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Synthesis and Characterization of Photolabile *o*-Nitrobenzyl Derivatives of Urea

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We present here the synthesis and characterization of four photolabile derivatives of urea in which α-substituted 2-nitrobenzyl groups are covalently attached to the urea nitrogen. These derivatives photolyze readily in aqueous solution to release free urea. The α-substituents of the 2-nitrobenzyl group strongly influence the rate of the photolysis reaction measured with transient absorption spectroscopy. Rates of photolysis at pH 7.5 and room temperature (approximately 22 °C) for *N*-(2-nitrobenzyl)urea, *N*-(α-methyl-2-nitrobenzyl)urea, *N*-(α-carboxymethyl-2-nitrobenzyl)urea, and *N*-(α-carboxy-2-nitrobenzyl)urea are, respectively, 1.7×10^4, 8.5×10^4 , 4.0×10^4, and 1.1×10^5 s^{-1}. The quantum yields determined by measurement of free urea following irradiation by a single laser pulse at 308 nm were 0.81 for *N*-(2-nitrobenzyl)urea, 0.64 for *N*-(α-methyl-2-nitrobenzyl)urea, and 0.56 for *N*-(α-carboxy-2-nitrobenzyl)urea. The caged *N*-(α-carboxy-2-nitrobenzyl)urea is not a substrate of the enzyme urease, while the photolytically released urea is. Also, neither this caged urea nor its photolytic side products inhibit hydrolysis of free urea by urease. Thus, the α-carboxy-2-nitrobenzyl derivative of urea is suitable for mechanistic investigations of the enzyme urease.

Introduction

Photolabile derivatives ("caged compounds") of many biochemically important molecules are now available and are exploited in applications for the temporal and spatial resolution that is achieved by their photolysis.[1−6] Many caged compounds release free products on a millisecond or even microsecond time scale when photolyzed by a pulse of UV light. This is important for activation of processes which are inherently rapid. For example, photolysis of caged compounds such as carbamoylcholine,[7−9] glycine,[10,11] NMDA,[12] kainate,[13] γ-aminobutyric acid,[14,15] and glutamate[16] initiates fast transmembrane ion currents mediated by neurotransmitter receptors on cell surfaces. The ability to spatially direct release of free products is useful where diffusional barriers exist that prevent transport of substrates to their targets. For example, caged calcium is widely employed to activate calcium-mediated processes occurring inside cells.[2] Caged carbamoylcholine photolysis has been used to determine the location of receptors on isolated BC_3H1 cells which express the muscle-type acetylcholine receptor.[17] Caged glutamate was employed to map glutamate-sensitive synaptic connections between neurons within a layer of mammalian neuronal tissue[18] and to probe functions of the neural network in the pharynx of a living organism, the nematode *Caenorhabditis elegans*.[19]

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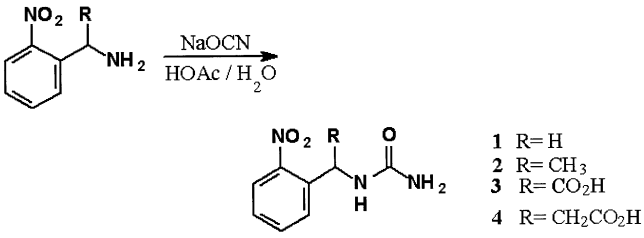
SCHEME 1



Photochemical protection used with many caged molecules involves coupling with a 2-nitrobenzyl group, and this strategy has now been used for a variety of functional groups, including carbamates,[7] amines,[15,20] carboxylates,[14,16] phosphates,[21] phenols,[22] and amides.[23] Photoprotection of amide precursors has also been used in general synthetic procedures[24] and has been applied in solid-phase peptide synthesis.[25,26] The temporal and spatial resolution achievable with photolysis of caged compounds is potentially useful in time-resolved X-ray crystallography,[25−29] and caged compounds have been used to release substrates by photolysis of precursors inside a protein crystal.[30] For example, synchrotron radiation and Laue diffraction were used to monitor structural changes that occur when free GTP is released in a crystal of a GTP-binding protein by photolysis of a caged phosphate ester precursor of GTP.[31,32]

A photolabile precursor of urea may be applicable in crystallographic and kinetic investigations of urease, an enzyme found in plants, fungi, and bacteria.[33] This nickel metalloenzyme catalyzes the hydrolysis of urea to ammonia and bicarbonate.[34,35] Urease from *Klebsiella aerogenes* has been crystallized and the structure determined at 2.0 Å resolution.[36] Models for the binding and hydrolysis of urea have been proposed and recently reviewed.[37] The goal of the work described here was to develop photolabile urea derivatives that rapidly release free urea upon photolysis. We report here the synthesis and properties of four urea analogues that release urea when exposed to a pulse of UV light. *N*-(α-Carboxy-2-nitrobenzyl)urea (compound **3**), which was soluble in aqueous solution, photolyzed rapidly, exhibited a high quantum yield, and was tested for compatibility with urease. Neither the caged compound itself nor its photolysis side products inhibit urease in solution. The caged compound is, therefore, expected to be useful in further studies of this enzyme.

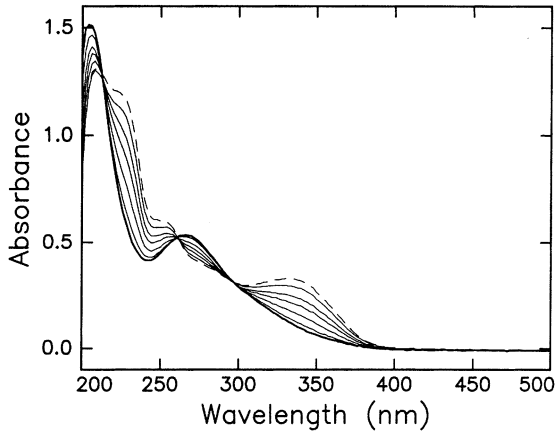


FIGURE 1. UV−vis spectra of the photolytic conversion of compound **3** to products. A 60 μL aliquot of a 1.0 mM solution of compound **3** in 100 mM phosphate buffer adjusted to pH 7.5 was photolyzed with 337 nm light produced by a pulsed nitrogen laser at room temperature. The laser was focused into a 2 × 2 mm cuvette to provide a beam with dimensions of approximately 2 × 6 mm. The energy of each shot was 4.5 mJ, and the individual spectra correspond to 0, 5, 20, 40, 60, 90, and 120 pulses. The optical path lengths for the laser and monitoring beams in the cuvette were 0.2 cm. The dark line (lower trace at 350 nm) corresponds to the spectrum of the starting material, and the isosbestic points are located at 258 and 295 nm**.** The photolysis was continued until no further spectral changes occurred; the dashed line is the final spectrum and corresponds to essentially complete conversion to products.

Results and Discussion

The structures of four photolabile urea derivatives and their synthesis are presented in Scheme 1. The derivatization is performed in a single step with the addition of an α-substituted 2-nitrobenzylamine to isocyanate. The reaction occurs under acidic conditions, and the derivatized urea product precipitates readily from the reaction mixture. The derivatives photolyze in aqueous solutions buffered at pH 7.4 and release free urea and a presumed nitroso side product[38] when exposed to a pulse of UV light in the range of 300−340 nm. The general mechanism for photohydrolysis of 2-nitrophenyl compounds in aqueous media has been discussed.[38,39] Figure 1 shows the changes which occur in the UV−vis spectrum of compound **3**, in which the urea nitrogen is protected by the α-carboxynitrobenzyl group,[7] as the photolysis reaction proceeds from starting material to products at pH 7.4. The spectral changes reflect the transformation of the 2-nitrobenzyl derivative (absorbance maximum at 268 nm) to the products, which for compound **3** are a nitrosophenyl

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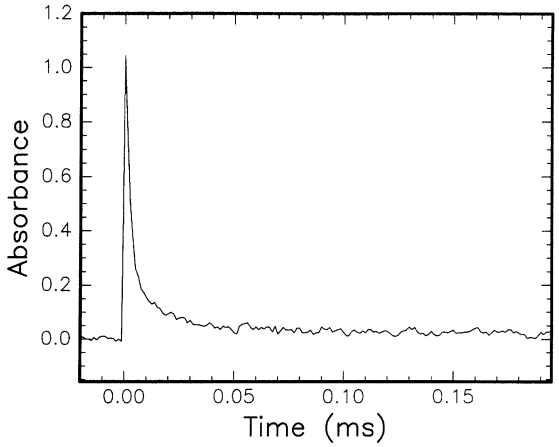
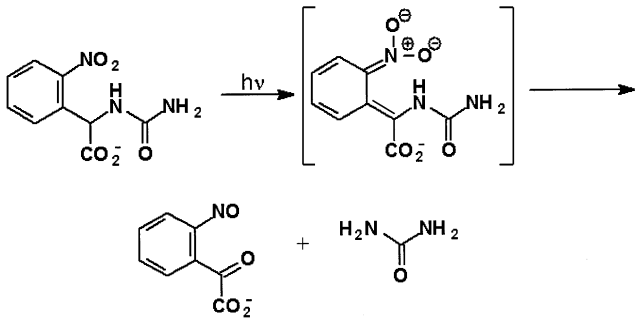


FIGURE 2. Absorbance transient produced by photolysis of compound **3**. The trace was produced by photolysis of a 3 mM solution of the caged urea in pH 7.5 phosphate buffer by a 10 ns pulse of 308 nm UV light (30 mJ per pulse) from an excimer laser at room temperature. The path length of the monitoring beam in the cuvette was 0.2 cm. The effective bandwidth of the detection system was 500 kHz. The absorbance decay of the photolysis intermediate was measured at 430 nm, near the absorbance maximum for the intermediate.

SCHEME 2



α-keto acid (absorbance maximum at 338 nm) and free urea (Scheme 2). The spectra share clear isosbestic points, so the spectral changes reflect the formation of the nitroso side product and no other absorbing species.

The rate and quantum yield of the photolysis reaction are important criteria in the selection of the most appropriate derivative for use in time-resolved crystallography. Photolysis of 2-nitrophenyl derivatives proceeds through an *aci*-nitro intermediate which has a characteristic near-UV absorbance.[38] This intermediate forms on a submicrosecond time scale immediately following excitation and then decays to products in 10−100 μs. The intermediate has a characteristic absorbance profile with a maximum between 420 and 440 nm,[38,40] and the intermediate disappearance can be followed spectrophotometrically to determine the reaction rate. Figure 2 shows the decay transient observed when compound **3** is photolyzed in buffer at pH 7.4; the half-life of the decay is 6.3 μs. Transient decay rates of the series of urea derivatives were determined by fitting a single-exponential function to the absorbance data and are shown in Table 1. Figure 3 shows the spectrum of this transient for compound **3** determined from the maximum of the absorbance signals at wavelengths between 350 and 500 nm measured 2 μs after the laser was triggered. The intermediates for compounds **2** and **4** have absorbance maxima at 420 nm, whereas **1** and **3** have maxima close to 430 nm. The spectra are consistent with the appearance of *aci*-nitro intermediates observed with similar 2-nitrobenzyl caged compounds[39] and are distinct from the spectra of the caged compounds themselves and the products of the photolysis reaction. *aci*-Nitro intermediate decay rates have been shown to be directly related to the rate of product release,[41,42] and free urea is, therefore, produced on a time scale of 10−100 μs for compounds **1**−**4**.

TABLE 1. Urea Derivatives: Photolysis Rate and Product Yield at pH 7.4 and Room Temperature

compd	photolysis rate[a] (s^{-1})	product quantum yield[b]	compd	photolysis rate[a] (s^{-1})	product quantum yield[b]
1	1.7×10^4	0.81	**3**	1.1×10^5	0.56
2	8.5×10^4	0.64	**4**	4.0×10^4	N.D.

[a] Measured with excitation by a pulsed excimer laser at 308 nm, 30 mJ per pulse. [b] Measured with excitation by a pulsed excimer laser at 337 nm, 5 mJ per pulse. Duplicate determinations were performed for each reported value; the relative error is estimated at 20%. N.D. not determined.

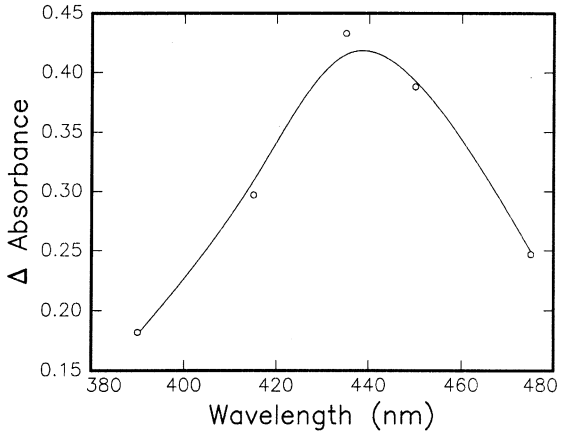


FIGURE 3. Spectral dispersion of the transient absorbance signal of compound **3**. Absorbance was measured at discrete wavelengths between 380 and 500 nm immediately after excitation by the laser pulse. The concentration, pH of the solution, and other conditions are those given in the caption for Figure 2.

The photolysis rate is also dependent on the pH. A plot of the *aci*-nitro intermediate decay rate for compound **3** versus pH is shown in Figure 4. The accelerated rates at lower pH are qualitatively similar to rates observed for a series of (2-nitrobenzyl)glutamine compounds which employed an analogous amide linkage.[23] However, compound **3** photoyzed 10−100 times faster than the corresponding (α-carboxy-2-nitrobenzyl)glutamine derivative at each pH where measurements were made. Quantum yield determinations for compounds **1**−**3** were performed in aqueous pH 7.4 buffer, and the results are presented in Table 1. Both the photolysis rates and quantum yields

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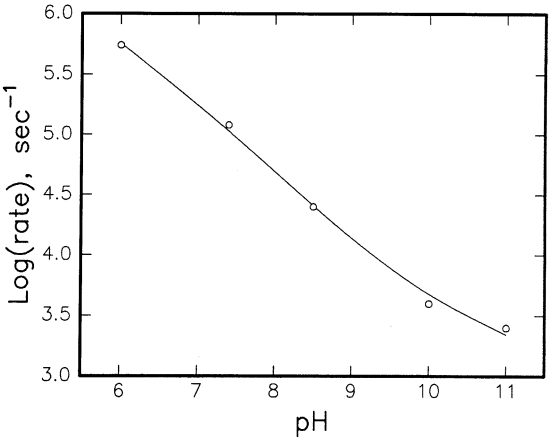

FIGURE 4. Variation of photolysis rate with pH at room temperature (22 °C). Solutions of 3 mM compound **3** in 100 mM buffers were photolyzed by 308 nm, 30 mJ pulsed light from the excimer laser, and the rates of the transient intermediate absorbance decay were determined from exponential approximations to the decay data. The solid line represents a spline fit to the data points. The buffers used were phosphate at pH 6.5 and 7.5, borate at pH 8.5 and 9.8, and bicarbonate at pH 11.0.

TABLE 2. Activity of Urease[a] in the Presence of Compound 3[b]

substrate	specific activity (U/mg)	normalized activity (%)
free urea	118 ± 2	100 ± 2
compound **3**	0	0
compound **3** + free urea	113 ± 8	96 ± 7
free urea	94 ± 3	100 ± 3
photolyzed compound **3**	5 ± 3	5 ± 3
photolyzed compound **3** + free urea	106 ± 5	110 ± 5

[a] Urease used in these assays was stored for 2 months at 4 °C and had a specific activity of 110 ± 10 U/mg at the start of the caged urea assays and 95 ± 10 U/mg at the beginning of the photolyzed caged urea assays. [b] When present, urea and/or caged urea were each at 10 mM. Given the efficiency of photolysis, the photolyzed samples would be expected to contain about 5 mM free urea and side products and 5 mM unreacted caged compound. Freshly prepared *K. aerogenes* urease has a specific activity of ~1800 U/mg (1 unit of urease is equivalent to the amount of enzyme required to hydrolyze 1 μmol of urea/min under the assay conditions). Upon storage at 4 °C for extended lengths of time, the enzyme activity decreases with no effect on K_m.

of the caged urea derivatives were greater than values found previously for an analogous series of amide derivatives of glutamine, asparagine, and glycine.[23]

Caged urea derivatives may be useful in investigations of the interaction of urea with the active sites in crystals of the enzyme urease. Compound **3** was chosen for evaluation with the enzyme because of its high photolysis rate and good quantum yield. To assess the compatibility of caged urea with urease, the specific activity of the enzyme in the presence of compound **3** and its photolysis products was measured. Table 2 shows that the activity of the enzyme is unchanged in the presence of 10 mM (α-carboxy-2-nitrobenzyl)urea, indicating that compound **3** does not itself inhibit urease. Furthermore, when compound **3** was incubated with the enzyme in the absence of urea, there was no indication that either urea or free ammonia was released by enzymatic hydrolysis or degradation of the caged compound itself. Thus, compound **3** is neither a substrate nor an inhibitor of *K. aerogenes* urease.

It was important to determine that urease is able to hydrolyze the free urea released by photolysis of compound **3**. To test this, a large-volume aliquot of compound **3** (0.5 mL, 10 mM solution in urease assay buffer) was photolyzed with the nitrogen laser. In contrast to the experiments shown above for determination of the quantum yield, many laser shots during an extended 4 min irradiation time were required for photolysis of the larger sample and complete conversion was not possible. The photolysis products absorb at the 337 nm nitrogen laser wavelength and may also undergo a photoreaction themselves. Nevertheless, a product of photolysis of compound **3** was turned over by urease to produce the color reaction of the assay (Table 2), which is consistent with release of free urea by the photolysis.

The rate of hydrolysis is only 5% of that expected if each photolyzed molecule produced one molecule of urea. The low activity is not due to inhibition of urease by other photolysis products since the presence of photolyzed products of compound **3** does not slow urease hydrolysis of added free urea (line 3 vs line 6 of Table 2). These results indicate that urease hydrolyzes urea released by photolysis of compound **3** and that byproducts of the photolysis reaction do not inhibit hydrolysis of urea by the enzyme.

Hadfield and Hadju[43] have proposed criteria which are desirable for a caged compound used in crystallographic experiments, and compound **3** conforms to several of these requirements: (1) it is water soluble at concentrations up to at least 50 mM, (2) photolysis occurs between 300 and 340 nm, which is above the typical optical absorbance of proteins, (3) it has a quantum yield of 0.6, and (4) the photolysis products do not inhibit urease. These characteristics of compound **3** are sufficient to justify its application in crystallographic studies of urease. However, because the absorbance of the photolysis products is greater than for the caged compound over the range of 300−400 nm (Figure 1), the extent to which the compound may be photolyzed in a crystal under conditions of continuous irradiation may be limited.

The caged urea derivatives shown here also serve as models for similar compounds which could be useful in situations where rapid, high-yield photolytic release of a free amide is required. For instance, it may be possible to adapt the procedures used here to produce light-activatable derivatives of hydroxyurea, an inhibitor of ribonuclease reductase used in clinical treatments of malignancies and potentially as an HIV virus inhibitor.[44] It may also be useful in studies of hydroxyurea in the treatment of sickle cell anemia.[45]

Experimental Section

For NMR, chemical shifts are referenced to the residual proton peak in D_2O at 4.64 ppm, and coupling constants (J) are measured in hertz. Elemental analysis was performed by

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a microanalytical laboratory, and high-resolution mass spectra were provided by the Mass Spectrometry Laboratory at the University of Illinois, Champaign. The amine hydrochlorides used as starting materials for the synthesis either were obtained from commercial sources or were synthesized according to reported methods for 1-amino-1-(2-nitrophenyl)ethane[40] and 3-amino-3-(2-nitrophenyl)propanoic acid.[46]

The synthetic procedure is depicted in Scheme 1. Portions of sodium cyanate (3.0 mmol) were added over a period of 6 h to a stirred mixture of the appropriate benzylamine hydrochloride complex (1.0 mmol), glacial acetic acid (3.0 mmol), and water (20 mL). A precipitate formed after 3 h, and stirring was continued overnight. For isolation of compound **1**, the reaction mixture was cooled on ice; the solid was filtered, washed with a water and acetic acid (1:1) mixture, and dried. For compounds **2**−**4**, the solutions were lyophilized and passed through a Sephadex LH-20 column twice using water as eluent.

Compound 1, *N*-(2-Nitrobenzyl)urea. Yield: 60%. Mp: 145 °C. ^{1}H NMR (D_2O, DMSO-d_6): 7.97 (d, 1H, J = 8.4, C_3–H), 7.60 (m, 1H), 7.42 (m, 2H) and 4.47 (s, 2H, CH_2). Anal. Calcd for $C_8H_9N_3O_3$: C, 49.23; H, 4.62; N, 21.59. Found: C, 49.46; H, 4.61; N, 21.25.

Compound 2, *N*-(α-Methyl-2-nitrobenzyl)urea.[47] Yield: 48%. Mp: 125−126 °C. ^{1}H NMR (D_2O, DMSO-d_6): 7.79 (d, 1H, J = 8.4 Hz), 7.53 (m, 1H), 7.33 (m, 2H), 5.05 (q, 1H, J = 6.8), 1.36 (d, 2H, J = 6.9). High-resolution mass spectrum (m/z): $(M + H)^+$ calcd for $C_9H_{12}N_3O_3$ 210.0879, found 210.0888.

Compound 3, *N*-(α-Carboxy-2-nitrobenzyl)urea. Synthesized according to Davis et al.[51] Yield: 55%. Mp: 138−145 °C dec. ^{1}H NMR (D_2O): 7.90 (d, 1H, J = 7.72, C_3–H), 7.47 (m, 1H), 7.42 (m, 2H) 5.46 and 5.45 (1H, CH). High-resolution mass spectrum (m/z): $(M + H)^+$ calcd for $C_9H_9N_3O_5$ 240.0620, found 240.0607.

Compound 4, *N*-(α-Carboxymethyl-2-nitrobenzyl)urea. Yield: 60%. Mp: 162−4 °C. ^{1}H NMR (D_2O): 7.85 (d, 1H, J = 7.9, C_3–H), 7.52 (m, 1H), 7.35 (m, 2H), 5.33 (m, 1H, C*H*CH_2), 2.56 (m, 2H, CHCH_2). High-resolution mass spectrum (m/z): $(M + H)^+$ calcd for $C_{11}H_{13}N_3O_6$ 254.0777, found 254.0763.

Urease Assay. The Bertholet ammonia assay[36,48] was used as a measure of enzyme activity achieved when urea is released from a caged precursor by photolysis and also to test whether urease is inhibited by compound **3**. The urease concentration used in each assay was 2×10^{-4} mg/mL as determined by the bicinchoninic acid protein staining method. All activity assays were carried out in triplicate, and steady-state initial rates were based on measurements made at 0.5, 10, 15, and 20 min. To provide aliquots of photolyzed caged compound, 500 μL of a 50 mM solution of compound **3** (in 10 mM phosphate buffer at pH 7.4) was irradiated with a pulsed nitrogen laser at 337 nm (5 mJ per pulse) at 5 Hz for approximately 4 min at room temperature. An 80 μL sample of this solution was then added to the reaction mixture. The change in the absorbance spectrum (Figure 1) was used to estimate the extent of photolysis and indicated that 53% of the starting caged compound was photolyzed under these conditions.

Laser-Flash Photolysis. Instrumentation used for transient absorption spectroscopy has been described.[23] A single 30 mJ pulse of 308 nm light from a XeCl excimer laser was used to initiate photolysis, and the decay of the transient intermediate absorbance was recorded by digitizing the photomultiplier output at rates up to 2 MHz. All measurements of photolysis rates and quantum yields were performed at room temperature. Nonlinear least-squares fitting of single-exponential decays were fit to the data to determine the photolysis rate constants. The remainder of the experiments used 5 mJ, 337 nm pulsed light produced by an excimer laser charged with N_2 and He gas instead of the Xe/HCl/He excimer mixture.

Quantum Yield Determination. Free urea produced by the photolysis reaction was determined by reversed-phase HPLC.[49] Urea was eluted under isocratic conditions from a 4.5 × 300 mm C-18 column[50] using buffer consisting of 50 mM phosphate at pH 6.4 and 2 mM tetraethylammonium chloride; free urea was detected by absorbance at 205 nm. Peak areas were compared to concentration standards in the range from 0 to 20 mM urea. Solutions (20 mM) of the compounds in 50 mM phosphate buffer at pH 7.4 were photolyzed by single 30 mJ pulses of 308 nm light from the XeCl excimer laser at room temperature. The energy of the laser light absorbed in the solution was measured with a pyroelectric thermopile energy meter.[23] Aliquots of the photolyzed solution were combined with HPLC running buffer and used for the chromatographic analysis. The quantum yield was calculated as the number of free urea molecules liberated by photolysis divided by the number of photons absorbed.

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