closely-related family of thio-DNA derivatives have been shown to be effective in the light-activated treatment of various cancers, such as efficient photodynamic therapy of malignancies in: (1) cultured cell lines;[29, 30] (2) 3-D skin models;[31] (3) *ex vivo* tissue biopsies;[32] and (4) animal models;[33] as well as a recent movement toward clinical trials.[34] Given that oxidatively generated damage of cellular RNAs is just as detrimental to cell viability as oxidatively generated damage of DNA,[35-38] phototherapeutics based on thio-RNA derivatives can be expected to perform as well, or better than, those currently based on thio-DNA derivatives. In fact, the ability to integrate thio-RNA photosensitizers into RNA aptamers for cancer cell targeting has already been demonstrated.[25] Furthermore, thio-RNA derivatives can be incorporated into emerging RNA interference therapies with an enhanced target binding efficiency through photocrosslinking reactions.[39-42]

A deeper understanding of the structure-photoreactivity relationships of thio-RNA derivatives is expected to

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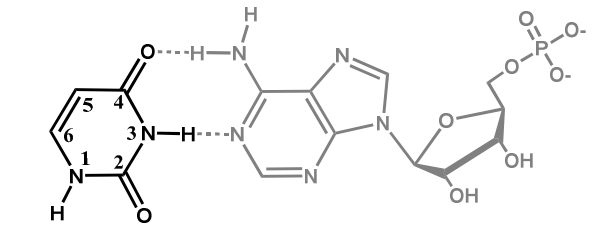
† Electronic Supplementary Information (ESI) available: Difference absorption spectra for the 4tUra-5′-AMP and 2,4dtUra-5′-AMP photoreaction solutions; extended discussion of the photoreaction mechanism of 4tUra and 2,4dtUra with 5′-AMP; and the methodology used to determine the enhanced near-visible absorption and the photoactive tissue depth. See DOI: 10.1039/x0xx00000x

Scheme 1. Structures and common ring numbering of the canonical RNA-base uracil and its three thionated derivatives



significantly advance their prospective use in phototherapeutic and photocrosslinking applications that specifically target RNA-RNA, RNA-DNA, and RNA-protein interactions. In particular, a thorough understanding of their photophysical and photochemical properties is essential to develop the next generation of thio-RNA derivatives exhibiting enhanced photosensitizing efficacy and photocrosslinking reactivity. This knowledge is required to enable their swift integration into phototherapeutic and structural-biology applications. With these goals in mind, we have employed advanced spectroscopic and photochemical methods to investigate the thio-RNA series 2-thiouracil (2tUra), 4-thiouracil (4tUra), and 2,4-dithiouracil (2,4dtUra) under equal experimental conditions (Scheme 1). From a fundamental perspective, the detailed set of experiments reported in this contribution provide important, new insights into the structural and electronic factors that control the photoreactivity and photosensitizing efficacy of this thio-RNA series. From an application-based standpoint, our results reveal that 2,4dtUra has the highest photosensitizing and photoreactivity efficacy when compared side-by-side to the widely used 4tUra. More importantly, 2,4dtUra provides a basis for developing novel RNA-targeting phototherapeutic agents, which can find applications in clinical settings.

Results

Steady-state absorption spectra

Figure 1 shows the molar absorptivity spectra of the thiouracil series investigated in this work and compares them to that of the canonical uracil nucleobase (Ura). Thionation at the C2 position of the uracil chromophore redshifts the absorption maximum by 11 nm and increases the molar absorptivity by 1.4-fold, whereas thionation at the C4 position redshifts the

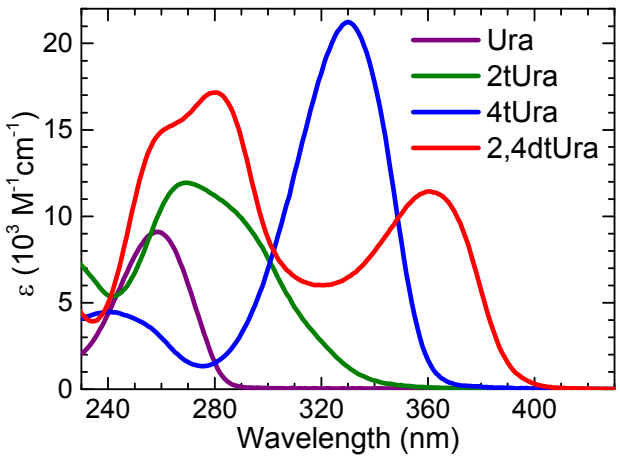


Figure 1. Molar absorptivity spectra of the canonical uracil nucleobase and the thiouracil series studied in aqueous phosphate-buffered saline solution, pH 7.4.

absorption maximum about 70 nm and more than doubles the molar absorptivity. Furthermore, substitution of the oxygen atoms by sulfur atoms in both carbonyl groups of uracil red-shifts the absorption spectrum by more than 100 nm (11,058 cm^{-1}) relative to that of the canonical nucleobase, while simultaneously increasing the molar absorptivity of the lowest-energy absorption band by 1.3-fold. These observations are in agreement with those reported in the early literature.[43, 44]

Measurement of intersystem crossing rates, triplet yields, and rates of triplet-state decay

Femtosecond to microsecond broadband transient absorption spectroscopy was used to measure the rates of intersystem crossing to the triplet manifold, the triplet yields, and the rates of triplet-state decay back to the ground state. The triplet state of the thiouracil derivatives can be selectively probed at wavelengths longer than 600 nm without the interference of other transient absorption species.[49] Figure 2a shows the triplet growth traces recorded at 600 nm, which were normalized to highlight their relative rates of triplet-state population. The population lifetimes (τ_{ISC}) obtained from a

Table 1. Triplet-state properties in aqueous buffer and singlet oxygen yields in O_2-saturated acetonitrile measured for 2tUra, 4tUra, and 2,4dtUra and compared to literature values for the uracil nucleobase

	τ_{ISC}[a] (fs)	Φ_T[b]	k_T[c] (10^6 s^{-1})	Φ_Δ[d]
Ura	< 10[e]	0.023[f]	2.9[f]	0.15 ± 0.02[g]
2tUra	360 ± 30	0.75 ± 0.20[h]	-	-
4tUra	240 ± 20	0.90 ± 0.15	1.7 ± 0.3	0.49 ± 0.02
2,4dtUra	220 ± 40	0.90 ± 0.15	4.3 ± 0.9	0.49 ± 0.02

[a] Intersystem crossing lifetime, [b] triplet quantum yield, and [c] triplet decay rate in aqueous phosphate-buffered saline solution, pH 7.4. The thiouracil triplet decay rates were collected in 24 µM solutions. [d] Singlet oxygen quantum yield in O_2-saturated acetonitrile. [e] Taken from ref. [45]. [f] Taken from ref. [46]. [g] The singlet oxygen yield of uracil was taken from ref. [47] in O_2-saturated acetonitrile. Its value is higher than that of the triplet yield of uracil in aqueous solution because the triplet yield is an order of magnitude greater in acetonitrile than in aqueous buffer solution,[46] thus enabling the greater singlet oxygen production. [h] Taken from ref. [48].

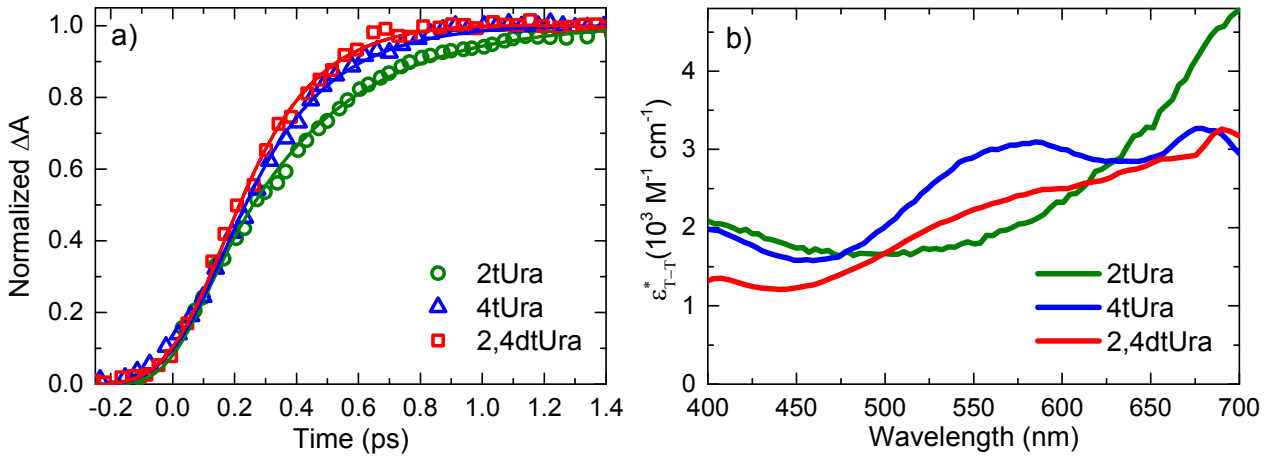


Figure 2. Growth of triplet-state population (a) and the corresponding triplet-triplet absorption spectrum (b) of 2tUra, 4tUra, and 2,4dtUra in aqueous buffer solution at pH 7.4[50] following femtosecond UVA excitation at 320, 335, and 350 nm, respectively. Triplet state populations were monitored at 600 nm to avoid overlap from other transient species. Growth traces are normalized and cropped at 1.4 ps to clearly show the relative rates of intersystem crossing. Triplet state extinction coefficients were determined using the singlet depletion method (see Experimental section for full details).

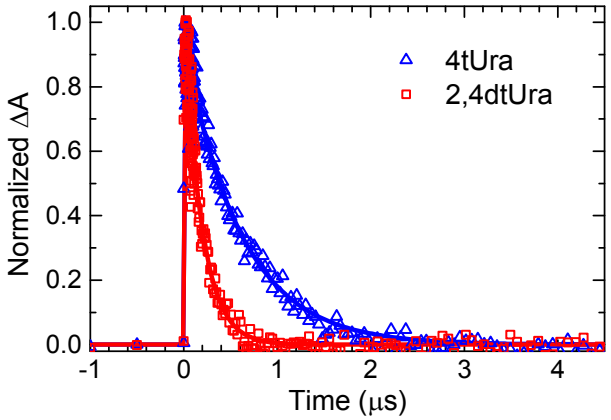


Figure 3. Triplet-state decay traces of 4tUra and 2,4dtUra following 350 nm excitation at concentrations of 24 μM in nitrogen-purged aqueous buffer solutions. Global fitting of several decay traces from the broadband transient spectra produced the fit lines shown and the rates of triplet-state decay (k_T) reported in Table 1.

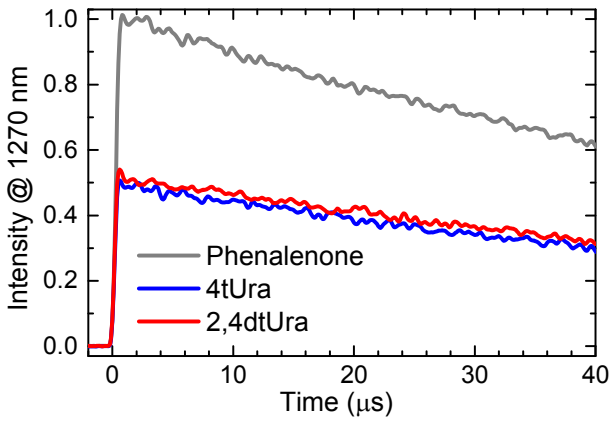


Figure 4. Singlet oxygen generation from 4tUra and 2,4dtUra following nanosecond excitation at 355 nm as monitored by the characteristic phosphorescence of singlet oxygen at 1270 nm in O_2-saturated acetonitrile solutions. Quantum yields were determined by comparison with the standard phenalenone (Φ_Δ = 0.98).[52]

global analysis of the broadband transient absorption data are presented in Table 1. Thionation at the C4 position results in faster intersystem crossing than thionation at the C2 position. Doubling thionation results in intersystem crossing with a shorter lifetime than that measured for 4tUra. Figure 2b shows that the triplet-triplet absorption spectrum of each derivative is broad and almost featureless; spanning the entire probe range from 400 to 700 nm.

Back-to-back transient absorption measurements of the 2tUra, 4tUra, and 2,4dtUra series were also performed under the same experimental conditions in order to determine their triplet-triplet extinction coefficients (ε^*_{T-T}) and triplet quantum yields (Φ_T). Analysis of these measurements indicate that all three thiouracil derivatives populate the triplet state with nearly unity yield (Table 1), in agreement with previous measurements for 4tUra[51] and its nucleoside.[13, 14] For 4tUra and 2,4dtUra, the triplet-triplet absorption band decays back to the ground state in hundreds of nanoseconds at thiouracil concentrations of 24 μM in N_2-saturated aqueous buffer solutions (Figure 3 and Table 1). The triplet-state of 4tUra decays on the same time scale as that measured for its nucleoside under similar experimental conditions.[13, 14] The rate of triplet-state decay of 2,4dtUra is reported for the first time in this work, whereas that of 2tUra decays on a similar time scale in N_2-saturated acetonitrile solution[53] and was not determined in aqueous solution in this work.

Measurement of singlet oxygen yields

The singlet oxygen ($^1O_2$) quantum yields of 4tUra and 2,4dtUra were measured by monitoring the characteristic phosphorescence of $^1O_2$ at 1270 nm with nanosecond time resolution. To the best of our knowledge, these yields are

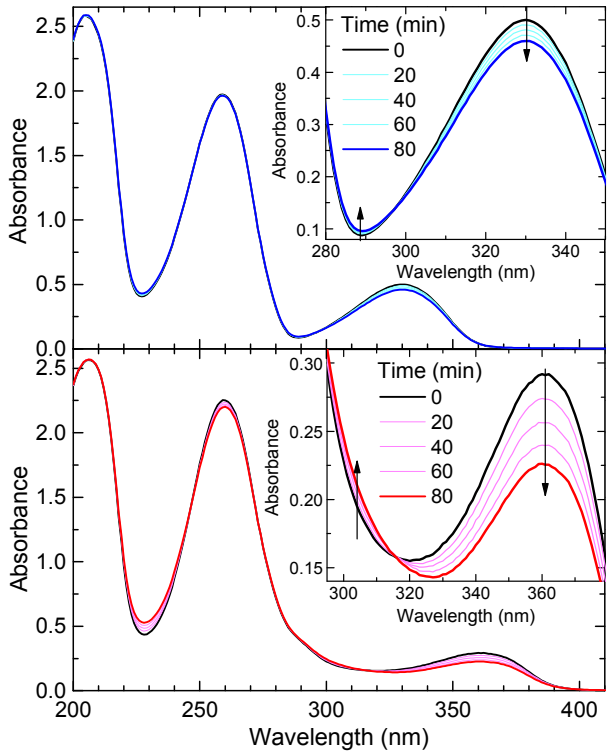


Figure 5. Changes in the steady-state absorption spectra used to monitor the reaction of 4tUra (top) or 2,4dtUra (bottom) with 5'-AMP upon 365 nm irradiation. Solutions were prepared at 1:5, thiouracil:5'-AMP ratios and continuously purged with ultrapure N_2. The decrease in absorption in the UVA region and simultaneous increase in absorption at shorter wavelengths (highlighted in the inset) provides direct evidence of the photoreaction between the thiouracil and 5'-AMP in each solution. The similar spectral changes suggest that the photoreaction mechanism and primary photoproduct are similar in both solutions mixtures.

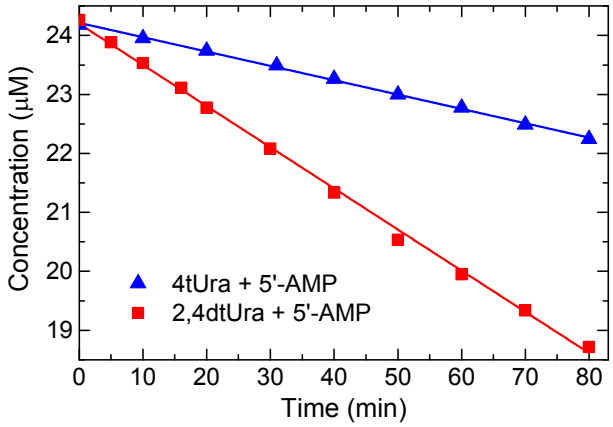


Figure 6. Relative photoreaction rates observed for 4tUra and 2,4dtUra in N_2-saturated aqueous buffer solutions containing 5'-AMP. Solutions were irradiated with 365 nm laser light and the decrease in thiouracil concentration was monitored by steady-state absorption spectroscopy. The photoreaction rates reported in the text were obtained from the slope of the linear regression.

reported for the first time for both thiobases. As shown in Table 1 and Figure 4, both derivatives exhibit a $^1O_2$ quantum yield of 0.49 ± 0.02 in O_2-saturated acetonitrile solutions. The yield measured for 4tUra in this work is in excellent agreement with that determined previously for the 4tUra nucleoside under similar experimental conditions.[12] No attempt was made to measure the $^1O_2$ yield of 2tUra because this derivative has limited absorption at 355 nm; the excitation wavelength used for these measurements.

Photoreactivity of 4tUra and 2,4dtUra with adenosine 5'-monophosphate

In order to scrutinize the light-induced reactivity of these sensitizers toward biomolecules, aqueous phosphate-buffered saline solutions of either 4tUra or 2,4dtUra were prepared in the presence of the RNA monomer adenosine 5'-monophosphate (5'-AMP, see Scheme 1) at a 1:5 molar ratio. 5'-AMP is used as a model biomolecule compound because 5'-AMP is the natural Watson-Crick base-pairing partner of uracil in RNA (Scheme 1). The experimental conditions were chosen in order to facilitate the extraction of relative photoreaction rates from the changes in the steady-state absorption spectra with irradiation time. Specifically, (1) the changes in absorption of the solutions with irradiation time were monitored during the initial, linear regime of the photoreaction process where the slope of the data can be related directly to the reaction rate; (2) the concentrations of the thiobase and 5'-AMP were selected in such a way as to enhance the rate of the thiobase-5'-AMP bimolecular reaction, while simultaneously minimizing triplet self-quenching and self-reaction pathways in the thiouracil derivatives;[11, 54] and (3) the solutions were irradiated with monochromatic UVA-laser light at (365 ± 1) nm and were continuously purged with ultrapure nitrogen gas in order to eliminate quenching of the triplet state of the thiobase by molecular oxygen and to avoid any putative[55] side reactions of 5'-AMP or the thiobase with reactive oxygen species. Finally, we remark that the photoproducts formed between the thiouracil derivatives and 5'-AMP do not absorb significantly at wavelengths longer than 320 nm and, therefore, the progression of the bimolecular thiobase-5'-AMP photoreactions can be monitored selectively by following the decay of the UVA absorption band in each solution (see the electronic supporting information (ESI) for details).

Figure 5 shows changes in the absorbance of these solutions upon irradiation at 365 nm, as monitored by steady-state absorption spectroscopy. Both solutions exhibit a linear decrease in their UVA absorption band with a simultaneous increase in their absorption at wavelengths shorter than ~320 nm. Isosbestic points occur at 246, 270, and 300 nm in the spectra of the 4tUra-5'-AMP mixture, whereas they appear slightly red-shifted in the 2,4dtUra-5'-AMP mixture at 246, 275, and 318 nm. The isosbestic points are indicative of the formation of photoproducts. A further spectral analysis described in the ESI shows that only one major photoproduct is formed in each solution, which is depicted in the difference absorption spectrum presented in Figure S2 for each solution mixture. As discussed in detail in the ESI, the difference absorption spectra obtained for the 4tUra-5'-AMP and 2,4dtUra-5'-AMP mixtures are both comparable to the absorption spectrum of the major photoproduct formed

between the structurally-similar thiobase, 4-thiothymidine, and adenosine.[56]

Figure 6 shows the change in concentration of 4tUra and 2,4dtUra as a function of irradiation time in solutions containing 5'-AMP. The relative photoreaction rates were estimated from the slopes of linear regression fittings of the data reported in Figure 6. Significantly, 2,4dtUra exhibits a photoreaction rate of $(1.2 \pm 0.2) \times 10^{-9}$ M/s, approximately three times faster than the photoreaction rate measured for 4tUra, $(0.39 \pm 0.04) \times 10^{-9}$ M/s, under the same experimental conditions. The importance of this threefold increase in the photoreaction rate is highlighted in the discussion section below.

Discussion

Photophysical characterization of the thiouracil series

An important property in the characterization of the photosensitizing potential of the thiouracil series is the determination of their ground-state molar absorptivity spectra. The molar absorptivity spectra shown in Figure 1 demonstrate that the electronic structure of the canonical uracil nucleobase is greatly perturbed upon thionation and that the extent of the perturbation depends sensitively on the carbon position at which the double-bonded oxygen atom is substituted for a sulfur atom. In comparison to the uracil nucleobase, each of the thionated derivatives displays a redshifted absorption spectrum into the UVA region (315 to 400 nm). The maximum of the lowest-energy absorption band is shifted considerably further to the red (60 nm, 6,734 cm^{-1}) upon sulfur substitution of the oxygen atom at the C4 position compared to when the substitution is made at the C2 position. Doubling thionation induces the greatest redshift in absorption, with 2,4dtUra exhibiting an absorption tail that extends into the visible region of the spectrum. In fact, based on the molar absorptivity spectra, the absorption efficiency of 2,4dtUra at near-visible wavelengths (380 to 400 nm) is about 27-fold greater than that of 4tUra (see ESI for details). The ability of these thiouracil derivatives to absorb longer wavelengths of light than uracil facilitates their selective excitation over the natural DNA and RNA nucleobases. This photophysical property is essential for their prospective use in *in vitro* and *in vivo* photosensitizing applications.

Absorption at longer wavelengths and with larger absorption coefficients than the natural DNA and RNA bases is an important property of effective photosensitizers. However, a photosensitizer must also populate long-lived, highly-reactive excited states upon light absorption in order to be truly effective. A hallmark of an efficient photosensitizer is population of the triplet state in high yield. The data shown in Figure 2 and Table 1 provide direct evidence that intersystem crossing to the triplet manifold occurs in hundreds of femtoseconds and in nearly unity yield in all three of the thiouracil derivatives. Assignment of the transient absorption growth traces (Figure 2a) to the population of the triplet state in both 2tUra and 4tUra is supported by the excellent agreement of the absorption spectra associated with these transient species (Figure 2b) with those previously reported for the triplet states of 2tUra[48] and the 4tUra nucleoside.[13, 14] The triplet-triplet absorption spectrum of 2,4dtUra has not been reported previously. However, several experimental observations point toward the assignment of the transient spectrum shown in Figure 2b to the triplet state absorption of 2,4dtUra. In particular, the slow rate at which this transient species decays to repopulate the ground state (Figure 3) strongly supports its assignment as the lowest energy triplet state. Furthermore, this transient species is readily quenched by molecular oxygen, producing a $^1O_2$ yield of ca. 50%.

The triplet yield of all three thiouracil derivatives is more than thirtyfold higher than the parent nucleobase uracil (Table 1). The thirtyfold increase in triplet-state population upon thionation of the uracil chromophore originates from the presence of the heavier sulfur atoms. Thionation increases the density of states and the spin-orbit coupling interaction between the singlet and triplet manifolds, while simultaneously reducing the relevant singlet-triplet energy gaps of the uracil chromophore.[57, 58] Hence, intersystem crossing becomes highly favored over internal conversion back to the ground state.[49]

The nearly unity triplet yields should also increase the photoreactivity of these thiouracil derivatives, and therefore, their efficacy as photosensitizers. One established method for quantifying the reactivity of a sensitizer's triplet state is to determine its ability to generate $^1O_2$.[52, 59] The results shown in Figure 4 demonstrate that, both 4tUra and 2,4dtUra generate $^1O_2$ with ca. 50% yield following UVA excitation. This is 3.3-fold higher than the amount of $^1O_2$ generated by uracil when these nucleobases are directly excited to their lowest-energy absorption band. The increase in the $^1O_2$ yield is also consistent with the more than thirtyfold increase in the triplet yield of 4tUra and 2,4dtUra compared to uracil under equal experimental conditions (Table 1).

Photoreactivity of 2,4dtUra versus 4tUra with 5'-AMP

The generation of $^1O_2$ by a photosensitizer and the subsequent reaction of this highly oxidizing species with biomolecules is an indirect mode of photochemical reaction known as Type II photosensitization. Oxidatively generated damage to cellular components mediated by Type II photosensitization and the formation of other reactive oxygen species can eventually lead to cell death and is a common mode of photodynamic therapy. Above, we have shown that UVA excitation of 2,4dtUra results in ca. 50% $^1O_2$ yield, evidencing the unsurpassed potential of 2,4dtUra in photosensitization applications compared to the other members of the series. This qualification warrants further investigation into the reactivity of 2,4dtUra as a photocrosslinking agent.

Direct photocycloaddition reaction between the excited-state of a sensitizer and a biomolecule is also an important mechanism that is widely used in photocrosslinking structural-biology studies based on 4tUra.[16-25, 60] Photoaddition reactions can play an equal or greater role than $^1O_2$ generation in phototherapeutic applications, especially in oxygen-deprived

environments such as hypoxic solid tumors.[61, 62] Therefore, it is important to evaluate the potential of 2,4dtUra as a photocycloaddition sensitizer for both structural biology and phototherapeutic applications.

To investigate the ability of 2,4dtUra to participate in photocycloaddition reactions with nucleic acid bases, we have measured the photoreactivity of 2,4dtUra with 5'-AMP in N_2-saturated aqueous solutions—experimental conditions that prevent $^1O_2$generation. Similar photoreactivity experiments were performed using 4tUra as the sensitizer in order to determine the relative efficacies of 2,4dtUra and 4tUra in undergoing these reactions. Efficient photocycloaddition between 4-thiouridine and adenosine has been reported,[54] and the mechanism of photoproduct formation between 4-thiothymidine and adenosine has been characterized in-depth.[63] Furthermore, both 4-thiouridine and 4-thiothymidine are structurally-similar to 2,4dtUra and their reaction with adenosine is expected to be similar to that of 2,4dtUra with 5'-AMP. We present evidence in the ESI that all three of these photocycloaddition reactions are analogous and propose a mechanism for the formation of the primary photoproduct between 2,4dtUra and 5'-AMP.

Figure 5 shows that irradiation of the 2,4dtUra-5'-AMP and 4tUra-5'-AMP solutions at 365 nm results in a decrease of the UVA absorption band of the thiobase and a corresponding increase in absorption at shorter wavelengths. The spectral changes observed for the 2,4dtUra-5'-AMP mixture closely resemble those of the 4tUra-5'-AMP mixture, showing that the photoreaction mechanism between 2,4dtUra and 5'-AMP and 4tUra and 5'-AMP are comparable, as articulated above. The photoproduct absorption spectra shown in Figure S2 reinforce the idea that 4tUra and 2,4dtUra undergo a similar photoreaction with 5'-AMP and that it is analogous to the one previously reported between 4-thiothymidine and adenosine (see ESI for further discussion).[63] More important to the present discussion is the fact that the photoproduct formed in this reaction has negligible absorption in the UVA region where 4tUra and 2,4dtUra absorb, allowing the relative rates of photoreaction to be quantified directly from the steady-state absorption data. The results reveal that the photocycloaddition observed between 2,4dtUra and 5'-AMP is threefold greater than that between 4tUra and 5'-AMP upon irradiation with monochromatic, 365 nm laser light in solutions containing the same concentrations of reactants (Figure 6). Furthermore, the photoreactivity experiments show that 2,4dtUra (and 4tUra) can react with 5'-AMP by a photocycloaddition mechanism. They also suggest the potential of using doubly-thionated RNA derivatives for near-visible phototherapeutic applications in oxygen-deprived biological environments where Type II photosensitization may not be effective.

On the enhancement of photocrosslinking and phototherapeutic applications using 2,4dtUra as the sensitizer

The photochemical characterization of the thiouracil series presented above shows that 2,4dtUra can outperform 4tUra in its current uses as a photocrosslinking agent[16-26] and can

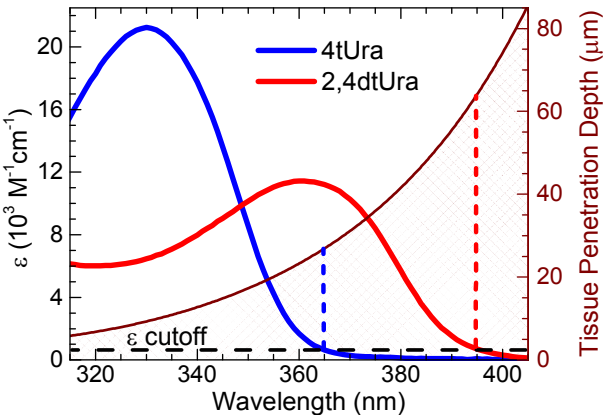


Figure 7. Molar absorptivity spectra of 4tUra and 2,4dtUra overlaid with the function of the wavelength-dependent penetration depth of light into unpigmented tissue.[64, 65] The ε cutoff is defined by the molar absorptivity of 4tUra at 365 nm; the excitation wavelength typically used in its photosensitization applications.

potentially enable phototherapies based on thio-RNA derivatives. Importantly, many of the current applications employing 4tUra and other UVA photosensitizers (e.g. psoralen derivatives) use radiation at 365 nm as the excitation source.[16-24, 26, 27, 30, 31, 66, 67] The use of longer UVA wavelengths reduces direct excitation of other biomolecules, thereby increasing photosensitization efficiency and minimizing unwanted side reactions.[16-20, 22-24, 26, 27] However, the requirement of using relatively high concentrations of the sensitizer and/or long irradiation times are two major drawbacks in the current use of 4tUra in photocrosslinking studies[20, 21, 25, 26, 68] as well as in PUVA (psoralen + UVA) photochemotherapies.[30, 31, 69, 70] The threefold higher photoreaction rate of 2,4dtUra as compared to 4tUra at 365 nm (Figure 6) can facilitate the use of lower sensitizer concentrations and/or shorter irradiation times in photocrosslinking and phototherapeutic applications. More importantly, 2,4dtUra enables the use of lower-energy excitation wavelengths, as long as 395 nm (see Figure 7), considering a molar absorptivity cutoff criterion equal to that currently used for 4tUra (see ESI for a more thorough justification of this cutoff criterion). In other words, the use of 2,4dtUra enables excitation at 395 nm while maintaining the same absorption efficiency and photoreactivity as that of 4tUra at 365 nm. The ability to use longer irradiation wavelengths afforded by 2,4dtUra should also improve the selective excitation of the sensitizer, while simultaneously enabling photosensitization deeper within the skin and other tissues. This is because irradiation at a wavelength of 365 nm leads to a photosensitization depth of about 27 μm into tissues, whereas radiation at 395 nm can penetrate as deep as 65 μm (Figure 7).[64, 65] Hence, the replacement of 4tUra by 2,4dtUra in current *in vivo* photocrosslinking studies[18, 24, 25, 27] is expected to increase the effective photosensitization depth by up to 140%. The greater tissue depths at which 2,4dtUra can be photoactivated, together with its enhanced photosensitization properties, have the potential to move thiouracil derivatives into mainstream phototherapeutic

application and could offer a viable substitute for psoralen derivatives in PUVA treatment without the late-stage side effects.[30, 31, 69, 70]

Structure-photoreactivity relationships between the DNA and RNA thiopyrimidine series and with other pyrimidine monomers

A comparison of the results presented in this work for the thiouracil series with those presented recently for the thiothymine series[71] shows that the methyl group at the C5 position of the pyrimidine ring plays a considerable role in modulating the photophysical properties of these thiopyrimidine series. For instance, the ground-state absorption spectra of the thiothymine series are slightly red-shifted (~5 nm) and show a moderate decrease in molar absorptivity as compared to those of the thiouracil series. These observations mirror earlier reports between the canonical uracil and thymine nucleobases.[11, 72] Similarly, the rate of intersystem crossing in the thiothymine series is more sensitive to the degree of thionation than is the intersystem crossing rate in the thiouracil series. That is, the thiothymine series exhibits a 3.5-fold increase in the rate of intersystem crossing in going from 2-thiothymine to 2,4-dithiothymine (2,4dtThy),[71] whereas a 1.6-fold increase is observed in going from 2-thiouracil to 2,4-dithiouracil in this work. The rate of triplet-state decay has also been shown to be modulated by C5-functionalization, being faster in 2-thiouracil than in 2-thiothymine.[53] This is consistent with their relative rates of intersystem crossing[48] and with the slightly different magnitude of singlet-triplet energy gaps between the two families of thiobases.[73] The observation that the intersystem crossing lifetime reported in this work for 2,4dtUra (220 ± 40 fs) is the same as that reported recently for 2,4dtThy (180 ± 40 fs),[71] within the experimental uncertainties, lends strong support to the idea that spin-orbit coupling in these compounds is saturated upon doubling thionation.[11] Moreover, the magnitude of the $^1O_2$ yields for 2,4dtThy and 4-thiothymidine (4tThd) are 6 and 14% lower, respectively, than those measured for the corresponding thiouracil derivatives. These trends in the triplet-state properties of the thiopyrimidine series are in line with previous works, where the C5-substituent is shown to modulate the photophysical properties of uracil in comparison to thymine and other uracil derivatives.[11, 53, 72, 74, 75]

The results presented in this work and elsewhere for the thiothymine series[71] also show that the functional groups at the C2 and C4 positions of the pyrimidine ring play important roles in modulating the photochemical properties of the thiothymine and thiouracil series. The absorption spectra, the rate of intersystem crossing, and the triplet and $^1O_2$ yields are uniquely sensitive to the specific position at which the thymine and uracil nucleobase are thionated. In particular, the structure-photoreactivity relationships observed in the thiopyrimidine series lend further support to the idea that functionalization at the C4 position of the pyrimidine ring, plays a more important role than functionalization at the C2 position in enhancing the photoreactivity of the pyrimidine nucleobases in solution.[76] However, a detailed investigation of the excited-state dynamics of both thiopyrimidine series,[49] as well as of other pyrimidine analogues, is necessary before these structure-photoreactivity relationships can be further generalized.

Finally, an important distinction between the thiouracil and the thiothymine series is their specific role in biochemistry; particularly, the targeted incorporation of thiouracil derivatives into cellular RNAs rather than DNA.[18, 23, 27] This biological distinction enables an alternative, broader range of intracellular sites for photochemical and photocrosslinking reactions. This biochemical distinction has facilitated the continued use of 4tUra as an informative photocrosslinking probe in RNA structural-biology studies over the past five decades.[15, 16, 22] It is surprising, however, that while thio-DNA derivatives have been shown to be effective sensitizers in the photodynamic treatment of various cancers,[29-34] the use of thio-RNA derivatives in phototherapeutic applications have received considerably less scrutinity.[18, 27, 28] Thio-RNA derivatives are not limited to interactions with DNA and proteins within the nucleus, but can also interact with proteins and other RNAs throughout the cell. Furthermore, highly-targeted therapeutics based on short, interfering RNA sequences (siRNAs) and on RNA aptamers that bind to intra- and extra-cellular proteins are rapidly emerging.[77] These therapies can readily incorporate 2,4dtUra to promote irreversible binding of their cellular targets through photocrosslinking reactions.[39-42] Indeed, RNA aptamers containing 4tUra are able to target and photocrosslink with extracellular marker proteins in live breast cancer cell cultures.[25] The results presented in this work suggest that 2,4dtUra could be even more effective in these types of applications. Furthermore, the increased targeting ability and higher $^1O_2$ yield of 2,4dtUra, as compared to 4tThd and 2,4dtThy,[71] suggest that this sensitizer could have greater photodynamic activity than these thio-DNA sensitizers,[29-34] or could be used as a complementary photosensitizer to target DNA and RNA simultaneously.

Conclusions

The detailed spectroscopic and photochemical investigation presented in this work reveals that doubling thionation of the uracil chromophore offers an attractive route to enhance the photocrosslinking and phototherapeutic potential of the RNA thiopyrimidine derivatives. Importantly, it is shown that 2,4dtUra is an effective photosensitizer, acting both in O_2-rich and O_2-depleted environments, which is able to absorb near-visible radiation with an efficiency that is almost thirtyfold greater than 4tUra. This could enable the effective use of 2,4dtUra in structural-biology investigations as well as in the photodynamic treatment of various tissues. Specifically, excitation of 2,4dtUra with near-visible radiation (~380 to 400 nm) leads to ultrafast population of the reactive triplet state in nearly unity yield. The triplet state can efficiently transfer its energy to molecular oxygen resulting in ca. 50% singlet oxygen generation or can react directly with 5'-AMP (and potentially with other nucleic acid bases) under N_2-saturated conditions

through a photocycloaddition mechanism. These observations suggest that 2,4dtUra can serve as a "drop-in" replacement in the wide range of photocrosslinking applications in which 4tUra is currently used,[16-26] offering enhanced photoreaction rates and the ability to be excited at longer wavelengths that penetrate approximately 140% deeper into tissues.

While thio-DNA bases have already shown great potential in phototherapeutic applications,[29-34, 71] thio-RNA bases have yet to be developed to such an extent. The photosensitizing properties afforded by doubling thionation of the uracil chromophore are expected to accelerate the use of 2,4dtUra derivatives in highly selective therapeutic applications that target RNA. In fact, preliminary experiments in our group,[78] in which epidermoid carcinoma cells are treated with 100 μM solutions of 2,4dtUra for 48 hours and subsequently irradiated with a UVA lamp (30 kJ/m^2 at 370 ± 25 nm), show a more than 50% decrease in cell survival rate relative to the control experiments (i.e., cells kept in the dark or cells exposed to UVA without the sensitizer). Optimization and mechanistic investigations of the photodynamic activity of 2,4dtUra is currently underway in our group.

Experimental

Chemicals

2-Thiouracil (2tUra, ≥99% purity), 4-thiouracil (4tUra, 97% purity), 2,4-dithiouracil (2,4dtUra, 98% purity), and adenosine 5'-monophosphate (5'-AMP, ≥99% purity) were obtained from Sigma-Aldrich and used as received. Phenalenone (97% purity) was also from Sigma-Aldrich and purified by recrystallization from ethanol. Aqueous phosphate-buffered saline solutions were freshly prepared in 100 mL of ultrapure water at pH 7.4 (0.12 g of sodium dihydrogen phosphate and 0.089 g of disodium hydrogen phosphate) and pH 5.4 (0.185 g of sodium dihydrogen phosphate and 0.009 g of disodium hydrogen phosphate). Solutions were adjusted to their specified pH by drop-wise addition of 2 M aqueous NaOH. Acetonitrile (≥99.9% purity) was obtained from Fisher Scientific and used as received.

Steady-state spectroscopy

Steady-state absorption spectra were measured using a Cary 100 Bio spectrometer. All absorption spectra were background corrected by subtracting the absorption of the neat solvent. Molar absorptivity coefficients were determined from the absorption spectra of serial dilutions of stock solutions with known concentrations.

Transient absorption spectroscopy

A detailed description of the transient absorption instrumentation used in this work has been described previously.[71, 79] Briefly, 800 nm, 100 fs fundamental pulses were generated with a Ti-Sapphire regenerative amplifier laser system (Libra-HE, Coherent, Inc.: 4.0 W, 1 kHz). The fundamental beam was used to produce pump wavelengths of 320, 335, and 350 nm via an optical parametric amplifier (TOPAS, Quantronix/Light Conversion). Unwanted wavelengths were removed from the pump beam using a reflective wavelength filter and a Glan-Taylor polarizer. A fraction of the remaining fundamental beam was focused into a continuously moving CaF_2 plate (2 mm thick) to generate broadband white light probe pulses (320 – 700 nm). The intensity of the excitation beam was attenuated to 1 μJ/pulse at the sample position. The polarization of the pump beam was randomized before being focused into the sample to overlap with the white light continuum probe at a 3:1 beam diameter ratio. Pump-probe experiments were collected using a Helios spectrometer (Ultrafast Systems, LLC) and a home-made data acquisition software (LabView, National Instruments, Inc.).

Solutions of the 2tUra, 4tUra, and 2,4dtUra derivatives were prepared in aqueous phosphate-buffered saline at pH 7.4. In the case of 2,4dtUra, solutions were also prepared at pH 5.4 due to its lower pK_a (7.4)[80] as compared to those of 2tUra (7.74)[81] and 4tUra (8.0).[82] The absorption spectrum of the excited deprotonated species of 2,4dtUra contributed slightly to the transient absorption spectra observed at pH 7.4. However, the intersystem crossing lifetime of 2,4dtUra at both pHs was identical within experimental uncertainties (220 ± 40 vs. 210 ± 50 fs). Solutions were investigated in 2 mm optical path length quartz cells and the irradiated volume was continuously refreshed by stirring with a Teflon-coated magnetic stir bar. Contributions to the transient data from any putative photoproduct formation were prevented by replacing the solution with fresh stock solution every 5 scans, or before 6% sample degradation (as monitored by a decrease in the steady-state absorbance of the lowest-energy absorption band). The UVA excitation wavelength used was varied depending on the absorption spectrum of the specific thiouracil derivative investigated. For 2tUra, an excitation wavelength of 320 nm was used, whereas 2,4dtUra was excited at 335 and 350 nm, and 4tUra was excited at all three of these UVA wavelengths. The dynamics did not display any excitation wavelength dependence in this range.

Decay of the triplet state of 4tUra and 2,4dtUra to the ground state was monitored using the same excitation and detection setups but probing with an electronically triggered broadband white light source (Eos, Ultrafast Systems, LLC) that has been conveniently integrated into the Helios spectrometer. This probe source has a spectral window from ~375 to 800 nm, a time resolution of about 400 ps, and a temporal window of up to 120 μs.[79] Aqueous buffer solutions of each thiouracil derivative were prepared at 24 μM in 1 cm path length septum-topped cuvettes and purged with ultrapure nitrogen for 30 min prior to testing. Data were collected with the solutions under a constant nitrogen flow, and exciting at 350 nm with 3 μJ per pulse for 10 min, corresponding to less than 5% degradation.

Transient absorption data analysis

A home-made LabView program (National Instruments, Inc.) was used to correct all transient absorption data for group velocity dispersion of the white light probe, as described in detail elsewhere.[79] For each data set, between 50 and 100

traces were selected across the entire range of probe wavelengths and globally analyzed in Igor Pro 6.32A (WaveMetrics, Inc.). Specifically, data sets for all thiouracil derivatives fit well to a sequential kinetic model, which was convoluted with an instrument response function of ~200 fs (FWHM), as determined by the coherence signal of methanol at the sample position.[83] The uncertainties reported for the triplet population lifetimes are twice the standard deviation from the average fitting of at least three independent datasets (i.e., recorded on three different days). Triplet-triplet absorption spectra were extracted from the global fitting analysis.

Determination of the triplet-triplet extinction coefficients and triplet yields

The extinction coefficients for the triplet-triplet absorption bands of 4tUra and 2,4dtUra were determined using the singlet depletion method.[84] This method is convenient for the thiobases, as previously shown for 4-thiothymidine[85] and 6-thioguanosine[86], because the bleaching signal can be selectively probed for both compounds within our probe wavelength range. Under these experimental conditions, the excited triplet state concentration, $[^3M^*]$, at any given delay time can be obtained using equation 1, where $\Delta A_{GS}(\lambda_1)$ is the intensity of the ground state bleaching signal at λ_1, $\varepsilon_{GS}(\lambda_1)$is the molar absorptivity of the ground state absorption at λ_1, and ℓ is the path length of the cuvette (0.2 cm).

$$\Delta A_{GS}(\lambda_1) = -\varepsilon_{GS}(\lambda_1)[^3M^*]\ell \quad (1)$$

Equation 2 can then be used to determine the triplet-triplet extinction coefficient, $\varepsilon^*_{T-T}(\lambda_2)$, at λ_2 using $[^3M^*]$ from equation 1 and the absorption intensity of the triplet band at λ_2, $\Delta A_T(\lambda_2)$. The wavelength of triplet-triplet absorption, λ_2, must be well-separated from the ground state bleaching signal.

$$\Delta A_T(\lambda_2) = \varepsilon^*_{T-T}(\lambda_2)[^3M^*]\ell \quad (2)$$

Using the singlet depletion method, the triplet-triplet extinction coefficients of 4tUra and 2,4dtUra were determined to be 3000 ± 600 M^{-1} cm^{-1} and 2500 ± 600 M^{-1} cm^{-1} at 600 nm, respectively. With these ε^*_{T-T} values, the triplet quantum yields can be determined using the relative actinometry method and equation 3.[84]

$$\Phi_T(U) = \frac{\Delta A_U(\lambda_4)\,\Phi_T(R)\varepsilon^*_R(\lambda_3)}{\Delta A_R(\lambda_3)\,\varepsilon^*_U(\lambda_4)} \quad (3)$$

This method requires back-to-back transient absorption data collection with a triplet reference compound that has a known triplet quantum yield, $\Phi_T(R)$. The back-to-back experiments must be performed using sample and reference solutions having the same optical density at the excitation wavelength and pumping with the same power. In this particular case we used 4-thiothymidine as the reference compound, which has a reported triplet yield of 1.0 ± 0.1.[87] It should be noted that our determination of the triplet-triplet extinction coefficient for 4-thiothymidine using the above singlet depletion method is in agreement with that previously reported (2500 ± 700 M^{-1} cm^{-1} at 520 nm),[86, 87] further supporting the use of this method herein. From the back-to-back transient absorption experiments, the intensity of the triplet-triplet absorption of the reference compound, $\Delta A_R(\lambda_3)$, and the unknown, $\Delta A_U(\lambda_4)$, are obtained. These can be taken at different wavelengths (λ_3 and λ_4, respectively) for the reference (R) and unknown (U), as long as the wavelengths chosen are well separated from the ground state bleaching signal and the triplet-triplet extinction coefficient is respectively known, $\varepsilon^*_R(\lambda_3)$ and $\varepsilon^*_U(\lambda_4)$. Having all this information, equation 3 is then used to find the triplet quantum yield of the unknown, $\Phi_T(U)$.

The triplet-triplet extinction coefficient of 2tUra was not determined using the singlet depletion method because the ground state absorption of 2tUra does not extend into the probe wavelength region used in this work. However, the triplet-triplet extinction coefficients and triplet yield of the structurally-similar 2-thiothymine have recently been reported.[88] Hence, the triplet yield of 2tUra was estimated from back-to-back experiments with the 2-thiothymine by assuming they have equal triplet-triplet extinction coefficients. This seems to be a good assumption given the comparable shapes and intensities of the triplet-triplet absorption spectra of these two compounds when testing solutions with identical optical densities at the excitation wavelength.[48, 71]

Singlet oxygen quantum yields

Solutions of 4tUra, 2,4dtUra, and the singlet oxygen standard (phenalenone; Φ_Δ = 0.98)[52] were prepared in acetonitrile, each with an absorbance of 0.3 at 355 nm in a 1 × 1 cm quartz cuvette. The solutions were purged with oxygen for 30 min, followed by the determination of quantum yields from back-to-back measurements of the singlet oxygen phosphorescence intensity at 1270 nm. The O_2-saturated solutions were excited at 355 nm (Spectra Physics GCR-150-30: 7 ns pulse width) and the phosphorescence at 1270 nm was detected with a NIR sensitive photomultiplier tube (H10330A-45, Hamamatsu).[71]

Photoreactivity measurements

Aqueous phosphate-buffered saline solutions, pH 7.4, containing either 4tUra or 2,4dtUra were prepared with and without 5'-AMP and loaded into 1 × 1 cm septum-top quartz cuvettes. The concentrations of the thiouracil derivatives and 5'-AMP were 24 and 120 μM, respectively. These concentrations were chosen in order to favor the bimolecular photoreaction between the thiouracil derivative and 5'-AMP, while simultaneously maintaining the absorbance of the solutions within the linear dynamic range limit of the UV-visible spectrophotometer used. All solutions were purged with ultrapure nitrogen for 30 min and kept under constant nitrogen flow throughout the irradiation period. An optical parametric amplifier (TOPAS, Quantronix/Light Conversion) pumped by our Libra laser system (described above) was used for monochromatic (±1 nm) irradiation of the samples. The irradiation beam diameter was approximately 7 mm at $1/e^2$

and had a power of 10 mW at the sample position, corresponding to an average intensity of 0.26 J/m^2 at the sample. Absorbance changes in the thiouracil solutions, with and without 5'-AMP, were monitored periodically during 365 nm irradiation for up to 90 min and while stored in the dark for up to three and a half days using UV-visible spectroscopy (Cary 100 Bio).

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