ARTICLES

Time-Resolved Spectroscopy of the Excited Singlet States of Tirapazamine and Desoxytirapazamine

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Laser flash photolysis (LFP, 400 nm excitation) of the anti-cancer drug tirapazamine (TPZ) in acetonitrile produces the singlet excited-state S_1 with $\lambda_{max} = 544$ nm. The lifetime of this state is 130 ps, in good agreement with the reported fluorescence lifetime. The excited state is reduced to the corresponding radical anion by KSCN or KI. The spectrum of the radical anion is in good agreement with previously reported pulse radiolysis studies and time-dependent density functional theory (TD-DFT) calculations. LFP of desoxytirapazamine (dTPZ) also produces the first excited singlet state, S_1. The fluorescence quantum yield and lifetime (5.4 ns) of the dTPZ singlet excited state are both much greater than the corresponding values of TPZ. This is explained by DFT calculations that predict that cyclization of TPZ to form an oxaziridine is thermodynamically facile but that cyclization of dTPZ to form an oxadiaziridine is not. Thus, the S_1 state of TPZ has a short lifetime and low fluorescence quantum yield due to ready cyclization whereas the cyclization of the S_1 state of dTPZ is unimportant and does not limit either the fluorescence quantum yield or the fluorescence lifetime. This conclusion is confirmed by studies of dTPZ′, an isomer of dTPZ containing the C═N−O moiety which has a low quantum yield and short fluorescence lifetime similar to that of TPZ.

1. Introduction

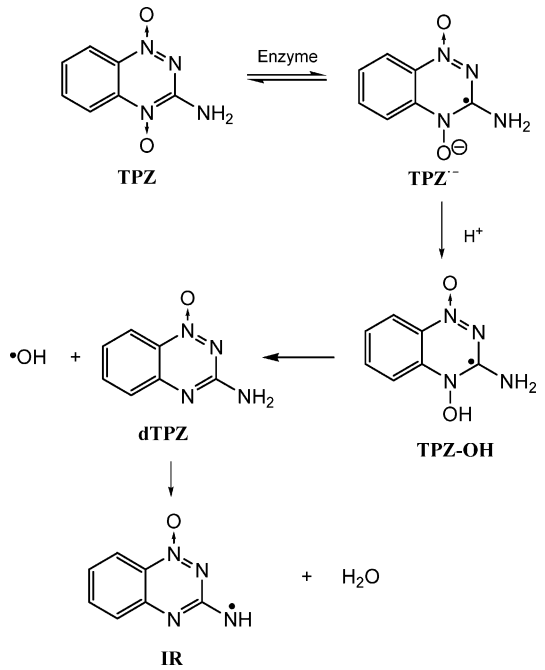
Tirapazamine (TPZ) is a promising anti-cancer drug that is currently in Phase-III clinical trials.[1] It is generally believed that TPZ is enzymatically reduced to the corresponding radical anion (Scheme 1).[2] Protonation of the TPZ radical anion produces a neutral radical, TPZOH, which is believed to fragment to form the inactive metabolite desoxytirapazamine (dTPZ, 3-amino-1,2,4-benzotriazine-1-*N*-oxide) and hydroxyl radical.[3] The hydroxyl radical or the iminyl radical (IR),[4] or a tautomer, formed by reaction of hydroxyl radical with dTPZ is likely responsible for abstracting a hydrogen atom from a DNA sugar and subsequent strand nicking. The sensitivity of the TPZ radical anion to oxygen accounts for the greater potency of the drug under hypoxic (oxygen-deficient) conditions (Scheme 1).[1]

We are interested in initiating these reactions photochemically to develop a source of hydroxyl radical using visible light.[5] Here we report our studies of the excited singlet states of TPZ, desoxytirapazamine and its isomer dTPZ′, quinoxaline di-*N*-oxide (QXNO), and phenazine di-*N*-oxides (PNNO).

2. Experimental Section

Picosecond time-resolved measurements were performed at the Ohio Laboratory for Kinetic Spectrometry at Bowling Green State University and the Center for Chemical and Biophysical Dynamics of The Ohio State University. The setup used for the ultrafast transient absorption spectrometric experiments at BGSU has been detailed elsewhere.[6] Briefly, the output of a

SCHEME 1



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Ti:sapphire laser (Spectra-Physics, Hurricane) (fwhm = 150 fs) was coupled into a second-harmonic generator (CSK Super Tripler) to obtain the 400 nm excitation wavelength. The energy of the probe pulse was less than 1 μJ/cm^2 at the sample. The pump beam used was approximately 5 μJ/pulse with a spot size of 1−2 mm diameter. After the sample cell both beams were coupled into 200 μm fiber optic cables and input to a CCD spectrograph (Ocean Optics, S2000−UV−vis) to obtain time-resolved spectral information (425−800 nm). Five thousand excitation pulses were averaged to obtain the transient spectrum at a particular delay time. From the accumulated spectral data kinetic traces at different wavelengths were assembled. The sample flow cell had an optical path of 2 mm and was connected to a solution reservoir and pump system. Sample solutions were prepared with an absorbance of 0.2−1.0 at the excitation wavelength and studied under an argon atmosphere. All measurements were conducted at ambient temperature, 22 ± 2 °C. The ground-state absorption spectra of the compounds studied in this work are given in the Supporting Information (SI, Figure S1)

The Ohio State University Center for Chemical and Biophysical Dynamics consists of a short pulse oscillator (Coherent/Positive Light, Mira) generating ~30 fs pulses at ~800 nm that seeds a high-energy regenerative amplifier (Coherent/Positive Light, Legend HE USP). The regenerative amplifier produces ~2.5 mJ, ~40 fs pulses at 1 kHz. The remaining fundamental is used for harmonic and white light generation. The pump beam is chopped with a frequency of 333 Hz. The probe beam is derived from the small portion of the fundamental output (800 nm). It is passed through an optical delay line consisting of a retroreflector mounted on a computer-controlled motorized translation stage. The probe beam is then used to generate a white light continuum in a 1- mm sapphire plate. This is followed by interference filtering. Transmission signals were detected by Si photodiodes and measured with a digital lock-in amplifier (SRS 830). The samples were circulated in a flow cell; the optical path length was 1 mm. To avoid polarization effects, the angle between polarizations of the pump beam and the probe beam was set to the magic angle by a $\lambda/2$ plate.

Nanosecond time-resolved measurements were performed at The Ohio State University using the instrument described previously.[7] Samples in 1-cm quartz cuvettes were excited with a Spectra Physics LAB-150-10 (~5 ns) water-cooled Nd:YAG laser at the third harmonic frequency (355 nm) with an energy of ~0.045 J/pulse.

Fluorescence quantum yield (Φ_F) and fluorescence quenching experiments were carried out using a Spex Fluorolog 1680 double spectrometer (JY Horiba Inc., Edison, NJ). Measurements of the fluorescence quantum yield were performed as described in the JobinYvon Ltd. website.[8] Acridine orange (Φ_F = 0.46, λ_{ex} = 475 nm, integrated from 500 to 750 nm, in ethanol) was used as the standard for TPZ and PNNO, and harmane ((Φ_F = 0.81, λ_{ex} = 390 nm, integrated from 405 to 650 nm, in 0.1 M H_2SO_4) was used as the standard for dTPZ, dTPZ′, and QXNO. A series of fluorescence intensities were plotted versus the corresponding absorptions at the excitation wavelengths to obtain the slopes (S) of the samples and standards. The quantum yields of samples were calculated by $\Phi_X = \Phi_{ST}(S_X/S_{ST})(\eta_X/\eta_{ST})^2$, where η is the refractive index of the solution.

The fluorescence lifetime of dTPZ was measured by TCSPC at Bowling Green State University as described in the literature.[9]

TPZ and dTPZ were prepared according to the procedure of Fuchs et al.[2] dTPZ is a synthetic precursor to TPZ. dTPZ′ was prepared by the deoxygenation of TPZ. A 30 mg amount of TPZ was dissolved in dioxane in the presence of 150 μL of azo-*tert*-butyl and refluxed overnight. The reaction was monitored by HPLC until all the staring material was consumed. The reaction solution was then evaporated under reduced pressure and eluted with ethyl acetate/hexane through a silica column. dTPZ′ was the third main fraction (the first two fractions were dTPZ and 3-amino-1,2,4-benzotriazine, respectively) to yield 15 mg of final product (55% yield). The spectroscopic properties (UV−vis, NMR, MS) of the product were measured and were identical to literature reports.[3d]

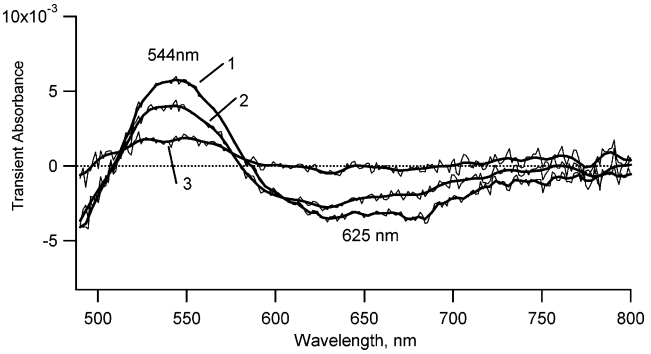


Figure 1. Transient absorption spectrum produced upon excitation (400 nm) of TPZ in acetonitrile under an argon atmosphere (1) 8.3, (2) 53, and (3) 218 ps after the laser pulse.

Quinoxaline di-*N*-oxide and phenazine di-*N*-oxide were prepared by the oxidation of parent quinoxaline and phenazine by acetic acid and hydrogen peroxide according to the literature.[10]

DFT[11] and TD-DFT[12] calculations were performed using the Gaussian 98 suite of programs[13] at The Ohio Supercomputer Center. Geometries were optimized at the unrestricted B3LYP/6-31G* level of theory with single-point energies obtained at the B3LYP/6-31+G**//B3LYP/6-31G* level of theory. Vibrational frequency analyses at the B3LYP/6-31G* level were utilized to verify that stationary points obtained corresponded to energy minima. The zero-point vibrational energy correction, scaled by a factor of 0.9804, was also obtained from the frequency analysis. Solution structures and energies were computed using the polarizable continuum model (PCM)[14] provided by the Gaussian 98 suite of programs. The electronic spectra were computed using time-dependent density function theory of Gaussian 98 at the B3LYP/6-31G* level, and 10 allowed electronic transitions were calculated.

3. Results

Tirapazamine. The absorption spectra, emission spectra, and fluorescence lifetime of TPZ have been studied as a function of solvent.[5] The interaction of ground and excited (S_1) state TPZ with amino acids and nucleosides has also been reported.[5]

Laser flash photolysis (LFP, 400 nm) of TPZ in acetonitrile produces the transient absorption spectrum of Figure 1. Bleaching of TPZ is evident below 500 nm. The negative band observed between 600 and 700 nm is attributed to TPZ stimulated fluorescence. The fluorescence has been previously reported in the 530−650 nm region.[5] The quantum yield of TPZ fluorescence was measured to be 0.002 in acetonitrile. The positive peak at 544 nm decays with the same time constant (130 ps) as the stimulated fluorescence decay and the recovery of the ground-state absorption below 500 nm. Thus, the peak at 544 nm is attributed to the S_1 state of tirapazamine. This assignment is consistent with our previous report that the fluorescence lifetime of TPZ in acetonitrile is 110 ps.[5]

The addition of the neutral electron donor DABCO increases the pseudo-first-order rate constant of decay of the transient

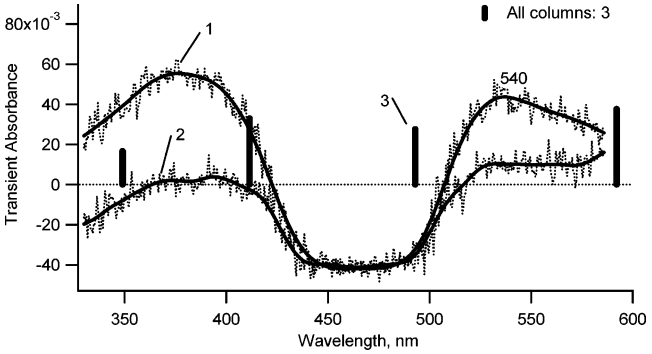


Figure 2. Transient spectrum produced upon excitation (355 nm) of 0.9 mM TPZ in water containing 4 M KSCN (1) 10 ns after the laser pulse, (2) 5 μs after the laser pulse, and (3) calculated UV−vis of the TPZ radical anion (B3LYP/6-31G*).

absorption of TPZ and its fluorescence, as expected (see Supporting Information, Figure S2). However, the transient spectrum of the TPZ radical anion is not observed. Presumably, reverse electron transfer between the TPZ radical anion and DABCO radical cation is extremely rapid, leading to undetectably short lifetimes and low concentrations of these species.

Nanosecond time-resolved LFP (355 nm) of 1.9 mM TPZ in water containing either 4−5 M KSCN or 3−5 M KI produces the transient spectrum shown in Figure 2. The bleaching observed between 430 and 500 nm is due to the ground-state absorption of TPZ in this region of the spectrum. The transient absorptions at 385 and 540 nm decay with the same time constant (1.1 μs), arguing that a common intermediate is responsible for both spectral features. The carrier of the transient absorption is assigned to the TPZ radical anion, and the spectrum itself is consistent with the spectrum of this species obtained by pulse radiolysis.[15] The pulse radiolysis studies demonstrated that the pK_a of TPZ-OH, the conjugate acid of the radical anion, is 5.6 ± 0.2.[15] The transient spectrum of TPZ radical anion is also consistent with TD-DFT calculations, which predict vertical absorptions of the following wavelengths: 592 (f = 0.0376), 493 (f = 0.0276), and 411 nm (f = 0.0329), see Figure 2 and the Supporting Information. The lifetime of the radical anion is greatly shortened in the presence of oxygen (Supporting Information, Figure S3). This result is consistent with the mechanism of action of TPZ and in particular its selectivity toward hypoxic cells.

4-Desoxytirapazamine. The visible absorption band of dTPZ has a maximum at 413 nm in water (log ϵ = 3.68), 404 nm in acetonitrile (log ϵ = 3.63), and 402 nm in dichloromethane (log ϵ = 3.70) (SI, Figure S1). The solvatochromic behavior of this band is much less pronounced than that of the visible band of tirapazamine and follows the opposite trend: the absorbance maximum is lower in energy in polar, hydrogen-bonding solvents.

Desoxytirapazamine fluoresces more intensely than does TPZ under comparable experimental conditions (Figure 3). We measured the fluorescence quantum yield of dTPZ as 0.12 in acetonitrile.

The rates of electron transfer from various donors to singlet excited state dTPZ were determined by fluorescence quenching experiments, and the data obtained are summarized in Table 1. The measured quenching rate coefficients have magnitudes approaching the diffusion limit but are, in general, slightly smaller than the corresponding values obtained with TPZ.[5] There is only marginal evidence to suggest that there is any significant curvature in the Stern−Volmer behavior, and so it cannot be determined whether dTPZ forms hydrogen-bonded complexes

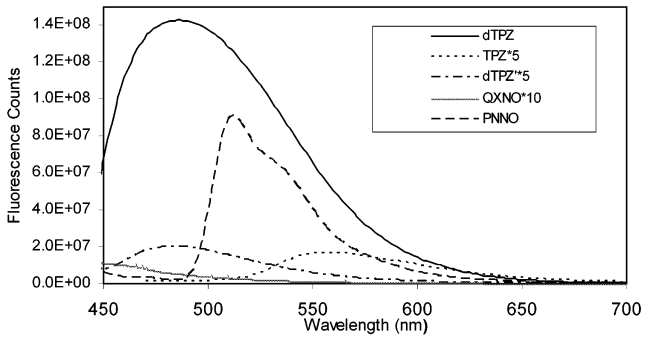


Figure 3. Fluorescence spectra of TPZ (λ_{ex} = 432 nm, A = 0.302), dTPZ (λ_{ex} = 422 nm, A = 0.301), dTPZ′ (λ_{ex} = 420 nm, A = 0.310), QXNO (λ_{ex} = 400 nm, A = 0.305), and PNNO (λ_{ex} = 422 nm, A = 0.308) in acetonitrile at ambient temperature.

TABLE 1: Rates of Reaction for Electron Transfer between Desoxytirapazamine and Various Substrates Determined from Stern−Volmer Analysis of Fluorescence Quenching

quencher, Q	solvent[a]	[Q] (M)	linear fit $k_Q\tau_f$[b] (M^{-1})	linear fit R^2[c]	k_q ($M^{-1}\,s^{-1}$)
AMP[d]	W	0.0−0.10	22.8 ± 0.4	0.995	(4.22 ± 0.07) × 10^9
NaN_3	W	0.0−0.3	16.4 ± 0.3	0.994	(3.03 ± 0.05) × 10^9
KSCN	W	0.0−0.17	20.5 ± 0.6	0.990	(3.80 ± 0.10) × 10^9
tyrosine[e]	W	0.0−0.15	21.6 ± 0.5	0.993	(4.00 ± 0.09) × 10^9
GMP[f]	W	0.0−0.09	21.8 ± 0.5	0.992	(4.04 ± 0.09) × 10^9
tryptophan[e]	W	0.0−0.06	33.4 ± 1.0	0.987	(6.2 ± 0.2) × 10^9

[a] W = aqueous phosphate buffer, 0.025M, pH 6.9. [b] Errors quoted are standard deviations on the line of best fit. [c] Goodness-of-fit parameter. [d] Adenosine monophosphate disodium salt. [e] Amino acids used as methyl ester hydrochlorides. [f] Guanosine monophosphate, monosodium salt.

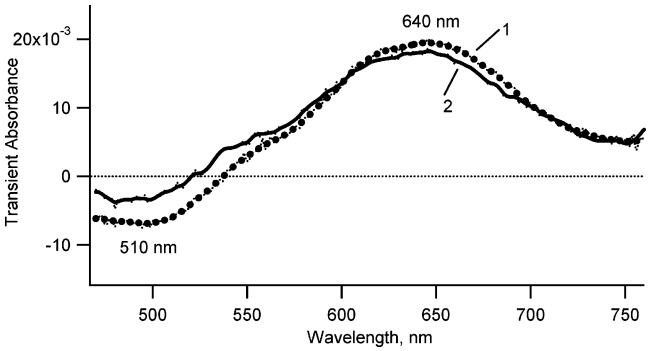


Figure 4. Transient absorption spectrum produced upon LFP (400 nm) of dTPZ in acetonitrile (1) 1.29 and (2) 1033 ps after the laser pulse.

with some quenchers in the same manner as TPZ.[5] The larger rate constants obtained with TPZ may reflect the greater tendency of this drug to form complexes with quenchers.

Adenosine monophosphate is an exception to the rule. It can be seen that unlike TPZ, dTPZ will undergo photochemical electron transfer with adenosine monophosphate. This result may be due to the fact that the photochemical energy available from excitation of dTPZ (assuming that the $S_{0,0} \rightarrow S_{1,0}$ electronic transition for dTPZ in water is approximately 450 nm or 2.8 eV) is greater than that available from excitation of TPZ ($S_{0,0} \rightarrow S_{1,0}$ approximately 510−520 nm or 2.4 eV).

Picosecond time-resolved LFP (400 nm) of dTPZ in acetonitrile produces the transient spectrum of Figure 4. The carrier of the transient absorption at 640 nm is assigned to the S_1 state of dTPZ. The lifetime of the transient absorption is much greater than 200 ps, the limit of the spectrometer. This is consistent with the fluorescence lifetime measurements. The fluorescence lifetime of dTPZ, obtained by TCSPC technique, was deter-

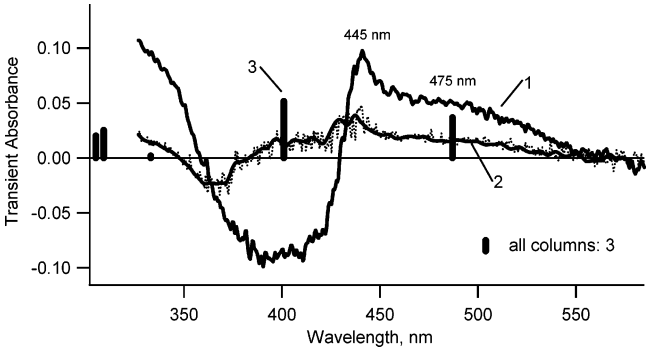


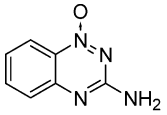
Figure 5. Transient absorption spectrum produced upon LFP (355 nm) of 0.9 mM dTPZ and saturated KSCN (~0.15 M) in acetonitrile (1) 10 ns after the laser pulse, (2) 1 μs after the laser pulse, and (3) calculated radical anion of the UV−vis spectrum of dTPZ (B3LYP/6-31G*, gas phase).

mined to be 5.4 ns, which is about 50-fold longer than that of TPZ.[5]

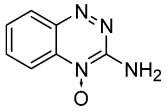
Nanosecond time-resolved LFP (355 nm) of dTPZ in acetonitrile containing either 0.15 M KSCN or 0.08 M NaN_3 produces new transient absorptions which decay with microsecond lifetimes (Figure 5). The transient absorption is rapidly quenched by oxygen (Supporting Information, Figure S4). The carrier of the transient spectrum is attributed to the radical anion of dTPZ based on TD-DFT calculations and by analogy to the TPZ studies and the oxygen dependence of the transient species.

1-Desoxytirapazamine. A second, minor metabolite of tirapazamine is dTPZ′, an isomer of dTPZ.[3]

After photoexcitation of dTPZ′, we observe stimulated fluorescence decay (Figure 6a) at 450 nm; the S1 lifetime is 1.6 ± 0.3 ps. The fluorescence quantum yield of dTPZ′ was measured to be 0.0024 in acetonitrile, which is similar to that of TPZ and much smaller than that of its isomer dTPZ.



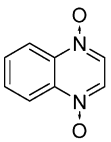
dTPZ



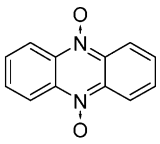
dTPZ'

Related experiments were performed with quinoxaline di-*N*-oxide (QXNO) and phenazine-di-*N*-oxide (PNNO), Figure 6b and c, respectively.

The excited S_1 states of QXNO and PNNO have fluorescence quantum yields of less than 0.001 and 0.011 in acetonitrile, respectively, and lifetimes of 3.1 and 400 ps respectively.



QXNO



PNNO

4. Discussion

Photolysis of TPZ and dTPZ produces the analogous excited singlet states which can be detected by picosecond transient absorption spectroscopy. The excited singlet states can be reduced by anionic electron donors to form observable radical anions, key species in the cascade of reactions involved in the biological activity of TPZ.

A key finding is that the excited singlet state of dTPZ is about 50 times longer lived than that of TPZ. Previous workers have

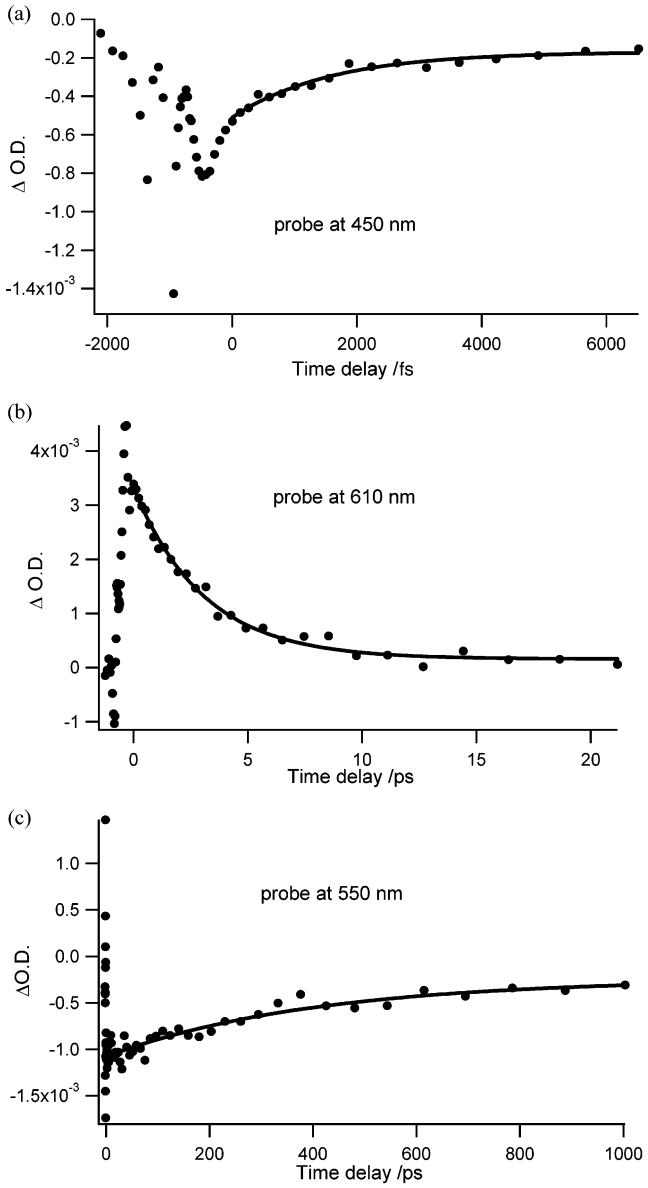
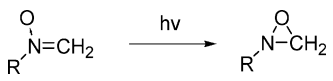


Figure 6. Transient absorption kinetic traces after photoexcitation of dTPZ′ (a), QXNO (b), and PNNO (c) in acetonitrile at 400 nm.

often noted interesting differences between the photochemistry of a C=N−O unit in aromatic *N*-oxides and the analogous N=N−O units in a comparable aromatic system.[16−18]

The photochemistry of aromatic *N*-oxides is complex, and it is unlikely that a single mechanism can explain all known observations.[16−18] Photolysis of nitrones produces oxaziridines, which can be isolated as stable compounds.[18]



Photolysis of aromatic *N*-oxides does not produce isolable bicyclic heterocyclic species. The evidence suggests that oxaziridines are formed as reactive intermediates by cyclization of the excited singlet state.[18]

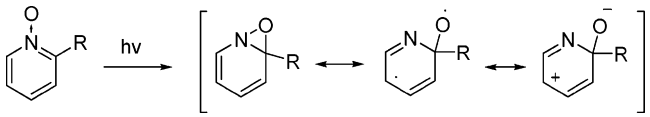
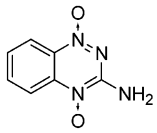
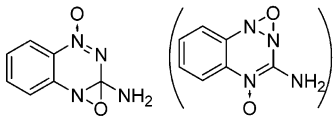
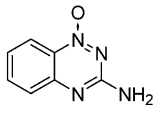
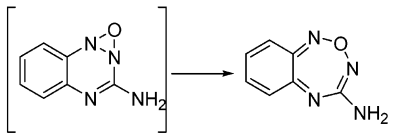
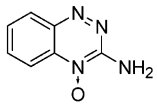
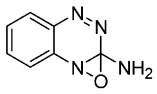
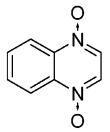
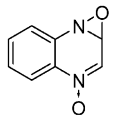
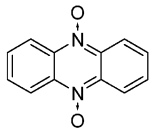
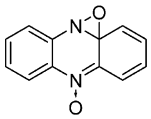


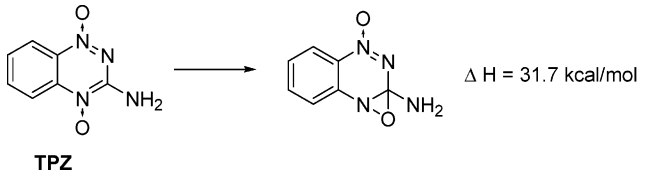
TABLE 2: Summary of Computational and Photophysical Data

Compound	Ground State	Cyclization Product	Calculated ΔH of cyclication (kcal/mol)	τ_{S1}[b] (ps)	E(S_1)[a] kcal/mol	Fluorescence quantum yield[b]
Tirapazamine (TPZ)			31.7 (68.0)	110-130[c]	54	0.0020
4-Desoxy-tirapazamine (dTPZ)			62.9	5400[d]	65	0.12
1-Desoxy-tirapazamine (dTPZ')			34.2	1.6[c]	64	0.0024
Quinoxaline di-N-oxide (QXNO)			30.1	3.1[c]	69	<0.001 (0.00088)
Phenazine di-N-oxide (PNNO)			42.6	400[c]	57	0.011

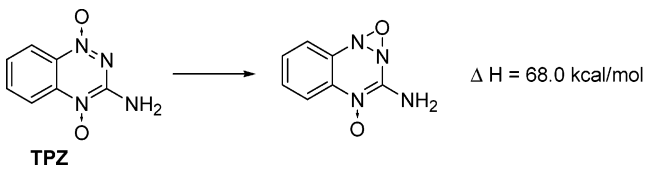
[a] Estimated by the UV−vis and fluorescence spectra. [b] Measured in acetonitrile. [c] Measured by picosecond transient absorption. [d] Measured by fluorescence decay.[9]

A complex mixture of stable products is then formed depending on the specific reaction conditions and the aromatic *N*-oxides employed.

To better understand our data DFT calculations were performed on the *N*-oxides and di-*N*-oxides of this study (Table 2). The cyclization of TPZ to form an oxaziridine is predicted by DFT to be endothermic by 31.7 kcal/mol. The singlet (S_1) energy of TPZ is 54−55 kcal/mol.

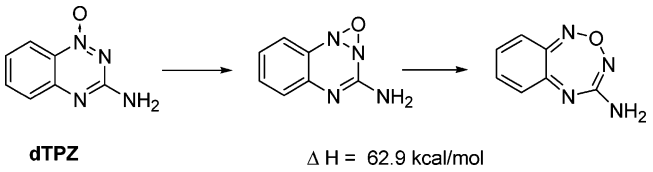


The cyclization of TPZ to form an oxadiaziridine is a much more endothermic process, 68.0 kcal/mol.

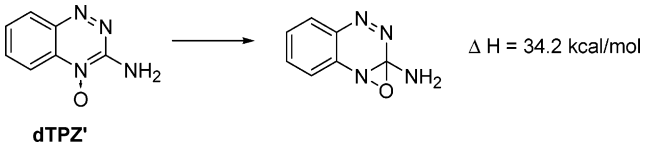


Thus, the S_1 state of TPZ should readily cyclize to from an oxaziridine, and this will result in a low fluorescence quantum yield and a short fluorescence lifetime.

We attempted the analogous calculation with dTPZ. However, the oxadiaziridine is no longer predicted to be a stationary point. Attempts to find a minimum for the oxadiaziridine always led to ring opening.



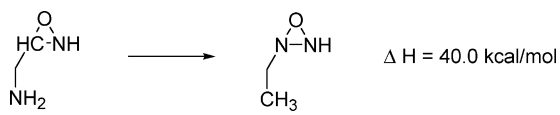
Cyclization of dTPZ′ produces an oxaziridine, which exists in a well-defined minimum.



The energy cost of cyclization of dTPZ′ (34.2 kcal/mol) is well below the singlet energy (64 kcal/mol) of the *N*-oxide. Thus, the fluorescence lifetime and quantum yield are lower than its isomer dTPZ.

The endothermicities of cyclization of quinoxaline-di-*N*-oxide and phenazine-di-*N*-oxide are 30.1 and 42.3 kcal/mol, which compare with their singlet energies of 69 and 57 kcal/mol, respectively. The short singlet lifetimes are consistent with these results.

Oxadiaziridine itself is not a known compound but has been studied previously by computational methods.[19] We used DFT calculations to study the following isomeric reaction in which both the oxaziridine and oxadiaziridine isomers are stable minima.



DFT calculations predict that oxadiaziridines are 40 kcal/mol less stable than isomeric oxaziridines. Thus, we conclude that cyclization of the excited state of dTPZ ($S_1 = 63-64$ kcal/mol) to an oxadiaziridine is endothermic by at least 7 kcal/mol and as a result is a slow and unimportant process. Consequently, the excited state of dTPZ does not enjoy a rapid deactivation process and thus has a greater fluorescence quantum yield and longer fluorescence lifetime than TPZ.

5. Conclusions

Laser flash photolysis of tirapazamine (TPZ) and desoxytirapazamine (dTPZ) produces their excited singlet states. The S_1 states have been observed by picosecond time-resolved absorption spectroscopy. The lifetime of TPZ is much shorter than that of dTPZ, and the quantum yield of TPZ fluorescence is much smaller than that of dTPZ. DFT calculations indicate that the S_1 lifetime of TPZ is controlled by reversible cyclization to an oxaziridine. The corresponding process in dTPZ forms a higher energy oxadiaziridine. This latter process is endothermic and does not influence the photophysics of the singlet state of dTPZ. The S_1 states of TPZ and dTPZ are reduced to radical anions by KSCN, KI, and NaN_3. Studies of the S1 studies of 4-desoxytirapazamine (dTPZ′), which can cyclize to form an oxaziridine, are consistent with this rule.

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Supporting Information Available: The absorption spectra of the compounds studied, the influence of oxygen on the lifetimes of transient species, and tables of energies, Cartesian coordinates, and vibrational frequencies related to the calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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