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Lifetimes and Reactivities of Some 1,2-Didehydroazepines Commonly Used in Photoaffinity Labeling Experiments in Aqueous Solutions[†]

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ABSTRACT: The reactive 1,2-didehydroazepine (cyclic ketenimine) intermediates produced upon photolysis of phenyl azide, 3-hydroxyphenyl azide, 3-methoxyphenyl azide, and 3-nitrophenyl azide in water and in HEPES buffer were studied by laser flash photolysis techniques with UV−vis detection of the transient intermediates. The lifetimes of the 1,2-didehydroazepines were obtained along with the absolute rate constants of their reactions with typical amino acids, nucleosides, and other simple reagents present in a biochemical milieu. The nitro substituent greatly accelerates the bimolecular reactions of the cyclic ketenimines, and the 3-methoxy group greatly decelerates the absolute reactivity of 1,2-didehydroazepines. The intermediate produced by photolysis of 3-hydroxyphenyl azide is much more reactive than the intermediate produced by photolysis of 3-methoxyphenyl azide. We propose that the hydroxyl-substituted 1,2-didehydoazepines rapidly (<10 μs) tautomerize in water to form azepinones and much more rapidly than the corresponding 3-methoxy-substituted cyclic ketenimines undergo hydrolysis. Azepinones react more rapidly with nucleophiles than do methoxy-substituted 1,2-didehydroazepines and are the active species present upon the photolysis of 3-hydroxyphenyl azide in aqueous solution.

Photoaffinity labeling (PAL)[1] was invented by Westheimer, Singh, and Thornton (*1*) more than 40 years ago. Their original studies used diazo esters and carbene intermediates to label amino acids in the binding pocket of α-chymotrypsin. Bayley and Knowles introduced aromatic azide reagents for photoaffinity labeling shortly afterward (*2*). Aryl azides are generally easier to synthesize and handle than diazo and diazirine precursors, but they also suffer some complications (*3*). Photolysis of phenyl azide **1** promotes nitrogen extrusion and the release of singlet phenyl nitrene **2**, but unlike its carbene analogue, **2** cyclizes rapidly (∼1 ns in organic solvents) to form benzazirine **3** which immediately opens to form 1,2,4,6-azacycloheptatetraene (a 1,2-didehydroazepine or cyclic ketenimine) **4** (Scheme 1) (*4*). Although the lifetime of singlet phenylnitrene in water is not currently known, McClelland and co-workers have demonstrated that the photochemistry of Scheme 1 also largely proceeds in aqueous solution (*5*). It therefore seems likely that cyclic ketenimines and not aryl nitrenes are the reactive intermediates generated in typical PAL experiments utilizing aryl azides.

Herein, we investigate the photochemistry of phenyl azide, 3-hydroxyphenyl azide, 3-methoxyphenyl azide, and 3-nitrophenyl azide in water and in HEPES buffer solution using laser flash photolysis (LFP) techniques. The lifetimes of the intermediates produced by photolysis of these azides are measured, absolute rate constants with simple amino acids and nucleosides determined, and the identities of the labeling intermediates proposed with the aid of time-dependent density functional theoretical (TD-DFT) calculations (*6*).

MATERIALS AND METHODS

General Supporting Information. Unless stated otherwise, all reactions were run under argon. All chemicals were purchased from Aldrich and Acros Organics. Phenyl azide (*7*), 3-hydroxyphenyl azide (*8*), 3-methoxyphenyl azide (*9*), 3-nitrophenyl azide (*10*), and 3,3′-dinitroazobenzene (*11*) were prepared as described in the literature. An HP 6890 Series GC−MS spectrometer was used to analyze samples by gas chromatography. MS spectra were obtained using a 5973 Mass Selective detector. UV−vis spectra were obtained using an HP 8452 diode array spectrophotometer. An electrothermal melting point apparatus was used to determine melting points. IR spectra were obtained using a Perkin-Elmer λ-19 spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC-250 MHz spectrometer.

LFP Studies. Each of the samples prepared for LFP studies contained 2 mL of the aryl azide in either water, 50 mM HEPES solution (pH 7.6), methanol, or acetonitrile with concentrations such that the optical density at the excitation wavelength was between 0.5 and 1.0. The excitation wavelengths used were either 308 or 355 nm. In each kinetic study, a few microliters of the quencher was added to the initial 2 mL of the aryl azide solution. The LFP spectrometer has been described in the literature (*12*). Briefly, the LFP system employed a Lambda Physik LPX-100 excimer (308 nm) and a Spectra-Physics Quanta-Ray (355 nm) laser. The transient absorption spectra were collected using an EG&G PARC 1460 optical multichannel analyzer with an EG&G 1304 PARC pulse amplifier, an EG&G PARC 1024 UV detector, and a Jarrel-Ash 1234 grating.

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[1] Abbreviations: DFT, density functional theory; PAL, photoaffinity labeling; TD-DFT, time-dependent density functional theory.

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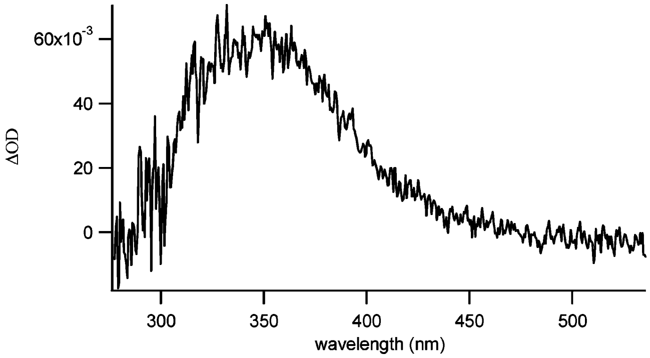


FIGURE 1: Transient spectrum produced by LFP (308 nm) of phenyl azide **1** in water. The spectrum was recorded immediately after the laser pulse over a window of 30 ns.

TD-DFT Calculations. UV−vis spectra of the ketenimines and other intermediates were calculated in the gas phase using the TD-DFT B3LYP/6-31G* TD level of theory (*6*) using the Gaussian 98 suite of programs (*13*). The energies of some intermediates were calculated in water using the solvent polarizable continuum model (*14*).

RESULTS

Phenyl Azide. LFP of phenyl azide in acetonitrile, water, and 50 mM HEPES buffer produced very similar transient spectra. In all three solvents, the transient spectra show a broad peak starting at ~300 nm and ending at ~450 nm with λ_{max} at ~360 nm (see Figure 1 and Figures S1 and S2 of the Supporting Information).

The transient spectrum of Figure 1 is assigned to ketenimine **4** on the basis of extensive prior study of this system (*4*). In the most relevant study, the McClelland group (*5*) reported a 72% yield of products derived from cyclic ketenimine **4** upon photolysis of phenyl azide in water. The yield of azobenzene, the product formed by dimerization of triplet phenylnitrene, was not reported and most likely is very small. Protonation of singlet phenylnitrene does not proceed in neutral water and only becomes an important process in the presence of strong acid (*5*). Our spectroscopic assignment is strongly supported by TD DFT calculations which predict that the cyclic ketenimine has vertical absorption bands at 342 and 371 nm (see Figure 2 and Tables S1 and SI).

The lifetime of ketenimine **4** in water is several microseconds. The observed first-order rate constant of disappearance (k_{obs}), measured at 400 nm, increases in the presence of nucleophiles. For example, a plot of k_{obs} versus the concentration of adenosine is linear (Figure 3) with a slope equal to the absolute second-order rate constant of the reaction of the nucleophile with cyclic ketenimine **4**. Several absolute rate constants were determined in this manner and are collected in Table 1.

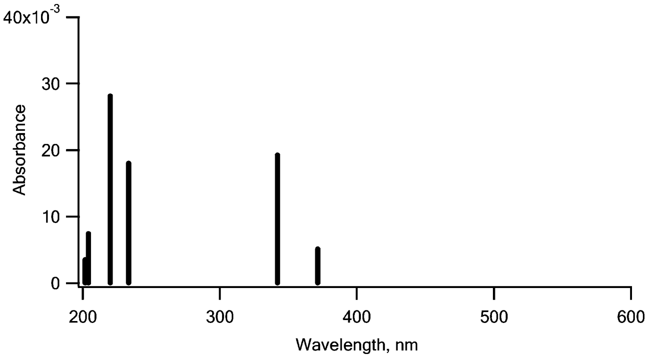


FIGURE 2: Calculated UV−vis spectrum of cyclic ketenimine **4** by TD-DFT.

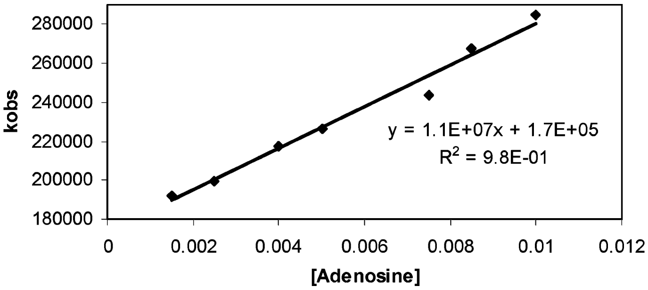


FIGURE 3: Adenosine quenching of ketenimine **4** in water measured at 400 nm.

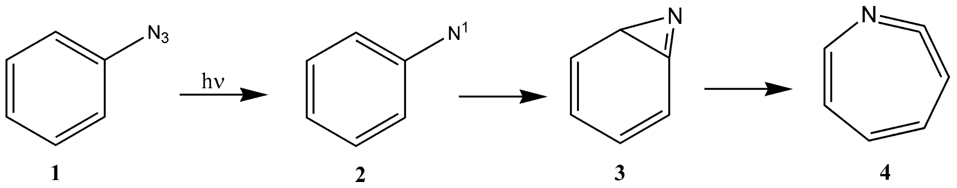
Table 1: Summary of Quenching Studies of Cyclic Ketenimine **4** Determined at Ambient Temperature

quencher	k_Q ($M^{-1}\ s^{-1}$)	solvent
adenosine	1.1×10^7	water
uridine	3.3×10^7	water
GMP	2.2×10^8	water
cytidine	*a*	water
adenosine	1.3×10^8	50 mM HEPES
uridine	3.7×10^8	50 mM HEPES
GMP	4.2×10^9	50 mM HEPES
D-ribose	*a*	50 mM HEPES
sodium hydroxide	3.9×10^7	water
sodium acetate	1.1×10^7	water
methionine methyl ester	1.9×10^6	water
sodium phenoxide	9.0×10^7	water
glutathione	1.5×10^7	water
phenol	*a*	water
ammonium chloride	*a*	water
urea	*a*	water
methionine methyl ester	1.5×10^7	50 mM HEPES
sodium acetate	4.3×10^7	50 mM HEPES
glutathione	6.8×10^8	50 mM HEPES
phenol	*a*	50 mM HEPES

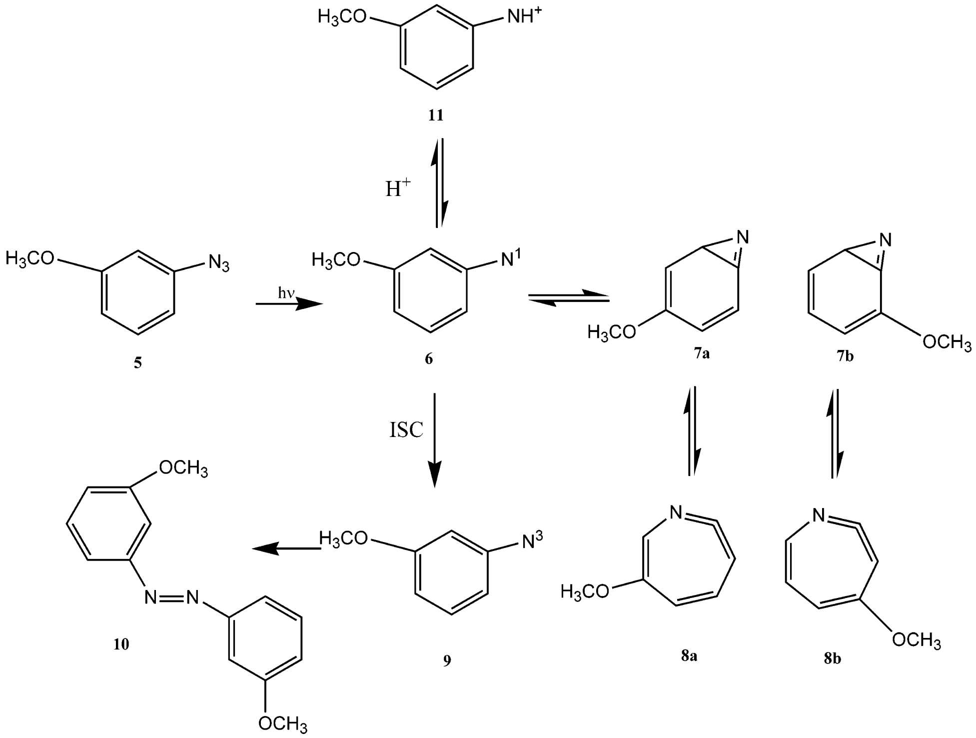
[a] The reaction is too slow to measure.

Didehydroazepine **4** reacts very rapidly with guanosine monophosphate (GMP), adenosine, and uridine. Reaction rate constants are always larger in buffer than in water. Cyclic ketenimine **4** reacts rapidly with negatively charged nucleophiles such as sodium hydroxide, sodium phenoxide, and sodium acetate. Sulfur nucleophiles also react rapidly with **4**. Phenol, urea, D-ribose, cytidine, and ammonium chloride do not react with **4** at a measurable rate.

Scheme 1: Photolysis of Phenyl Azide



Scheme 2: Photolysis of 3-Methoxyphenyl Azide in Aqueous Solution



3-Methoxyphenyl Azide. Photolysis of 3-methoxyphenyl azide **5** is expected to produce singlet 3-methoxyphenylnitrene **6**. We expect that **6** reacts in a manner similar to that of singlet phenylnitrene **2** to form the ketenimines **8a** and **8b** through azirine intermediate **7a** (or **7b** by cyclization in the other direction). Singlet 4-methoxyphenylnitrene abstracts protons from water to form a highly stabilized nitrenium ion (*15*). The corresponding reaction of the meta isomer should be much less facile because the 3-methoxy group can no longer interact with the nitrenium ion by resonance. The singlet nitrene can, in principle, also relax to lower-energy triplet nitrene **9** which will eventually form 3,3′-dimethoxyazobenzene (*10*).

LFP of 3-methoxyphenyl azide **5** was studied in acetonitrile and in aqueous HEPES buffer. The transient spectrum so produced contained a peak with a λ_{max} of 375 nm. Even 10 μs after the laser pulse, the peak intensity is almost unchanged. The carrier of the transient spectrum is attributed to a mixture of cyclic ketenimines **8a** and **8b** because of their similarity to the spectrum of parent cyclic ketenimine **4**. TD DFT calculations (Figure 5) predict that the methoxy group will have little influence on the spectrum of the 1,2-didehydroazepine.

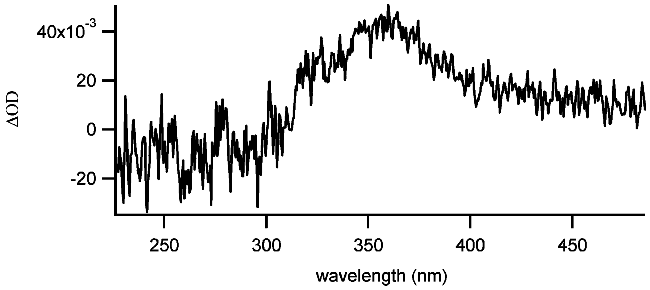


FIGURE 4: Transient spectra produced by 308 nm LFP of 3-methoxyphenyl azide in 50 mM HEPES. The spectrum was recorded over a 30 ns window, 10 μs after the laser pulse.

We attempted to study the kinetics of reaction of ketenimines **8a** and **8b** with numerous quenchers in a manner similar to that of parent cyclic ketenimine **4**. The kinetics were monitored at 375 nm. The quenchers used were 1,3-dimethyluracil in methanol and uridine triacetate in aceto-

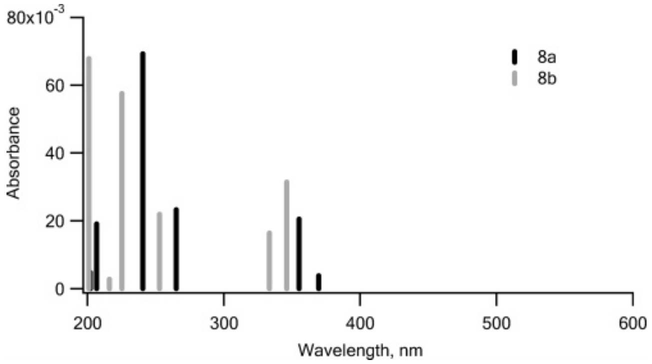


FIGURE 5: Calculated UV−vis spectrum of ketenimines **8a** and **8b** by TD-DFT.

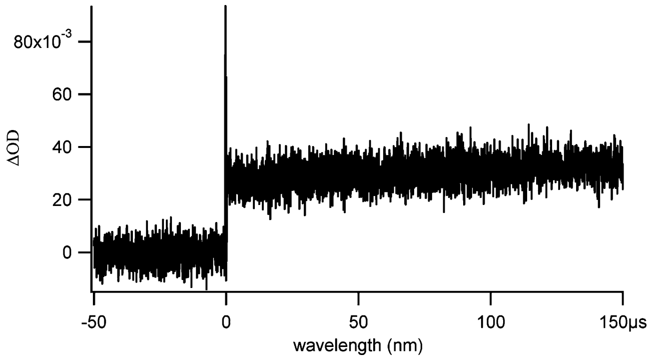
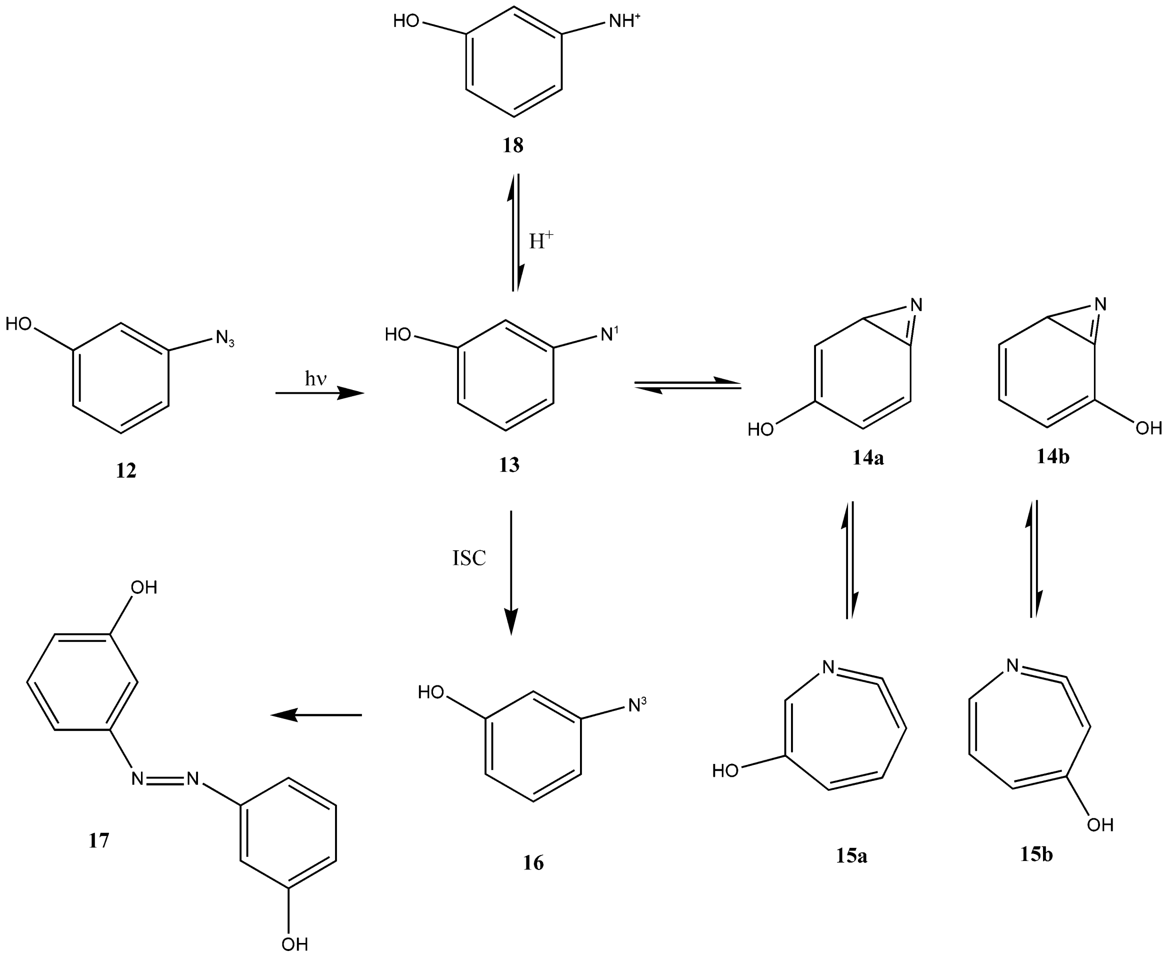
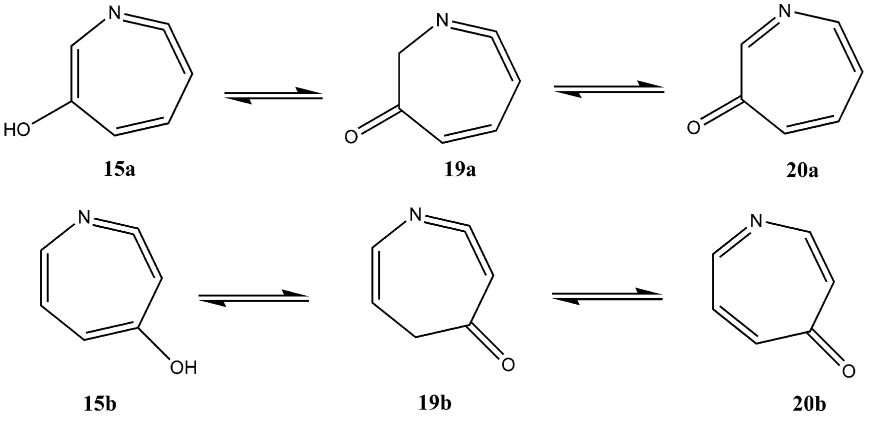


FIGURE 6: Formation of ketenimines **8a** and **8b** in water in the presence of 0.18 M sodium acetate. There is no evidence of decay of the ketenimines. The slight growth is an artifact due to baseline drift.

Scheme 3: Photolysis of 3-Hydroxyphenyl Azide **12** in Solution



Scheme 4: Rearrangement of Cyclic Ketenimines **15**



nitrile. Uridine, guanosine monophosphate, sodium acetate, sodium hydroxide, and sodium phenoxide were studied in aqueous solution. No measurable decay of the mixture of ketenimines **8a** and **8b** was observed even at very long time delays (tens of microseconds) and at very large concentrations of the quenchers.

The low reactivity of ketenimines **8a** and **8b** was surprising because LFP of 3-hydoxyphenyl azide produces intermediates with reactivity comparable to that of parent cyclic ketenimine **4** (*16*). This prompted our further investigation of the photochemistry of 3-hydroxyphenyl azide.

3-Hydroxyphenyl Azide. Photolysis of 3-hydroxyphenyl azide **12** produces singlet 3-hydroxyphenylnitrene **13**. The expected chemistry of this singlet nitrene is summarized in Scheme 3.

Upon photolysis of 3-hydroxyphenyl azide (308 nm excimer laser) in water and in 50 mM HEPES buffer, a transient UV spectrum is observed which exhibits two peaks. Buchmueller and co-workers identified the peak with a λ_{max} at 314 nm as triplet 3-hydroxyphenylnitrene **16**, and the peaks at 340−360 nm were thought likely, but not definitively, to be a mixture of ketenimines **15** (*16*). In this work, the transient UV spectrum shows a small peak at ∼300 nm, corresponding to triplet 3-hydroxyphenylnitrene **16**, and a much stronger and broader peak with a λ_{max} at ∼350 nm presumably corresponding to ketenimines **15**.

Table 2: Summary of Reaction Rate Constants of the Intermediates Produced by LFP of 3-Hydroxyphenyl Azide at Ambient Temperature

quencher	k_Q (M^{-1} s^{-1})	solvent
uridine	8.0×10^5	water
uridine[a]	7.4×10^5	water
sodium hydroxide	9.6×10^6	water
sodium acetate	1.3×10^6	water
methionine methyl ester	2.2×10^5	water
sodium phenoxide	7.8×10^6	water
glutathione	1.4×10^6	water
phenol	1.4×10^5	water
methionine methyl ester	1.6×10^7	50 mM HEPES
sodium acetate	3.6×10^7	50 mM HEPES
glutathione	4.7×10^8	50 mM HEPES
phenol	1.5×10^7	50 mM HEPES

[a] Value obtained by Buchmueller and co-workers (*16*).

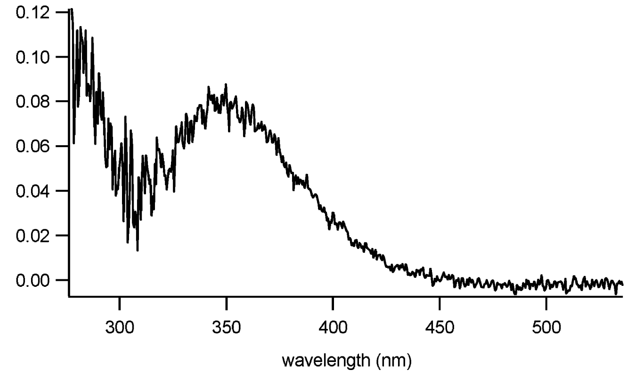


FIGURE 7: Transient spectrum produced by 308 nm LFP of 3-hydroxyphenyl azide **12** in 50 mM HEPES. The spectrum was recorded over a 30 ns window, 1 μs after the laser pulse.

Laser flash photolysis (308 nm excimer laser) kinetic experiments were performed to study the rate of reaction of the transient absorbing between 350 and 500 nm with several different quenchers. The wavelength chosen to monitor the decay was 400 nm. A typical quenching plot is shown below.

The uridine quenching rate constant is 8.0×10^5 M^{-1} s^{-1}. This is in excellent agreement with the previously reported value of 7.4×10^5 M^{-1} s^{-1} obtained by Buchmueller et al. (*16*). Several absolute rate constants were determined and are given in Table 2. It is obvious that the intermediate or intermediates produced upon LFP of 3-hydroxyphenyl azide in water are much more reactive than the intermediates produced by LFP of 3-methoxyphenyl azide in aqueous solution.

Although the transient spectra produced by LFP of 3-hydroxyphenyl azide **12** and 3-methoxyphenyl azide **5** in aqueous solution are very similar, the kinetics of the transient intermediates they produced upon LFP are very different. It seems likely that we were not quenching ketenimines **15** but rather another intermediate that it rearranges to form, species such as **19** and then **20** (Scheme 4). The UV−vis spectra of **20a** and **20b** predicted by TD DFT calculations have significant absorption above 300 nm, and their UV−vis spectra may well resemble those of ketenimines **15a** and **15b**. Such rearrangements are predicted by DFT to be highly exothermic. Similar results are predicted for the gas phase (Figure 11a) and in water (Figure 11b).

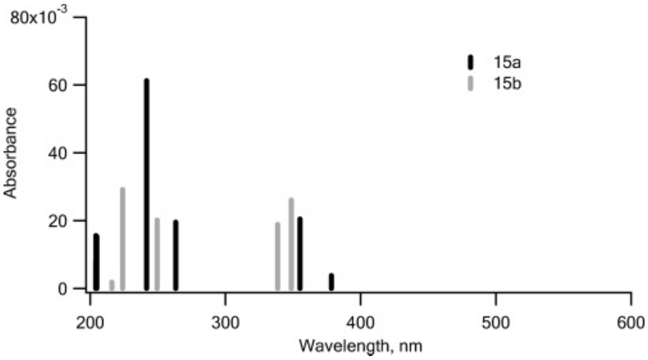


FIGURE 8: Calculated UV−vis spectra of cyclic ketenimines **15a** and **15b** by TD DFT.

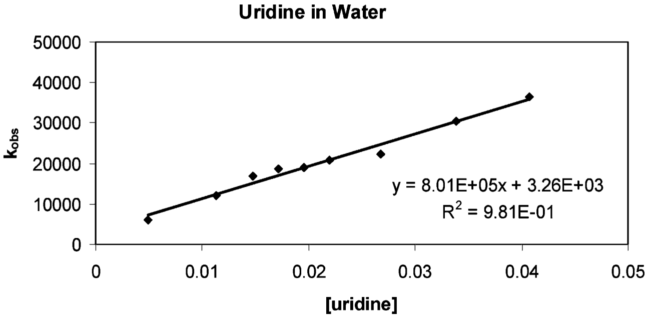


FIGURE 9: Uridine quenching of the reactive intermediates produced upon LFP of 3-hydroxyphenyl azide at ambient temperature, in water, measured at 400 nm.

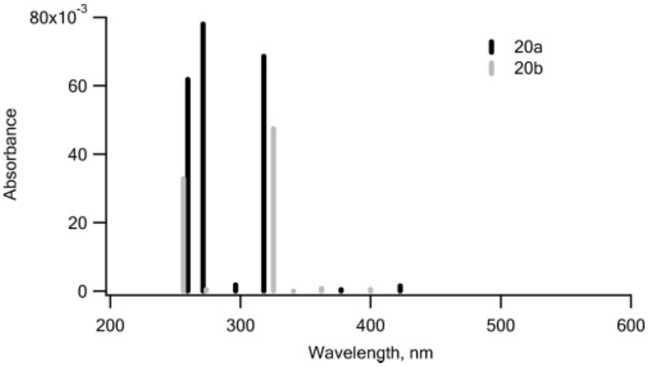


FIGURE 10: Calculated UV−vis spectra of **20a** and **20b** by TD DFT.

Rapid (microseconds) tautomerization of enols and their analogous ketones has been reported by Kresge, Wirz, and co-workers (*17*). We expect that the **15**−**20** isomerizations will be as fast or faster than those previously reported because of their greater exothermicity and the possibility of a proton shuttle mechanism involving a hydrogen bond water network involving the nitrogen atom of the azepinones.

Azepinones (isomeric to **20a** and **20b**) have been observed by Dunkin and co-workers (*18*) using matrix IR spectroscopy following photolysis of *p*-hydroxyphenyl azide in solid argon. Thus, there is a precedent for this rearrangement even at very low temperatures.

We conclude that azepinones such as **20a** and **20b**, rather than singlet nitrenes or ketenimines, are the reactive intermediates involved in our previous study (*16*) and in PAL studies using the 3-hydroxyphenyl azide family of reagents.

3-Nitrophenyl Azide. The photochemistry of 3-nitrophenyl azide **21** has been thoroughly studied by Schuster and co-workers in typical organic solvents (*19*). The nitro substituent is an attractive moiety in PAL studies because it shifts the absorption spectrum of the aryl azide and thereby allows its

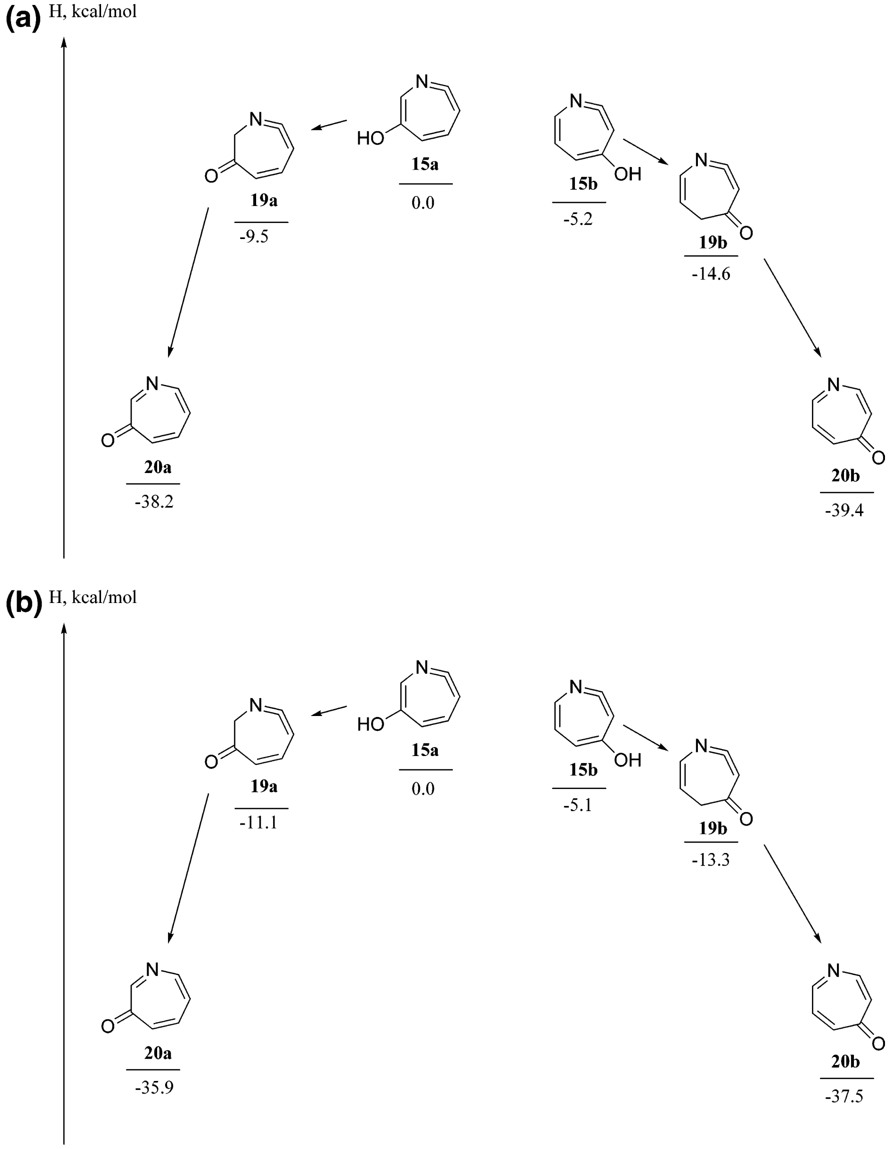


FIGURE 11: (a) Comparison of energies of products derived from ketenimine **15** in the gas phase as predicted by DFT calculations. (b) Comparison of energies of products derived from cyclic ketenimines **15** in water as predicted by DFT calculations.

selective excitation in the presence of proteins and nucleic acids.

Photolysis of 3-nitrophenyl azide **21** produces singlet 3-nitrophenylnitrene **22**. Singlet 3-nitrophenylnitrene can react, in principle, with sulfoxides and alkenes. It can possibly react with proton donors, yielding 3-nitrophenylnitrenium ion **27**, but this seems highly unfavorable. Moreover, **22** can reversibly (*19*) form the azirine intermediates **23**, which can form the 1,2-didehydroazepines (ketenimines) **24**. In addition, **22** can irreversibly intersystem cross to triplet 3-nitrophenylnitrene **25**. Triplet 3-nitrophenylnitrene reactions include reactions with oxygen to give nitrobenzene and abstract electrons from reducing agents. Moreover, **25** can react with itself or with azide **21** to form 3,3′-dinitroazobenzene **26** (Scheme 5).

Laser flash photolysis of 3-nitrophenyl azide **21** (308 nm excimer laser) in both water and 50 mM HEPES buffer produces transient UV spectra with two major peaks (Figure 12). The first has a λ_{max} of 320 nm corresponding to triplet 3-nitrophenylnitrene **25** (*19*). The second has a λ_{max} of 400 nm corresponding to cyclic ketenimines **24**. This band was previously observed but was not assigned (*19*). The cyclic ketenimines have a shorter lifetime than the triplet nitrene does in water.

TD-DFT calculations predict that the nitro groups shifts the spectra of the cyclic ketenimines to longer wavelengths (Figure 13), as seen in Figure 12 as observed in LFP studies.

Laser flash photolysis (308 nm excimer laser) kinetic experiments were performed in an attempt to study the rates of reaction of ketenimines **24** with several different quenchers. The wavelength chosen to monitor the decay was 400 nm. The kinetics at 400 nm show a fast decay of the ketenimine and a slow growth of 3,3′-dinitroazobenzene **26**, which is formed by the reaction of the triplet nitrene with itself or with azide. The UV−vis spectrum of **26** (Figure S6 of the Supporting Information) demonstrates that it has substantial absorption at 400 nm; the extinction coefficient of **26** in water at 400 nm is 570 L mol^{-1} cm^{-1}. The time dependence of the absorbance at 400 nm is shown in Figure 14a,b.

The ketenimine mixture was quenched with several nucleophiles. The glutathione reaction rate constant in water is 2.7 × 10^8 M^{-1} s^{-1}, for example (Figure 15).

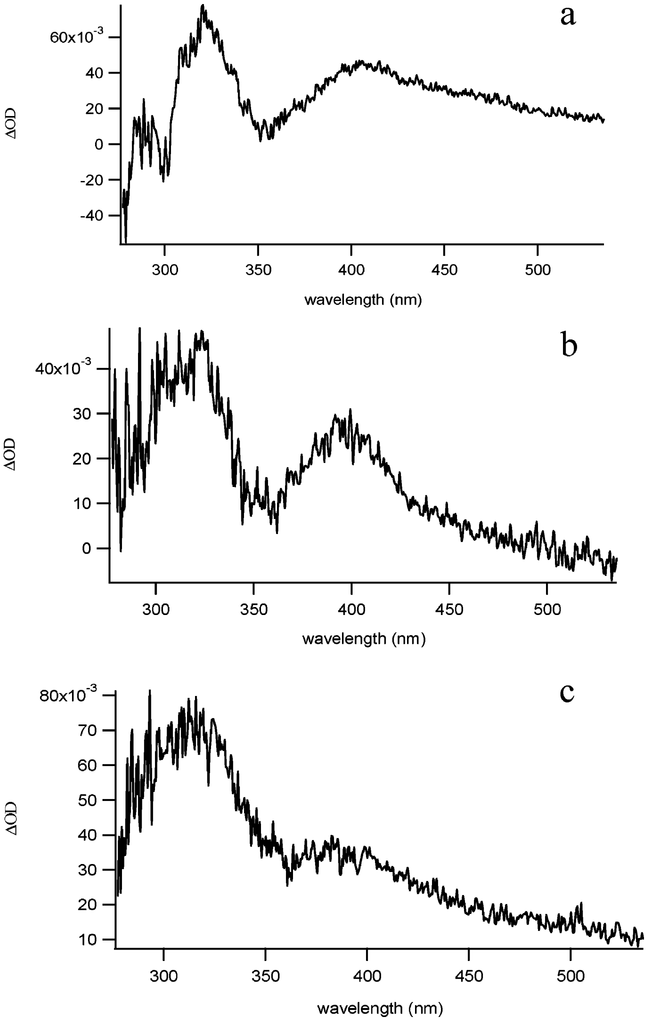


FIGURE 12: Transient spectra obtained by 308 nm LFP of 3-nitrophenyl azide **21** in water. Spectra were recorded over 30 ns widows (a) immediately after the laser flash, (b) 1 μs after the laser flash, and (c) 10 μs after the laser pulse.

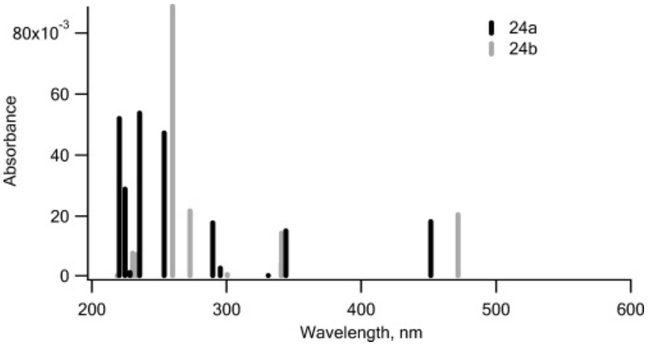


FIGURE 13: Calculated UV−vis spectra of the cyclic ketenimines **24a** and **24b**.

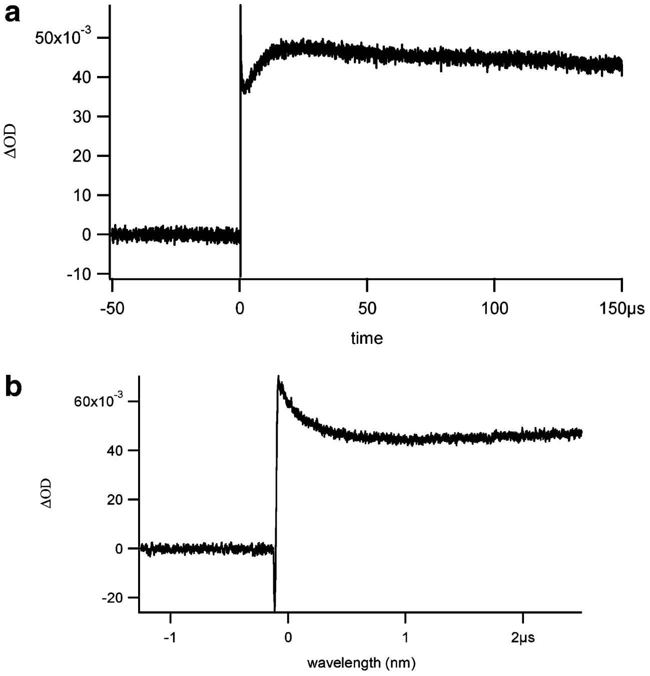


FIGURE 14: (a) Kinetics monitored at 400 nm showing the fast decay of ketenimines **24a** and **24b** and the slow growth of 3,3′-dinitroazobenzene, which is formed by the reaction of the triplet nitrene with azide. (b) Decay curve of ketenimines **24a** and **24b** measured at 400 nm.

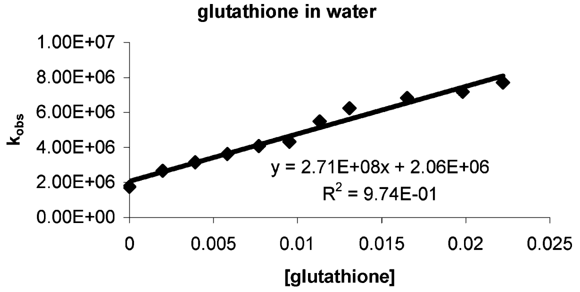


FIGURE 15: Glutathione quenching of ketenimine **24** in water measured at 400 nm.

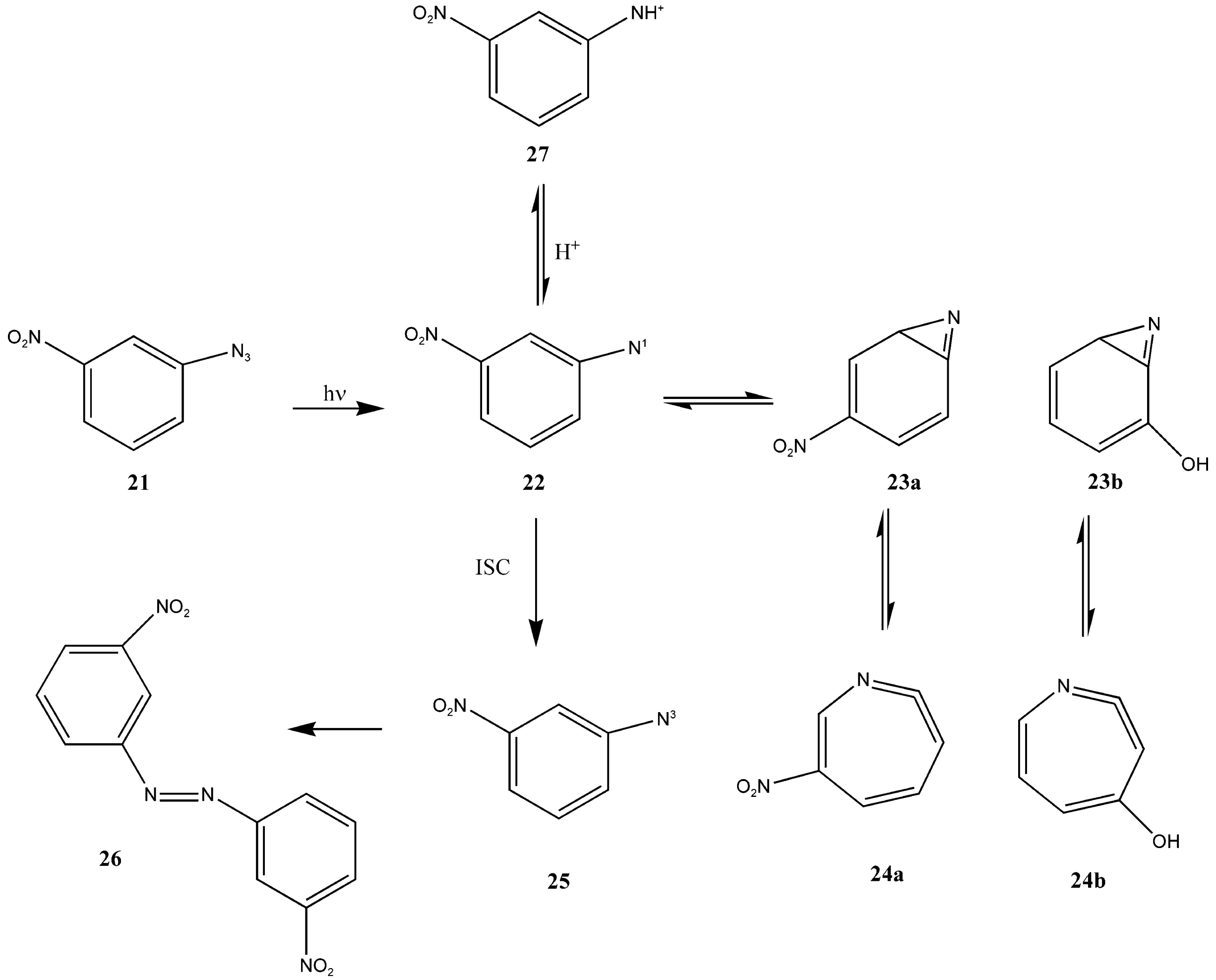
Table 3: Summary of Quenching Studies with Nitro-Substituted Ketenimines **24**

quencher	k_Q (M^{-1} s^{-1})	solvent
sodium hydroxide	1.6×10^9	water
sodium acetate	2.5×10^7	water
methionine methyl ester	6.3×10^5	water
sodium phenoxide	1.8×10^9	water
glutathione	2.7×10^8	water
phenol	3.9×10^5	water
GMP	4.6×10^9	water
uridine	1.2×10^8	water
AMP	3.0×10^8	water
methionine methyl ester	8.9×10^5	50 mM HEPES
glutathione	4.0×10^9	50 mM HEPES
phenol	5.6×10^5	50 mM HEPES

The optical yield of 3,3′-dinitroazobenzene **26** decreased in the presence of methionine. The sulfur atom of methionine methyl ester likely reacts with singlet nitrene **22**, a reaction known for polyfluorinated singlet arylnitrenes (*20*). Methionine also reacts with ketenimines **24**. The lifetime of ketenimine **24** is much shorter than that of **4** in aqueous solution. The nitro group is a powerful electron-withdrawing group and increases the electrophilicity of **24** (*21*). Ketenimines **24** were quenched with sodium hydroxide, sodium phenoxide, glutathione, uridine, guanosine monophosphate, adenosine monophosphate, and sodium acetate. It seems that adenosine quenches **24** but at a modest rate. Adenosine monophosphate (AMP) quenched ketenimines **24a** and **24b** with a rate constant similar to that of uridine.

Nitro-substituted ketenimines **24** are generally more reactive than parent ketenimine **4**. Ketenimine **24** reacts more rapidly with sodium hydroxide, sodium phenoxide, and phenol than does **4**. Sodium acetate and glutathione trap **24**

Scheme 5: Photolysis of 3-Nitrophenyl Azide **21** in Solution



and **4** with comparable rates. Curiously, methionine methyl ester reacts with **4** approximately twice as rapidly as **24**.

CONCLUSIONS

Photolysis of phenyl azide, 3-methoxyphenyl azide, 3-hydroxyphenyl azide, and 3-nitrophenyl azide in aqueous solution promotes nitrogen extrusion and release of the corresponding singlet aryl nitrenes. Singlet phenylnitrene isomerizes cleanly to the corresponding didehydroazepine (cyclic ketenimine) **4**, which was monitored by nanosecond time-resolved UV spectroscopy. Absolute rate constants of the reaction of **4** with amino acids, nucleosides, and other reagents found in a biological milieu were obtained. Reaction rate constants are generally larger in HEPES buffer than in pure water.

Singlet 3-methoxy- and 3-nitrophenylnitrenes relax to form a mixture of triplet nitrenes and ketenimines in aqueous solution. The 3-methoxy substituent greatly reduces the reactivity of the ketenimine. The nitro substituent shortens the lifetime of the ketenimine in aqueous solution and generally increases its biomolecular reactivity. These effects are not surprising as Li et al. (*21*) found that electron-donating groups decrease and electron-withdrawing groups increase the reactivity of ketenimines toward diethylamine in cyclohexane.

Singlet 2-hydroxyphenylnitrene also isomerizes to form a mixture of ketenimines. In aqueous solution, these hydroxy-substituted ketenimines tautomerize to azepinones. The azepinones have a reactivity with nucleophiles comparable to that of the parent ketenimine **4**.

In summary, the ketenimine and azepinone intermediates produced upon photolysis of phenyl azide and 3-methoxy, 3-hydroxy, and 3-nitrophenyl azide have been observed in aqueous solution by laser flash photolysis techniques. The lifetimes and bimolecular reactivities of these intermediates have been determined under conditions relevant to photoaffinity labeling experiments.

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SUPPORTING INFORMATION AVAILABLE

Figures depicting transient spectra of ketenimines in aqueous solvents and in organic solvents along with the Cartesian coordinates of the optimized geometries of the reactive intermediates and the exact positions of their vertical transitions and oscillator strengths are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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