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Complete List of Authors:	<p>Nonoshita, Daiki; Aoyama Gakuin University College of Science and Engineering Graduate School of Science and Engineering, Chemistry and Biological Sciences</p> <p>Kashihara, Wataru; Aoyama Gakuin University College of Science and Engineering Graduate School of Science and Engineering, Chemistry and Biological Sciences</p> <p>Tanabe, Kazuhito; Aoyama Gakuin University - Sagamihara Campus , Department of Chemistry and Biological Science, College of Science and Engineering</p> <p>Isozaki, Tasuku; J F Oberlin University, Division of Natural Sciences, College of Arts and Sciences</p> <p>Xu, Yao; The Open University, School of Life, Health and Chemical Sciences</p> <p>Suzuki, Tadashi; Aoyama Gakuin University College of Science and Engineering Graduate School of Science and Engineering, Chemistry and Biological Sciences</p>

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**Excited States of Thio-2'-deoxyuridine Bearing an Extended
 π -Conjugated System:
3',5'-Di-*O*-acetyl-5-phenylethynyl-4-thio-2'-deoxyuridine**

Daiki Nonoshita[†], Wataru Kashiara[†], Kazuhito Tanabe[†], Tasuku Isozaki[‡], Yao-Zhong Xu[§], and Tadashi Suzuki^{†, *}

[†] Department of Chemistry and Biological Science, Aoyama Gakuin University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5258, Japan

[‡] Division of Natural Sciences, College of Arts and Sciences, J. F. Oberlin University, Tokiwa-machi, Machida, Tokyo 194-0294, Japan

[§] School of Life, Health and Chemical Sciences, The Open University, Milton Keynes MK7 6AA, United Kingdom

*Corresponding author e-mail: suzuki@chem.aoyama.ac.jp (Tadashi Suzuki)

ABSTRACT

A new thio-2'-deoxyuridine with an extended π -conjugated group was successfully synthesized: 3',5'-di-*O*-acetyl-5-phenylethynyl-4-thio-2'-deoxyuridine. The thio-2'-deoxyuridine derivative has a large red-shifted absorption band in the UVA region and also shows a fluorescence, a rare photo-property among thionucleobase/thionucleoside derivatives. Triplet-triplet absorption spectrum and the rate constants (the intrinsic decay rate constant of the triplet state, the self-quenching rate constant, and the quenching rate constant of the triplet state by an oxygen molecule) of the thio-2'-deoxyuridine were obtained by transient absorption spectroscopy. The quantum yield of intersystem crossing and the quantum yield of singlet molecular oxygen formation (ϕ_{Δ}) under oxygen atmosphere were also determined. The ϕ_{Δ} value of the new thio-2'-deoxyuridine was found to be substantially higher than all reported values of other thio-2'-deoxyribonucleosides in low oxygen concentrations similar to cancer cell environments. The fluorescence quantum yield depended on the excitation wavelength, revealing certain photochemical reactions in the higher excited singlet states. However, when excited into the higher excited state with non-resonant two-photon absorption, the ϕ_{Δ} of the thio-2'-deoxyuridine derivative was found to remain sufficiently large. These findings should be very useful for the development of thio-2'-deoxyribonucleoside-based pharmaceuticals as DNA-specific photosensitizers for photochemotherapy.

INTRODUCTION

Thionucleosides are sulfur-containing analogues of canonical nucleosides. As the replacement of an oxygen atom by a sulfur atom introduces a minimum structural change, these compounds can retain many of their original biological properties¹ and can be readily incorporated into synthetic DNA and RNA for structural studies.^{2–4} However, the introduction of a sulfur atom on the nucleobases gives thionucleosides certain unique photophysical characters, such as a prominent absorption band in the UVA region (320–400 nm) where native nucleosides are transparent.^{5–16} Whereas the relaxation process of native nucleosides in the excited singlet state is known to be ultrafast internal conversion (IC) to the ground state (the lifetime in the excited singlet state was reported to be within 1 ps),^{17–19} that of thionucleosides is highly efficient intersystem crossing (ISC) to the triplet manifolds, triggered by the thiocarbonyl group.^{5–12, 20–33} The triplet thionucleosides enable effective triplet energy transfer and/or electron transfer to generate singlet molecular oxygen ($^1\text{O}_2^*$), superoxide and other oxygen radical species through the photosensitization reaction,^{5–12} eventually causing cell death.^{34–38} Therefore, such a thionucleoside can be expected to act as a photosensitizer for photochemotherapy like photodynamic therapy (PDT)⁵. Currently, porphyrin derivatives such as *Photodyn* (Hematoporphyrin) and *Photofrin* (Porfimer Sodium) are widely used in the actual treatment for PDT.^{39, 40} On the other hand, metal-free triplet photosensitizers including a thiocarbonyl group have been, in recent years, investigated as alternative PDT agents since they have the advantages of lower molecular weight and easier dosage control compared with porphyrin derivatives.^{25, 41–45} Thus, thionucleosides, some of them have

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been already prescribed as a thionucleoside-based pharmaceutical, can be candidates of the new type photochemotherapy agents.

It is well known that the light penetration depth into biological tissues very much depends on the wavelength of light applied.^{46, 47} The penetration depth of the UVA light, which thionucleosides absorb, is relatively low. To develop desirable thionucleosides with an absorption band at longer wavelengths, one of the solutions would be to extend a π -conjugation of itself by the appropriate substitution. Some native nucleosides with a π -conjugated system at C5-position on the pyrimidine ring were synthesized⁴⁸ and their fluorescent properties and biological activities also investigated.^{49–52} 5-Phenylethynyl-2'-deoxyuridine (Figure 1, **2b**) is intrinsically fluorescent. Phenylethynyl is a large group, however, such a size of the π -conjugated system in DNA doesn't interfere the base-pairing.⁵³ Therefore, the combination of thionucleoside and the extended π -conjugation is well worth exploring and could bring new synergies.

In this article, we present our design and synthesis of a novel thio-2'-deoxyuridine with a π -conjugated system: 3',5'-di-*O*-acetyl-5-phenylethynyl-4-thio-2'-deoxyuridine (Figure 1, **1c**) and report our studies on the excited states of **1c** by steady state absorption/emission spectroscopy, transient absorption (TA), time-resolved near-infrared emission, time-resolved thermal lensing (TRTL) measurements, and quantum chemical calculations. We also describe photocytotoxic effects of **1c** on cancer cells, its excitation wavelength effects on $^1\text{O}_2^*$ formation quantum yield, and its two-photon absorption property. The potentials of the newly available thio-2'-deoxyribonucleoside are also discussed as a potent photosensitizer for photochemotherapy such as PDT.

EXPERIMENTAL SECTION

Materials

Commercially available reagents and solvents for syntheses were used without further purification. **2c** and 3',5'-di-*O*-acethyl-5-iodo-2'-deoxyuridine (**4**) were synthesized from the commercially available reagent, 5-iodo-2'-deoxyuridine (Tokyo Chemical Industry Co., Ltd.), according to the previous literature.^{54, 55} The synthetic route to **1c** is shown in Scheme 1. Initially, we tried the thiolation of **2c** using Lawesson's reagents following reported procedure⁸, however, only a trace amount of **1c** was afforded. Thus, we explored various conditions (Table S1 in the Supporting Information) to convert **4** into its 4-thio-anologue (**5**)^{56, 57} using Lawesson's reagents and then conducted Sonogashira coupling with phenylacetylene to obtain the desired phenylethynyl-substituted thio-2'-deoxyuridine **1c** in an acceptable yield. The characterization of **1c** is described in the Supporting Information (Figures S1–S4). Our synthetic route to the highly π -conjugated thio-2'-deoxyuridine would be applicable to other photo-sensitive thionucleobases/ thionucleosides. Human lung carcinoma cell line, A549, was purchased from JCRB cell bank (Japanese Collection of Research Bioresources Cell Bank).

Apparatus

Nanosecond TA measurements were carried out with a XeCl excimer laser (Lambda Physik, COMpex 102; 308 nm, 60 mJ per pulse, 20 ns pulse duration, repetition rate 2 Hz) as an excitation light source. White continuum light from a Xe flash lamp (Ushio

UXL-300D; 300 W) was used as a probe light to monitor the TA change. The monitoring light, passing through a flow cell (Tosoh Quartz T514M-ES-10; 10 mm optical path length), was dispersed by a monochromator (Nikon, P-250) and then detected by a photomultiplier tube (Hamamatsu Photonics, R928). The output signals from the photomultiplier tube were fed into a digital oscilloscope (Sony Tektronix, TDS380P; 400 MHz, 2 GS s⁻¹) and analyzed by a personal computer. These signals were averaged over 100 shots to improve the S/N ratio. The incident laser power and the concentration of solution were controlled to avoid triplet-triplet annihilation or any multiphotonic incidents, and the linearity of the signal intensity against the incident laser power was confirmed on each experiment. The sample solution flowed into the the cuvette to remove the influence of photoproducts. All measurements were carried out at room temperature.

TRTL measurements were carried out with a Nd³⁺:YAG laser (Continuum, Surelite II; pulse width 4–6 ns, repetition rate 2 Hz; 355nm) or the XeCl excimer laser as an excitation light source. The excitation light was focused into the cell with a 120 mm focal length lens. A cw beam from a He-Ne laser (NEOARK, HN-530P; 632.8 nm) for probing of the TRTL signal was focused in front of the cell with a 80 mm focal length lens coaxially to the excitation laser. The probe beam emerging from the cell was passed through a pinhole (100 μm diameter) and then detected by the photomultiplier tube.

Time-resolved near-infrared emission measurement was carried out with a thermoelectric cooled near-infrared photomultiplier tube (Hamamatsu Photonics, H10330-45; InP/InGaAsP, spectral response 950 to 1400 nm) combined with a longpass filter (Thorlabs, FEL1250; cut-on wavelength 1250 nm) and a bandpass filter (Edmund,

Hard-coated bandpass filter; 1275±50 nm). The Nd³⁺:YAG laser or the XeCl excimer laser was used as an excitation light source.

UV/vis absorption spectra were measured with a spectrophotometer (JASCO V-550), and emission spectra were obtained using a spectrofluorometer (JASCO FP-6500).

Quantum Chemical Calculations

Quantum chemical calculations for corresponding uracil derivatives were performed with the Gaussian 09W program package.⁵⁸ Ground-state geometries were optimized by the density functional theory (DFT) at the B3LYP/6-311+G(d,p) level. Vertical excitation energies were estimated by the time-dependent DFT (TD-DFT) at the TD-B3LYP/6-311+G(d,p) level. For the spectral simulation of two-photon absorption, the vertical transition energies, the transition dipole moments to the excited singlet states, the transition dipole moments between excited states, and the permanent dipole moments of the ground and the excited states necessary were estimated by the TD-DFT with Tamm-Dancoff approximation (TDA).⁵⁹ Simulation of the two-photon absorption spectra was performed using sum-over state formalism summing all intermediate and final states considered in the calculation (20 excited states).⁶⁰ The dumping constant of the Lorentzian linewidth function was taken to be 0.25 eV.⁶¹ Solvent effects were modeled with the polarizable continuum model (PCM) for the ground- and the excited-states.

Cell Culture and Cell Treatments

A549 cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were maintained at 37 °C in 5% CO₂ and were kept in the logarithmic growth phase by routine passages every 3–4 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer. A549 cells (2.0×10^3 cells) were plated into 96 well plate with or without 100 mM of **1c** and cultured for 48 h. After incubation of the cells, photoirradiation (365 nm) was conducted for 5 minutes (15 kJ/m²). Then, the medium was replaced to fresh medium. After 72 h incubation, the cells were washed with PBS and replaced to 100 mL fresh medium and 10 µL Cell Counting Kit 8 solution (Dojindo), and the cell viability assay was performed by xMark Microplate Spectrophotometer at 450 nm (Bio–Rad).⁶²

RESULTS AND DISCUSSION

Absorption and Emission Spectra

Figure 2a shows the absorption spectra of **1c**, acetylated 5-phenylethynyl-2'-deoxyuridine (**2c**), and 4-thiouracil (**3a**) in acetonitrile. The shapes of the absorption spectra of **3a** and 4-thiouridine have been found to be nearly identical,^{5,10} and the sugar moiety and its acetylated form had little effect on electronic states concerning with transitions in this spectral range of nucleobases/nucleosides.^{6,8,12} The prominent first absorption band of **1c** appeared at around 369 nm with the molar absorption coefficient $\epsilon_{1c}^{369\text{ nm}} = (4.39 \pm 0.04) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, which was assigned to $^1\pi\pi^*$, the LUMO $\leftarrow$ HOMO transition in Figures 2b, 3, and Table 1. Other transitions were also assigned and are listed in Table 1, Figure S5 and Table S2 in the Supporting Information. The calculated results for **2a** and **3a** are also shown in Figures S5 to S7, and Tables S3 to S6 in the Supporting Information. The energy diagrams of **1a** and **3a** are shown in Figure S8 in the Supporting Information. The transition to the lowest excited singlet state was the $^1n\pi^*$ transition at 2.99 eV, corresponding to the LUMO $\leftarrow$ HOMO-1 transition. The oscillator strength of the $^1n\pi^*$ transition was extremely low ($f=0.0001$). The absorption peak at 369 nm of **1c** was the longest wavelength among all thiopyrimidine derivatives reported (Table 2). The red-shift of **1c** should be ascribed to the conjugation of a phenylethynyl group attached to the thiouracil frame, which is also reflected in the molecular orbitals of HOMO and LUMO of **1a** as shown in Figure 2b.

The emission spectrum of **1c**, when excited at 355 nm, is shown in Figure 3a. The intense emission was detected at around 450 nm, and the fluorescence quantum yield (ϕ_f) was determined to be 0.09 ± 0.01 by comparing the fluorescence spectra of

9,10-diphenylanthracene as a standard.^{63, 64} It was a substantially high value despite the extremely small ϕ_f values of thiopyrimidine and thiopurine derivatives (10^{-5} – 10^{-4}).⁵ To the our best knowledge, this is the first report that a thionucleoside (such as **1c**) has a relatively high ϕ_f value. The phenylethynyl group in **1c** should have a large effect on the potential energy surface in the excited state, causing the relaxation process very different from that of other thionucleoside derivatives.

Triplet–triplet Absorption Spectra and Quantum Yield of Intersystem Crossing

Figure 4 shows the TA spectra for **1c** in Ar-saturated acetonitrile using a pulsed excimer laser of 308 nm. The bleach signal was detected at less than 330 nm. This would result from depletion of **1c** in the ground state. The observed TA spectra show a strong band at 330–600 nm, which can be assigned to the triplet–triplet absorption of **1c**. To confirm the assignment, the effect of the O₂ dissolved in acetonitrile was examined. The typical time profiles of TA monitored at 390 nm in Ar-saturated and N₂/O₂-mixed acetonitrile are shown in the inset of Figure 4. The time profiles of the TA intensity decreased single-exponentially, however, the lifetime in the presence of O₂ became shorter. Therefore, the band at 330–600 nm was safely assigned to the triplet–triplet absorption of **1c**.

To investigate the influences of dissolved O₂ on the excited triplet states of **1c**, the time profile of the TA was measured at various oxygen concentrations ([O₂]=9.1 mM under the O₂-saturated condition⁶⁵). Figure 5a illustrates the plots of the decay rate constant (k_{obs}) against the concentration of oxygen dissolved in acetonitrile, together with the

fitting by the single exponential function. The kinetics of the decay process could be described as follows,

$$k_{\text{obs}} = k_{\text{T}} + k_{\text{q}}[\text{O}_2] \quad (1)$$

where k_{T} and k_{q} denote the decay rate constant of the triplet state itself and the quenching rate constant of the triplet **1c** by O_2 , respectively. As shown in Figure 5a, these plots show a good linear relationship. By analyzing these plots using eq. (1), the quenching rate constant by O_2 (k_{q}) was estimated to be $(2.7 \pm 0.1) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. The k_{q} value is similar to the ones for other thiopyrimidine derivatives previously reported (Table 2). These results suggest that **1c** should be able to effectively generate $^1\text{O}_2^*$ (see below).

Figure 5b shows the time profiles of TA monitored at 390 nm under various concentrations of **1c** in Ar-saturated solution. The decay rate constant of the excited triplet state increases along with the concentration of **1c**, suggesting the existence of the self-quenching process by ground state of **1c**. Figure 5c indicates the plots of the decay rate constant of excited triplet state (k_{T}) against the concentration of **1c**. The plots show a good linear relationship. The triplet self-quenching kinetics can be written as follows,

$$k_{\text{T}} = k_0 + k_{\text{SQ}}[\textbf{1c}] \quad (2)$$

where k_0 and k_{SQ} denote the intrinsic decay rate constant of the triplet state of **1c** and the self-quenching rate constant, respectively. By analyzing with eq. (2), the k_0 and the k_{SQ} values for **1c** were determined to be $(8.5 \pm 0.3) \times 10^4 \text{ s}^{-1}$ and $(7.3 \pm 0.6) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The intrinsic lifetime of triplet for **1c** ($\tau_0 = 1/k_0$) was estimated to be $12 \pm 1 \mu\text{s}$. The triplet **1c** was found to have a relatively long lifetime in comparison with other thionucleosides (see Table 2).

Figure 6a depicts the time evolution of the TRTL signal for **1c** in Ar-saturated acetonitrile obtained by the 355 nm laser irradiation. The TRTL method can detect the temporal heat released by the excited molecule through non-radiative processes. The signal consists of two rising components: the fast (U_F) and the slow (U_S) components. The U_F component is derived from vibrational cooling, IC to the S_0 state, and ISC to the T_1 state, while the U_S component is due to ISC from the T_1 to the S_0 states. The U_T indicates total quantity of heat ($U_T=U_F+U_S$).

The quantum yield of ISC, Φ_{ISC} , was described as the following equation.^{6, 66}

$$\Phi_{ISC} = \frac{U_S}{U_T} \times \frac{\alpha \cdot E_{EX}}{E_T} \quad (3)$$

Here, α is the heat conversion efficiency, namely, the fraction of energy released as heat against the energy absorbed. E_{EX} is the excitation energy. E_T is the lowest triplet (T_1) energy. No phosphorescence of **1c** was observed even at 77 K, therefore, the T_1 energy was estimated from quantum chemical calculation to be 2.30 eV (Table 1). With benzophenone as a calorimetric standard,⁶⁶ the α value was obtained to be 0.90 ± 0.02 at 355 nm excitation. The detailed procedure was presented in Figures S9–S13 in the Supporting Information. Thus, as the ratio of U_S/U_T was obtained to be 0.41 ± 0.01 (Figure 6b), the ϕ_{ISC} value was successfully determined to be 0.55 ± 0.02 . This value of **1c** was found to be less than that of other thiopyrimidine derivatives reported (Table 2). In general, the dominant relaxation process for thionucleosides was reported to be ISC.⁵ Taking account of the results for $\alpha=0.90$, $\phi_I=0.09$, and $\phi_{ISC}=0.55$, the rest should be the IC process, and the quantum yield of IC for **1c** would be as large as 0.36 ± 0.02 . It suggested that the

introduction of a phenylethynyl group into the thio-2'-deoxyribonucleosides should increase the contribution of the IC in addition to the fluorescence processes.

Estimation of Quantum Yield of the $^1\text{O}_2^*$ Formation

The production of $^1\text{O}_2^*$ has been examined to check whether **1c** would be useful as a potential drug for photochemotherapy or not. The phosphorescence decay profiles of $^1\text{O}_2^*$ at around 1275 nm of **1c** and phenalenone (PN) as a reference compound⁶⁷ were measured in oxygen-saturated acetonitrile (Figure 7a). The decaying signals were analyzed with a single-exponential function, and the lifetime of $^1\text{O}_2^*$ was obtained to be 67 ± 2 μs , which agrees well with the reported lifetime of $^1\text{O}_2^*$ (about 65 μs).^{8, 68–70} The emission intensity maximum immediately after laser irradiation (I_S^0) was plotted against the laser fluence (I_L) (Figure 7a inset and Figures S14 to S16 in the Supporting Information). A good linear relationship was observed between I_S^0 and I_L . This finding reveals that $^1\text{O}_2^*$ was generated by one-photon process through photosensitization by **1c** and PN. The values of the slope obtained from these plots (I_S^0/I_L) were plotted against the ground-state absorbance ($1 - 10^{-A}$) where A denotes the absorbance at the excitation wavelength (Figure 7b). These plots also show good linear relationships. By comparing the slopes of **1c** with that of PN (the formation quantum yield of $^1\text{O}_2^*$, ϕ_Δ , by PN was reported to be 1.00 ± 0.02 ⁶⁷), we successfully determined the ϕ_Δ value for **1c** with a high degree of accuracy to be 0.40 ± 0.01 at 355 nm irradiation. The ϕ_Δ value for **1c** is close to that of other thiopyrimidine derivatives summarized in Table 2, indicating **1c** has a potential as a good photochemotherapeutic agent. **3a** has been reported to react with $^1\text{O}_2^*$, generating the photo-oxidation products such as uracil-6-sulfonate (U^{SO_3}).³⁷ Thus, it is highly likely

that **1c** will produce photo-oxidation products in which the sulfur atom at 4-position in the pyrimidine skeleton is oxidized, similar to 4-thiouracil.³⁷ Since the lifetime of $^1\text{O}_2^*$ obtained in the work is almost the same as the reported ones,^{8, 68-70} the photo-oxidation reaction of **1c** would not be a minor process.

Estimation of Quantum Yield of $^1\text{O}_2^$ in Carcinomatous Condition*

The important photophysical property for a good potential photosensitizer is to have a high ϕ_Δ value in the low oxygen concentration condition similar to the microenvironment for cancer cells (about 18 μM of O_2 is contained in general cancer cells⁷¹). The ϕ_Δ value largely depends on the O_2 concentration as follows.

$$\phi_\Delta = \frac{S_\Delta \cdot \phi_{\text{ISC}} \cdot k_q[\text{O}_2]}{k_0 + k_{\text{SQ}}[\mathbf{1c}] + k_q[\text{O}_2]} \quad (4)$$

Here, S_Δ denotes the fraction of the triplet state of **1c** quenched by dissolved oxygen which gives rise to the $^1\text{O}_2^*$ formation. Figure 8 shows the oxygen concentration dependence of the ϕ_Δ value at 21 μM of **1c**. The ϕ_Δ value gradually decreased as the concentration of O_2 decreased. The plots were analyzed with eq. (4) and the experimental results obtained above (ϕ_{ISC} , k_0 , k_{SQ} , and k_q), and the best fitting curve was calculated with S_Δ as a fitting parameter as shown by a blue solid line in Figure 8. The S_Δ value of **1c** was successfully estimated to be 0.73 ± 0.04 . This S_Δ value was found to be substantially high compared with other thiopyrimidine derivatives (see Table 2). This high S_Δ value is believed to result from two factors of the T_1 character. Firstly, the T_1 transition of **1c** was $^3\pi\pi^*$ (Tables 1 and S2). The $^3\pi\pi^*$ state of polynuclear aromatics was known to have a higher

value of S_{Δ} . For example, the S_{Δ} values for naphthalene and anthracene were reported to be about 1.0 and 0.7, respectively.^{72, 73} Secondly, the T_1 energy of **1c** was quite low among thiopyrimidine derivatives (Table 2). It was suggested that the smaller E_T tended to have a higher S_{Δ} value.⁷³ The ϕ_{Δ} value of **1c** was the largest at lower oxygen concentrations (lower than 100 μ M) among the thionucleosides examined.²¹ The highest value of ϕ_{Δ} even in low oxygen condition like those within cancer cells, was found to result from the high S_{Δ} value in addition to the low k_0 and k_{SQ} values.

Evaluation of UVA-Induced Toxicity

We examined the cytotoxicity induced by **1c** using a human cell line of lung adenocarcinoma, A549. As demonstrated in Figure 9, UVA irradiation to A549 cells in the presence of **1c** (100 μ M) led to cell death dramatically. By contrast, little cell death was observed in the control groups (non-treated, **1c**-treated only and light-irradiated only). This finding confirmed **1c** retains the cytotoxicity as other thionucleosides when combined with UVA irradiation.^{13, 34–36} 4-Thiothymidine is incorporated into DNA after 48 h of incubation and caused DNA damage when exposed to UVA light.³⁶ The acetylation of 2-deoxyribose moiety of **1c** might prevent incorporation into cellular DNA, however, according to the results, **1c** is likely to be incorporated into cells and the cytotoxicity would be caused by $^1O_2^*$. Thionucleosides/Thionucleobases have been found to undergo type I and type II reactions after light exposure.^{5, 74} The oxidation potentials of thionucleosides/thionucleobases (such as 6-thioguanosine and 4-thiouracil) have been reported to be lower than that of guanine, tryptophan, and tyrosine^{75, 76} and are lower than that of charge transfer target biomolecules such as guanine, tryptophan, and tyrosine.^{77–81}

Though the oxidation potential of **1c** has not been determined yet. Thus, it is not likely that triplet excited **1c** is capable of oxidizing such biomolecules through charge transfer. Since the triplet energy of **1c** is lower than that of normal pyrimidine derivatives,⁸² triplet-triplet energy transfer from **1c** to pyrimidine bases would not occur and the cyclobutane pyrimidine dimers would not be formed.⁸³ Based on these findings, the type II reaction will be appropriate for **1c**. Furthermore, as **1c** can absorb at longer wavelengths and have the highest value of ϕ_{Δ} , therefore it can be exploited as an ideal photosensitizer for photochemotherapy.

Excitation Wavelength Effects on the ϕ_{Δ} Value

Fluorescence excitation spectrum of **1c** was measured and a large difference between the excitation and the absorption spectra below 340 nm was observed (Figure S17). This finding suggests that the relaxation processes below 340 nm should be different from those at 355 nm and above wavelength where little difference was noted. The relaxation process of **1c** from the second prominent absorption was examined. When excited **1c** at 308 nm, the ϕ_f was determined to be 0.05 ± 0.01 . Other values were also determined to be $\alpha = 0.84 \pm 0.02$, $\phi_{ISC} = 0.48 \pm 0.02$, and $\phi_{\Delta} = 0.35 \pm 0.01$ with 308 nm excitation (see Figures S11, S12, S13, S15, and S16 in the Supporting Information). When excited at 355 nm, almost all **1c** in the S_2 state goes back to the ground state. In contrast, when excited at 308 nm, the sum of the quantum yields of ϕ_f , ϕ_{ISC} , and ϕ_{IC} was about 0.89, suggesting another process, namely a photochemical reaction, had taken place. However, the ϕ_{Δ} value of **1c**, even excited into the higher excited state, was found to remain sufficiently large ($\phi_{\Delta} \approx 0.35$).

The ϕ_{Δ} values in the higher-order relaxation process assuming two-photon excitation is important to evaluate the potential of two-photon PDT as a photosensitizer for thionucleosides.⁶¹ Two-photon PDT has attracted much attention due to significant benefits such as much longer wavelength compared with one-photon excitation and spatial selectivity.^{84–86} We simulated the two-photon absorption spectra of **1a**, **3a** (Figure 10), and other pyrimidine derivatives (Figure S18). The strong absorption band was obtained at 476 nm, and can be assigned to the $S_9 (\pi\pi^*) \leftarrow S_0$ transition. The two-photon absorption cross section value, $\sigma^{(2)}$, of **1a** was found to be far larger than that of any other thiopyrimidine derivatives. The large $\sigma^{(2)}$ can be attributed to the substitution of the phenylethynyl group.

Recently, we reported the two-photon absorption spectrum of a thionucleoside (2',3',5'-tri-*O*-acetyl-6,8-dithioguanosine).⁶¹ Our simulated two-photon cross section peak of **1a** was found to be about three times larger than that of the 6,8-dithioguanine.⁶¹ As **1a** is part of **1c**, then **1c** is expected to retain these desirable photo-properties, which would make **1c** an excellent agent to induce the apoptosis for more deep-seated tumors.

CONCLUSIONS

We synthesized a new type of thio-2'-deoxyuridine derivative (**1c**), and investigated its excited state dynamics and its cytotoxicity. The prominent first absorption band at around 370 nm of **1c** was the longest wavelength among all related thiopyrimidine derivatives. The fluorescence quantum yield (ϕ_f) was determined to be 0.09 at 355 nm. This is the first report that such a thio-substituted compound has a high fluorescence yield. The broad TA spectra of **1c** were observed in the visible region. These spectra were assigned to the triplet-triplet absorption. The quenching rate constant by the dissolved oxygen (k_q) was successfully determined. The excited triplet lifetime was found to depend on the ground state concentration, and the self-quenching rate constant (k_{SQ}) and the intrinsic decay rate constant of the triplet state (k_0) were successfully determined. The ϕ_{ISC} value of **1c** was determined to be 0.55 at 355 nm excitation by the TRTL measurement. The ϕ_Δ value (0.40) in the oxygen saturated condition revealed that **1c** could also be used as a potent photochemotherapeutic agent, and the ϕ_Δ value was found to be higher than those of other thio-substituted nucleobases even under hypoxic conditions like cancer cells. The effect of cytotoxicity was demonstrated by irradiating **1c** *in vitro*. The difference between absorption and fluorescence excitation spectra suggested certain photochemical reactions in the higher excited states, however, **1c** was still able to effectively generate $^1O_2^*$ at 308 nm excitation. The high two-photon absorption property will make **1c** excited at longer wavelengths. The characteristics of the excited states for **1c** offers the possibility to develop a more specific photochemotherapy for deep-seated tumors.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at

<http://>

Synthesis, characterization, molecular orbitals, vertical transition energies, energy diagrams, time evolution of TRTL signals, plots of the TRTL signal intensities for the U_T and U_S components against incident laser power, plots of the TRTL signal intensities for U_T/I_L and U_S/I_L components against absorbance, plots of the I_S^0 against I_L , phosphorescence decay profiles of $^1O_2^*$ measured at around 1275 nm, plots of the I_S^0/I_L value against the absorbance, fluorescence excitation spectrum, fluorescence spectra, and two-photon absorption spectra (PDF)

AUTHOR INFORMATION

Corresponding Authors

Tadashi Suzuki – *Department of Chemistry and Biological Science, Aoyama Gakuin University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5258, Japan;*
orcid.org/0000-0002-9145-8601;

Email: suzuki@chem.aoyama.ac.jp

Authors

Daiki Nonoshita – *Department of Chemistry and Biological Science, Aoyama Gakuin University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5258, Japan*

Wataru Kashiara – *Department of Chemistry and Biological Science, Aoyama Gakuin University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5258, Japan*

Kazuhito Tanabe – *Department of Chemistry and Biological Science, Aoyama Gakuin University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5258, Japan*

Tasuku Isozaki – *Division of Natural Sciences, College of Arts and Sciences, J. F. Oberlin University, Tokiwa-machi, Machida, Tokyo 194-0294, Japan*

Yao-Zhong Xu – *School of Life, Health and Chemical Sciences, The Open University, Milton Keynes MK7 6AA, United Kingdom*

Complete contact information is available at:

<https://>

Notes

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Table 1. Spectroscopic properties for singlet and triplet excited states of **1c**.

Transition from S_0	λ_{max} ^a / nm (eV)	ϵ ^b / $10^4 \text{ M}^{-1} \text{ cm}^{-1}$	E_{calc} ^{c, d} / eV	f_{calc} ^e
$S_1 \ n\pi^*$			2.99	0.0001
$S_2 \ \pi\pi^*$	369 (3.36)	0.44	3.37	0.1318
$S_3 \ \pi\pi^*$	321 (3.86)	0.93	3.89	0.7535
$S_4 \ n\pi^*$			3.97	0.0000
$S_5 \ \pi\pi^*$	309 (4.01)	1.17	4.21	0.1329
$S_6 \ n\pi^*$			4.48	0.0005
$S_7 \ n\pi^*$			4.69	0.0001
$S_8 \ n\pi^*$			4.70	0.0007
$S_9 \ \pi\pi^*$	267 (4.64)	2.15	4.83	0.4042
$T_1 \ \pi\pi^*$			2.30	
$T_2 \ \pi\pi^*$			2.73	
$T_3 \ n\pi^*$			2.88	
$T_4 \ \pi\pi^*$			3.27	

^a Wavelengths of the maximum absorption band. ^b Molar absorption coefficient at the absorption peak. ^c The values were calculated by the TD–DFT method for the molecular configuration optimized in the S_0 state at the PCM/B3LYP/6–311+G (d,p) level for **1a**. ^d Values are not scaled. ^e Oscillator strength was obtained by the TD–DFT calculation for **1a**.

Table 2. Photophysical characteristics of thiopyrimidine derivatives.

	$\lambda_{\text{max}}/\text{nm}$	$\epsilon/10^3 \text{ M}^{-1}\text{cm}^{-1}$	ϕ_{A}	ϕ_{ISC}	$\tau_0/\mu\text{s}$	$k_{\text{q}}/10^9 \text{ M}^{-1} \text{ s}^{-1}$	$k_{\text{SQ}}/10^9 \text{ M}^{-1} \text{ s}^{-1}$	S_{A}	E_{T}/eV	References
1c	369	4.39±0.04	0.40±0.01	0.55±0.02	12±1	2.7±0.1	0.73±0.06	0.73±0.04	2.30^a	This work
3a	330	16.7	0.49±0.02	0.90±0.15	2.0 ^b	6.8 ^b	5.1 ^b	0.54±0.09	2.76 ^a	10, 15
2-thiouracil	270	14.8	—	0.75±0.20	0.21±0.02 ^c	—	—	—	—	5, 10
2,4-dithiouracil	361	11.4	0.49±0.02	0.90±0.15	0.23 ^d	—	—	0.54±0.09	—	5, 10
4-thiothymidine	337	19.4	0.50±0.10	1.0±0.1	4.2±3.0	6.4±1.0	7.8±0.4	0.50±0.11	2.51 ^e	6
2-thiothymine	300	11.6	0.36±0.02	1.00±0.02	2.7±0.5	5.1±0.1	1.53±0.05	0.36±0.02	3.14 ^a	7, 16
2,4-dithiothymine	362	11.3	0.46±0.02	≥0.9	0.25±0.03 ^f	—	—	≥0.51	—	5, 11

^a The E_{T} values were estimated by the TD-DFT method. ^b These values were for the compound 2,3,5-tri-*O*-acetyl-4-thiouridine. ^c The value was reported under the concentration of 1.0 mM for 2-thiouracil. ^d The value was reported under the concentration of 24 μM for 2,4-dithiouracil. ^e The E_{T} value was determined by the phosphorescence spectrum. ^f The value was reported under the concentration of 0.4 mM for 2,4-dithiothymine.

Scheme 1. Reagents and conditions: (a) Lawesson's reagents, dioxane, 4 h reflux, 78%, and (b) ethynylbenzene, Pd(PPh₃)₄, CuI, triethylamine, DMF, 4 h 40 °C, 23%.

Figure 1. Molecular structures of uracil derivatives and their nucleosides.

Figure 2. (a) Steady-state absorption spectra of **1c** (blue), **2c** (green), and **3a** (red), and (b) molecular orbitals involved in transitions to the first, second, and third excited states of **1a** and **2a**.

Figure 3. (a) A steady-state absorption spectrum (blue solid-line) and an emission spectrum (blue broken-line) of **1c** in acetonitrile solution, and (b) computational vertical transition energies and oscillator strengths (f) of **1a**.

Figure 4. Transient absorption spectra of **1c** in Ar-saturated acetonitrile at several delay times after the laser irradiation. Inset: Time profiles of transient absorption for **1c** (34.0 μM) monitored at 390 nm in Ar-saturated (green) and N_2/O_2 mixed (blue) acetonitrile.

Figure 5. (a) Plots of the decay rate constant (k_{obs}) against the concentration of dissolved oxygen. (b) Time profiles of transient absorption monitored at 390 nm for various concentrations of **1c** in Ar-saturated acetonitrile. (c) Plots of the triplet decay rate constant (k_{T}) of **1c** against the ground-state **1c** concentration.

(a)

(b)

Figure 6. (a) Time evolution of the TRTL signal of **1c** in Ar-saturated acetonitrile excited at the 355 nm. (b) Plots of the TRTL signal intensities for U_T/I_L (yellow) and U_S/I_L (purple) components against absorbance of **1c**.

Figure 7. (a) Time profiles of $^1\text{O}_2^*$ phosphorescence measured at around 1275 nm of **1c** (blue) and phenalenone (PN) (green) in oxygen-saturated acetonitrile. Inset: Plots of the emission intensity maxima (I_s^0) immediately after laser irradiation for **1c** and PN against incident laser power (I_L). (b) Plots of the I_s^0/I_L value of **1c** and PN against the absorbance ($1-10^{-A}$) at excitation wavelength (355 nm).

Figure 8. Extrapolation of the ϕ_{Δ} values at infinitely low oxygen concentrations using the kinetics parameters estimated in this study. Each of the concentration (**1c**, **3a**, and 2-thiouracil) was set to 21 μ M in this simulation. The concentration of O_2 in general cancer cell is highlighted (purple).

Figure 9. Cell viability of A549 cells: (I) no-treated and no-UVA, (II) treated with **1c** (100 μ M) and no-UVA, (III) no-treated and UVA light for 5 minutes (15 kJ/m²), and (IV) treated with **1c** (100 μ M) and UVA light for 5 minutes (15 kJ/m²); bars, SD ($n=5$).

Figure 10. Simulated two-photon absorption spectra of **1a** (blue) and **3a** (red) in acetonitrile.

TOC Figure