Photochemical Coupling of 5-Bromouracil (BU) to a Peptide Linkage. A Model for BU-DNA Protein Photocrosslinking

Timothy M. Dietz, Robert J. von Trebra, Barry J. Swanson, and Tad H. Koch*

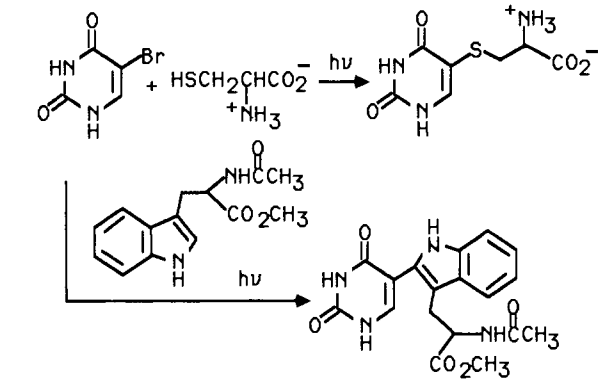
Contribution from the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215. *Received September 15, 1986*

Abstract: Photocrosslinking of DNA containing 5-bromouracil (BU) in place of some of the thymine to an associated protein has potential for studying protein nucleic acid interactions. Experiments with model compounds are described which indicate some of the electronic and molecular details of the crosslinking. Earlier experiments suggested that the BU chromophore in its π,π^* singlet state reacted by C–Br bond homolysis probably leading to BU-DNA single strand breaks and that the triplet state was responsible for crosslinking of BU-DNA to associated proteins probably via initial electron transfer. Only intermolecular coupling of BU to cysteine, glutathione, tryptophan, and tryptophan derivatives had been observed previously. The important conclusions established here are (1) that the BU chromophore has a poorly resolved, possibly $n-\pi^*$ transition in the region of 308 nm, (2) that the photochemistry of the BU chromophore is significantly wavelength dependent, (3) that the $^1n,\pi^*$ state can be selectively populated with a XeCl excimer laser or the 313-nm mercury band isolated with a monochromator, (4) that the $^1n,\pi^*$ state does not undergo C–Br bond homolysis in competition with intersystem crossing to the triplet manifold, and (5) that the triplet state couples to a peptide linkage in proximity with loss of HBr. These conclusions were established by studying the photoreduction of BU in 2-propanol-*d* and the photocyclization of (5-bromo-6-uracilyl)-*N*-ethylacetamide (**1**) and *N*-[(5-bromo-6-uracilyl)acetyl]-D,L-threonine *N*-ethylamide (**2**) to furans **3** and **6** in buffered water, both as a function of wavelength.

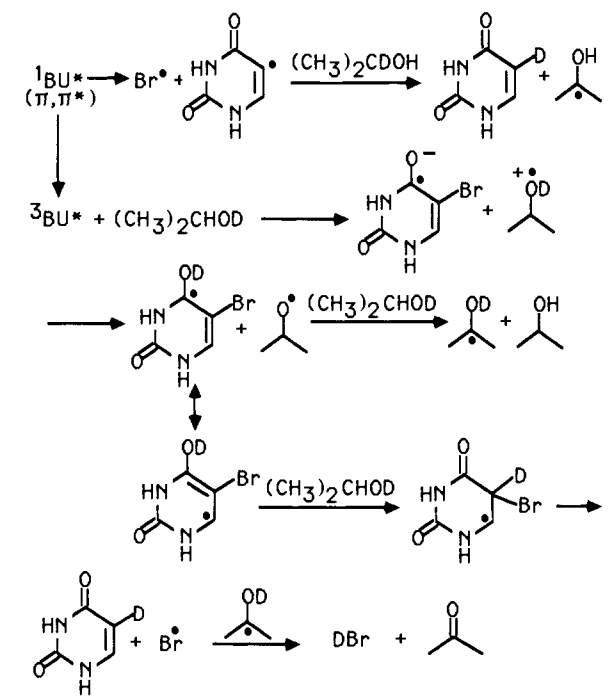
Replacement of thymine in DNA by 5-bromouracil (BU) yields BU-DNA which has enhanced photosensitivity with respect to single strand breaks, creation of alkali labile bonds, double strand breaks, and DNA-protein photocrosslinking.[1-3] Photocrosslinking of nucleic acids containing BU to associated proteins is potentially a useful method for studying protein nucleic acid interactions.[4] Although photocrosslinking of BU-DNA to histones,[5] RNA polymerase,[5] and lac repressor[6,7] have been observed, little is known about the molecular details of the crosslinking. Studies of photoreactivity of 5-bromouracil and 5-bromouridine with amino acids and amino acid derivatives have revealed only coupling to cysteine and glutathione[8] and tryptophan[9] and tryptophan derivatives[10] (Scheme I). Adduct formation with tryptophan derivatives was shown to occur via an electron-transfer reaction of the triplet state.[10] Thermal electron-transfer reactions from *N*-methylindole and *N*-methylphenothiazine to bromouracil derivatives have also been reported.[11]

We[12] and others[13,14] have established some of the primary photochemical properties of BU by studying the photoreduction of BU to uracil (U) in 2-propanol solvent. Earlier, Schulte-Frohlinde and co-workers showed that the photoreduction was wavelength, pH, temperature, and hydrogen donor concentration dependent.[14] We have demonstrated that photoreduction of BU to U in 2-propanol solvent occurs in two different excited states via two different mechanisms.[12] The π,π^* singlet formed by excitation in the region of 276 nm undergoes C–Br bond homolysis,

Scheme I



Scheme II



followed by hydrogen atom abstraction from 2-propanol and intersystem crossing to the triplet manifold. The lowest triplet

(1) Smith, K. C. In *Photochemistry and Photobiology of Nucleic Acids*; Wang, S.Y. Ed.; Academic Press: New York, 1976; Vol. II, p 187.
(2) Hutchinson, F.; Köhnlein, W. *Prog. Subcell. Biol.* **1980**, *7*, 1.
(3) Shetlar, M. D. *Photochem. Photobiol. Rev.* **1980**, *5*, 105.
(4) Schimmel, P. R.; Budzik, G. P.; Lam, S. S. M.; Schoemaker, H. J. P. In *Aging, Carcinogenesis, and Radiation Biology*; Smith, K. C., Ed.; Plenum: New York, 1976; p 123. Helene, C. In *Aging, Carcinogenesis, and Radiation Biology*; Smith, K C., Ed.; Plenum: New York, 1976; p 149.
(5) Weintraub, H. *Cold Spring Harbor Symp. Quant. Biol.* **1973**, *38*, 247.
(6) Lin, S. Y.; Riggs, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 947.
(7) Ogata, R.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4973.
(8) Varghese, A. J. *Photochem. Photobiol.* **1974**, *20*, 461.
(9) Saito, I.; Ito, S.; Matsuura, T.; Helene, C. *Photochem. Photobiol.* **1981**, *33*, 15.
(10) Saito, I.; Matsuura, T. *Acc. Chem. Res.* **1985**, *18*, 134. Ito, S.; Saito, I.; Matsuura, T. *J. Am. Chem. Soc.* **1980**, *102*, 7535.
(11) Sako, M.; Hirota, K.; Maki, Y. *Chem. Pharm. Bull.* **1983**, *31*, 3496.
(12) Swanson, B. J.; Kutzer, J. C.; Koch, T. H. *J. Am. Chem. Soc.* **1981**, *103*, 1274. The experimental procedures for this communication are available as supplementary material to work described here or in the Ph.D. Thesis of B. J. Swanson, University of Colorado, 1981.
(13) Hutchinson, F. *Quart. Rev. Biophys.* **1973**, *6*, 201.
(14) Campbell, J. M.; von Sonntag, C.; Schulte-Frohlinde, D. *Z. Naturforsch., B: Anorg. Chem., Org. Chem.* **1974**, *29B*, 750.

0002-7863/87/1509-1793$01.50/0

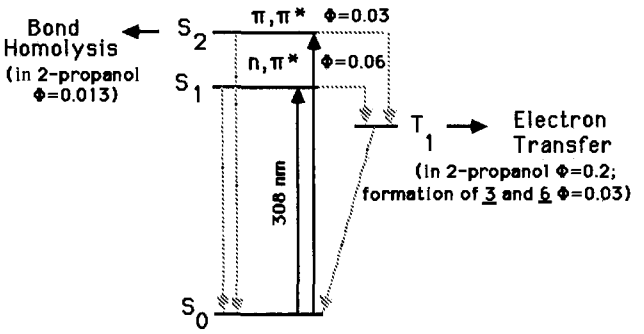


Figure 1. Jablonski diagram for the BU chromophore.

state also yields U, however, by initial abstraction of an electron from 2-propanol. Two-thirds of the U results from the π,π^* singlet state and one-third from the lowest triplet state. The quantum yields for these processes are summarized in Figure 1. The hydrogen atom abstraction and electron-transfer pathways were established by deuterium labeling of the 2-propanol on oxygen and on the 2-carbon in separate experiments coupled with sensitization and quenching measurements. Irradiation of BU in 2-propanol-*d* gives high deuterium incorporation at the 5-position of U via the BU triplet state and in 2-propanol-2-*d*, high deuterium incorporation via the π,π^* singlet state. The mechanisms proposed for deuterium incorporation as a function of state are shown in Scheme II. These deuterium labeling experiments can now be used to diagnose the reactive states or states of BU or BU derivatives under varying conditions, vide infra. A related state dependent photoreduction of 2-bromo-4,4-dimethyl-2-cyclohexenone has been reported by Hombrecher and Margaretha.[15]

The observation of electron-transfer photoreduction of BU in 2-propanol via the triplet manifold[12] coupled with the observation of Saito, Matsuura, and co-workers that BU adds to tryptophan derivatives via electron transfer in the triplet manifold[10] suggests that BU in DNA might react with amino acid residues in associated proteins much less easily oxidized than Trp. In fact triplet BU might even react with a peptide linkage. The proposal has now been tested with model compounds in which the bromouracil chromophore is covalently bound to peptides at the 6-position of the BU. The systems investigated here are (5-bromo-6-uracilyl)-*N*-ethylacetamide (**1**) and *N*-[(5-bromo-6-uracilyl)-acetyl]-D,L-threonine *N*-ethylamide (**2**). System **1** models a peptide linkage associated with the BU chromophore, and system **2** models a peptide bearing a 2-propanol substituent associated with the BU chromophore.

Results and Discussion

(5-Bromo-6-uracilyl)-*N*-ethylacetamide (**1**) and *N*-[(5-bromo-6-uracilyl)acetyl]-D,L-threonine *N*-ethylamide (**2**) were synthesized in 50 and 22% overall yields, respectively, by condensation of urea with diethyl 1,3-acetonedicarboxylate followed by amide–ester exchange and bromination as shown in Scheme III. BU derivatives **1** and **2** and all synthetic intermediates were characterized by spectroscopic and elemental analysis.

A major problem associated with achieving a high yield of photocrosslinking is the competitive C–Br bond homolysis of BU in the π,π^* singlet state which most likely leads to DNA single strand breaks.[13,16] Earlier, we determined that the intersystem crossing efficiency of the π,π^* singlet state, populated with 254-nm light, to the triplet manifold was only 0.03.[12] We also observed that the quantum yield of U formation in 2-propanol was an order of magnitude lower when BU was irradiated at 290 ± 20 nm by using a high pressure mercury lamp and a monochromator. Careful inspection of the UV absorption spectrum of BU revealed a weak, poorly resolved shoulder at 300 nm (ε 338) which we have assigned to the n–π^* transition based upon its intensity. This band is also apparent in the UV spectra of the BU derivatives **1** and

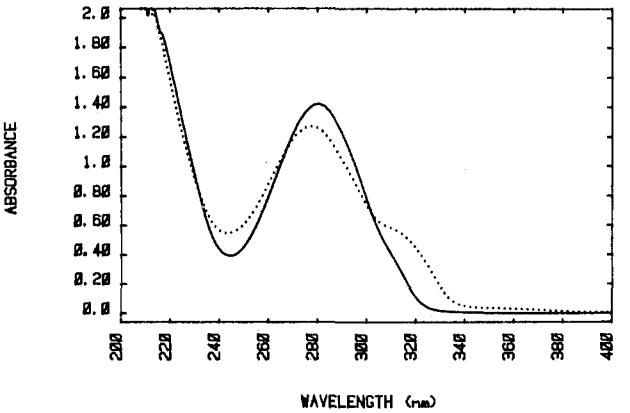
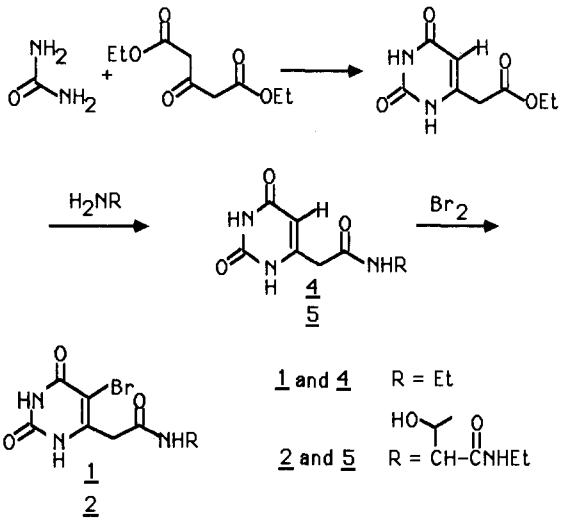


Figure 2. (—) UV absorption spectrum of 2.0×10^{-4} M (5-bromouracilyl)-*N*-ethylacetamide **1** in pH 7.0 buffered water; (···) UV absorption spectrum after irradiation with 0.35 Wh of 308 nm light. At this time the solution contained both **1** and furan **3**.

Scheme III



2 as shown for **1** in Figure 2. Unfocused XeCl excimer laser irradiation at 308 nm of the n–π^* band of BU in 2-propanol-*d* solvent gave a mixture consisting of 39% U and 61% BU. Integration of the olefinic ^{1}H NMR signals of the uracil indicated that the U had approximately 77% deuterium incorporation in the 5-position. This is more than the percent deuterium incorporation observed with acetone sensitized reduction of BU in 2-propanol-*d*.[12] Consequently, in the n,π^* singlet state, C–Br bond homolysis was not competitive with intersystem crossing to the triplet manifold and photoreduction occurred via the triplet state. The monochromicity of the XeCl excimer laser was useful in this experiment and in the cyclization experiments to be described for achieving specific population of the poorly resolved ^{1}n,π^* state and then subsequently the triplet state.

The quantum yield of photoreduction of 2-propanol with excitation at 308 nm was 1.2×10^{-2}. The reaction was efficiently quenched by *cis*-piperylene with $\phi_0/\phi_q = 6.1$ at 2.68×10^{-3} M quencher; a linear Stern–Volmer plot was obtained with a slope of 2000 M^{-1} (correlation coefficient = 0.99). Assuming diffusion controlled quenching, the Stern–Volmer slope indicates that the BU triplet lifetime is 400 ns in 2-propanol at ambient temperature.[17]

5-Bromouracil coupled to a peptide linkage, system **1** in, pH 7, phosphate buffered water was irradiated with the partially focused emission from a XeCl excimer laser. The sample consisting of 40 mg of **1** in 125 mL of buffered water was irradiated

(15) Hombrecher, H.; Margaretha, P. *Helv. Chim. Acta.* **1982**, *65*, 2313.
(16) Lion, M. B. *Biochem. Biophys. Acta* **1968**, *155*, 505; **1970**, *209*, 24.

(17) The rate of diffusion of 2-propanol at 23 °C was calculated to be 4.9×10^9 M^{-1} s^{-1} by using the method of Osborne and Porter: Osborne, A. D.; Porter, G. *Proc. R. Soc. London, Ser. A* **1965**, *284*, 9.

Table I. Quantum Yields of Photoreduction and Cyclization with 308-nm Laser Excitation[a]

starting material	solvent (ioniztn potntl, eV)[19]	$\phi_{destructn}$	% reductn	% cyclizatn
Bu	EtOH (10.50)	2.9×10^{-3}	100	
Bu	2-propanol (10.15)	1.2×10^{-2}	100	
1	MeOH (10.85)	3.7×10^{-3}	100	0
1	H_2O (12.59)	1.8×10^{-3}	0	100
1	90% H_2O 10% 2-propanol	1.8×10^{-3}	30	70
1	90% H_2O 10% MeOH	1.8×10^{-3}	30	70

[a] The product yields were determined by HPLC analysis assuming that reduction and cyclization were the only processes that occurred at low conversion (10%).

for 2 h at an average power of 1.5 W, and the progress of the reaction was monitored by C-18 reverse phase HPLC. The reaction could not be carried to completion because one of the products, furan **3**, absorbed competitively at 308 nm (see Figure 2). At 0.6 Wh 32% of **1** had been destroyed, and the product composition consisted of 7% 6-uracilyl-*N*-ethylacetamide (**4**) and 93% of the cyclized product 4,6-diaza-5,7-dioxo-2-(ethylamino)-4,5,6,7-tetrahydrobenzofuran (**3**); maximum furan was formed at 3 Wh and 52% destruction of **1**. Furan **3** was isolated by preparative HPLC, and the structure was assigned from the spectral data.

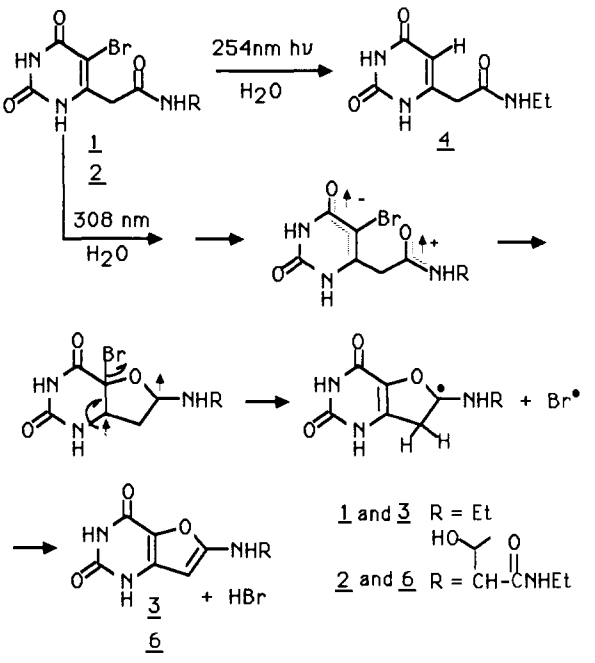
The quantum yields of photoreduction and cyclization of **1** are highly solvent dependent as shown in Table I. The quantum yield of destruction of **1** at 10% conversion upon 308-nm laser excitation in pH 7.0 buffered water was 1.8×10^{-3}, and only furan **3** was formed. When the solvent was changed to propanol, the quantum yield of destruction of **1** was also 1.8×10^{-3}, and both uracil **4** and furan **3** were formed. The solvent dependency established that the cyclization reaction is a triplet reaction since cyclization was replaced by photoreduction in 10% 2-propanol, and photoreduction of the BU chromophore with excitation at 308 nm in 2-propanol is a triplet reaction. The cyclization reaction in pH 7.0 water was also quenched by molecular oxygen; the quantum yield of destruction of **1** was 1.1×10^{-3} when the irradiation solution was saturated with pure oxygen giving an oxygen concentration[18] of 1.1×10^{-3} M ($\phi_o/\phi_q = 1.6$). The quantum yield of destruction of BU and **I** increased with decreasing ionization potential of the solvent consistent with the proposed electron-transfer mechanisms. The higher measured quantum yield for photoreduction of the BU chromophore determined with 308-nm laser excitation than that estimated earlier with mercury emission selected with a monochromator[12] probably resulted from more complete light absorption with the laser excitation. With the mercury emission the light intensity is highest where the chromophore absorbs least.

When **1** was irradiated with the emission from a medium-pressure mercury lamp filtered with two thicknesses of Pyrex glass, only formation of uracil **4** was observed. However, results similar to those achieved with the laser were obtained by excitation with the 313-nm emission from a high-pressure mercury lamp selected with a monochromator set at 310 nm with a band pass of 20 nm.

Similar laser irradiation of BU coupled to threoninamide, system **2**, at an average power of 1.6 W resulted in 35% destruction of **2** with no formation of *N*-(6-uracilylacetyl)-D,L-threonine *N*-ethylamide (**5**) and 100% yield of the analogous furan, *N*-(4,6-diaza-5,7-dioxo-4,5,6,7-tetrahydrobenzofuran-2-yl)-D,L-threonine *N*-ethylamide (**6**). The quantum yield of destruction of **2** with 308-nm excitation was 2×10^{-3}. Furan **6** was isolated by preparative HPLC and characterized from comparison of spectral data with those of **3**.

A mechanism for the formation of furans **3** and **6** is proposed in Scheme IV based upon the electron-transfer mechanism proposed earlier for photoreduction of triplet BU as shown in Scheme II[12] and the mechanism proposed by Saito, Matsuura, and Ito[10] for photocoupling of triplet BU to tryptophan and its derivatives. The initial step is electron transfer from the peptide linkage to the excited BU chromophore. Bond formation prior to intersystem crossing is proposed to minimize back electron transfer. The earlier quantum yield measurements of photoreduction[12] and the measurement of the quantum yield of photoreduction in 2-propanol solvent with 308-nm excitation indicate that intersystem crossing efficiency of the n,π^* singlet state of BU to the triplet manifold is 0.06; consequently, the quantum yield of cyclization within the triplet manifold is 0.03, assuming similar intersystem crossing efficiencies in water and 2-propanol. These efficiencies are summarized in Figure 1.

Scheme IV



The results of these experiments suggest that more selective triplet reactivity of BU can be achieved by initial specific excitation of the n,π^* singlet state and that the lowest triplet state of the BU chromophore can oxidize a peptide linkage in proximity. The resulting radical ions will couple with substitution of a bond to the peptide linkage for the bromine. In system **2** electron transfer could have occurred from the secondary alcohol or either of the amide functional groups. Only electron transfer from the proximal amide functional group was observed. The necessity of a peptide linkage in proximiy was tested by irradiation of BU in buffered water containing 0.01 M *N*-ethylacetamide at 308 nm. After 0.6 Wh less than 1% of the BU was destroyed. The observation of photocyclization of **1** in competition with photoreduction with 10% 2-propanol–90% water as the solvent suggests that photocrosslinking of BU-DNA to a peptide linkage in an associated protein via initial electron transfer from the peptide functional group might be competitive with intramolecular photoreduction via electron transfer from an oxygen on a nearby deoxyribose group. The probability of a 2-propanol molecule being immediately available for reaction with a triplet excited molecule of **1** is high when the amount of 2-propanol is 10% of the solvent by volume, and, yet, photocyclization was the predominant reaction pathway.

Earlier experiments of Weintraub suggested that more efficient crosslinking of BU-DNA to proteins could be achieved by using the 313-nm mercury band.[5] The explanation offered was preferential light absorption by the BU chromophore of the DNA at this longer wavelength. The results described here suggest that the 313-nm mercury band was preferential because it populated more of the n,π^* singlet state.

In summary, triplet reactivity of BU can be achieved more selectively by specific excitation of the poorly resolved n–π^*

(18) Landolt-Bornstein *Zahlenwerte Und Functionen Aus Physik-Chemie-Astronomie-Geophysik-Technich*, 6th ed.; Springer Verlag: Berlin, 1962; II Band 2. Teil Bandteil b, pp 1–74.
(19) Watanabe, K. *J. Chem. Phys.* **1957**, *26*, 542.

transition, irradiating in the region 310 ± 10 nm, than by excitation of the $\pi-\pi^*$ transition, and triplet BU will couple with a peptide linkage in proximity. Consequently, we predict that a preferred method for achieving BU–DNA protein photocrosslinking will be 308-nm laser excitation and that crosslinking may not require a specific amino acid residue to be adjacent to the BU.

Experimental Section

General Remarks. Melting points were determined with a Thomas Hoover apparatus and are uncorrected. Infrared spectra were recorded by using a Perkin-Elmer Model 337 spectrophotometer and UV spectra with a Hewlett-Packard 8450 spectrophotometer. NMR spectra were obtained with Varian EM 390 or Bruker 250-MHz instruments, and chemical shifts are reported in parts per million on the δ scale from internal tetramethylsilane, pentadeuteriodimethyl sulfoxide (δ = 2.49 ppm), or Tier's salt, trimethylsilylpropanesulfonic acid sodium salt. Mass spectra were recorded with Varian MAT CH-5 or VG Instruments 7070 EQ-HF mass spectrometers. Analytical HPLC was performed with a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array detector and data processing unit. The HPLC column was 4.6 mm × 240 mm packed with Alltech RSIL C-18 HL 10-μm reverse phase packing. The detector was set to monitor at 260, 278, and 308 nm, positions of maximum absorption of starting material and products. Yields were measured by using constant injections of 10 μL, and the detector responses of **1**, **2**, and **4** were determined with standard solutions. The yields of **3** and **6** were assumed to comprise the balance of the reaction mixture since no other product were formed. Preparative HPLC was performed with a Tracor 950 HPLC pump equipped with a 970A variable wavelength UV–vis detector set at 300 nm. The column was 9.52 mm × 240 mm of Alltech RSIL C-18 HL 10-μm reverse phase packing. HPLC solvents were Fisher HPLC grade, and reagents for synthesis of bromouracil derivatives were obtained from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Microanalyses were performed by Atlantic Microlab, Atlanta, GA.

Ethyl 6-Uracilylacetate. A 500-mL, round-bottom flask was charged with 50.0 g (0.247 mol) of diethyl 1,3-acetonedicarboxylate, 14.8 g (0.247 mol) of urea, and 3 mL of concentrated sulfuric acid. A magnetic stir bar and 400 mL of an anhydrous 68% benzene–32% ethanol (v/v) solution were added. A large soxhlet extractor and a drying tube containing Drierite were attached, and the cellulose thimble of the extractor was filled with 3-Å powdered molecular sieves. The solution was refluxed with stirring, and the sieves were replaced every other day for 10 days. At this time, the product was isolated by suction filtration to yield 23 g of white powder. The reaction was refluxed for an additional 35 days with weekly changes of the sieves. Suction filtration then yielded another 17 g of ethyl 6-uracilylacetate for a total yield of 40 g (82%) with mp 185–189 °C. An analytical sample was prepared by sublimation in a Kugelrohr oven at 170 °C (0.005 torr); it showed the following physical properties: mp 189–191 °C; IR (KBr) 3.40, 3.51, 5.77, and 5.99 μm; 90-MHz ^{1}H NMR (Me_2SO-d_6) δ 1.22 (t, J = 6.6 Hz, 3 H), 3.49 (s, 2 H), 4.13 (q, J = 6.6, 2 H), 5.46 (s, 1 H), 10.95 (s, 1 H), 11.08 (s, 1 H); MS, m/z (rel intensity) 198 (74), 153 (11), 126 (53), 125 (13), 109 (18), 99 (11), 98 (18), 97 (19), 82 (10), 68 (31). Anal. Calcd for $C_8H_{10}N_2O_4$: C, 48.49; H, 5.09; N, 14.13. Found: C, 48.54; H, 5.10; N, 14.15.

6-Uracilyl-*N*-ethylacetamide (4). A 100-mL, round-bottom flask equipped with magnetic stirrer was charged with 1.00 g (5.05 mmol) of ethyl 6-uracilylacetate and 60 mL of a 70% solution of ethylamine in water. The solution was stirred at ambient temperature for 4 h, at which time most of the solvent was removed on a rotary evaporator equipped with a vacuum pump. The resulting white solid was slurried in 60 mL of anhydrous chloroform with stirring for 1 h. Collection of the white solid by suction filtration gave 0.91 g (92%) of **4**, mp 235–240 °C (dec), which was spectroscopically pure: IR (KBr) 3.04, 3.40, 3.56, 5.78, 6.04, 6.41 μm; 250-MHz ^{1}H NMR (Me_2SO-d_6) δ 1.01 (t, J = 7.2 Hz, 3 H), 3.08 (d of q, J = 5.5, 7.2 Hz, 2 H), 3.18 (s, 2 H), 5.34 (s, 1 H), 8.01 (t, J = 5.5, 1 H), 10.82 (br s, 2 H); MS (70 eV), m/z (rel intensity) 198 (13), 197 (100), 127 (14), 110 (11), 98 (37), 97 (14), 82 (14), 72 (42), 68 (18), 55 (15), 44 (68), 43 (11), 42 (23), 30 (16). Anal. Calcd for $C_8H_{11}N_3O_3 \cdot 0.5H_2O$: C, 46.60; H, 5.87; N, 20.38. Found: C, 46.58; H, 5.88; N, 20.38.

(5-Bromo-6-uracilyl)-*N*-ethylacetamide (1). A 50-mL, round-bottom flask equipped with reflux condenser and magnetic stirrer was covered with aluminum foil and charged with 0.17 g (0.87 mmol) of *N*-ethyl-6-uracilylacetamide, 0.27 g (0.95 mmol) of pyridinium bromide perbromide, and 9 mL of water. The solution was stirred at ambient temperature for 1 h and at 90 °C in an oil bath for 1 h. Upon cooling, 0.16 g (66%) of **1** as a white solid, mp 240–244 °C, was collected by suction filtration; the material was spectroscopically pure: IR (KBr), 3.06, 3.20, 3.32, 3.40, 5.85, 5.92, 5.98, 6.10, 6.38 μm; 250-MHz ^{1}H NMR (Me_2SO-d_6) δ 1.04 (t, J = 7.5 Hz, 3 H), 3.12 (d of q, J = 3, 7.5, 2 H), 3.4 (s, 2 H), 8.12 (t, J = 3, 1 H), 11.33 (s, 1 H), 11.55 (br s, 1 H); UV (H_2O) λ_{max} 276 nm (ϵ 8600); MS (70 eV), m/z (rel intensity) 277 (7), 275 (10), 206 (28), 204 (29), 197 (14), 196 (100), 72 (24), 44 (27). Anal. Calcd for $C_8H_{10}N_3O_3Br$: C, 34.80; H, 3.65; N, 15.22. Found: C, 34.87; H, 3.66; N, 15.16.

D,L-Threonine *N*-Ethylamide. To a solution of 2.0 g (11.8 mmol) of D,L-threonine methyl ester hydrochloride in 12.5 mL of 70% ethylamine in water was added 4.9 mL of a 10% sodium hydroxide solution. After removal of the solvent via high-vacuum (0.1 torr) rotary evaporation, the white oily residue was extracted with 3 × 25 mL portions of chloroform. After suction filtration the chloroform was removed by rotary evaporation to yield 0.61 g (35%) of D,L-threonine *N*-ethylamide as a white powder, mp 114–116 °C, with the following spectral properties: IR (KBr) 2.93, 2.98, 3.04, 6.09, and 6.51 μm; 90-MHz ^{1}H NMR (Me_2SO-d_6) δ 1.01 (t, J = 7.5 Hz, 3 H), 1.04 (d, J = 6, 3 H), 2.88 (d, J = 5.7, 1 H), 1.7–4.8 (br, 3 H), 3.08 (p, J = 7.5, 2 H), 3.78 (d of q, J = 5.7, 6, 1 H), 7.90 (br s, 1 H); MS, m/z (rel intensity) 147 (2), 128 (2), 102 (86), 101 (12), 74 (100), 73 (15), 57 (19), 44 (15). Anal. Calcd for $C_6H_{14}N_2O_2$: C, 49.29; H, 9.65; N, 19.16. Found: C, 49.24; H, 9.70; N, 19.16.

***N*-(6-Uracilylacetyl)-D,L-threonine *N*-Ethylamide.** A 250-mL, round-bottom flask equipped with heating mantle, reflux condenser, magnetic stirrer, and nitrogen inlet and outlet bubbler was charged with 100 mL of anhydrous methanol, 2.11 g (10.7 mmol) of ethyl 6-uracilylacetate, 1.56 g (10.7 mmol) of D,L-threonine *N*-ethylamide, and 0.10 g (1.9 mmol) of sodium methoxide. The solution was refluxed with stirring and a flow of nitrogen for five days, at which time 5 mL of dimethylsulfoxide was added. The solution was refluxed with stirring and a flow of nitrogen for 10 additional days. Upon cooling, a slightly yellow precipitate formed, and 1.47 g (46%) of **5**, pure by ^{1}H NMR (mp 210–220 °C dec), was isolated by suction filtration. An analytical sample prepared by recrystallization from methanol showed the following spectral properties: IR (KBr) 3.07, 3.24, 3.38, 3.59, 5.88, 6.08, 6.42 μm; 250-MHz ^{1}H NMR (Me_2SO-d_6) δ 0.99 (t, J = 7.2 Hz, 3 H), 1.02 (d, J = 6, 3 H), 3.07 (d of q, J = 5.5, 7.2, 2 H), 3.31 (s, 2 H), 3.95 (m, 1 H), 4.07 (d of d, J = 4.0, 8.6, 1 H), 4.85 (d, J = 4.7, 1 H), 5.38 (s, 1 H), 7.80 (t, J = 5.5, 1 H), 8.01 (d, J = 8.6, 1 H), 10.80 (s, 1 H), 10.96 (s, 1 H); MS (70 eV); m/z (rel intensity) 298 (6), 280 (19), 262 (12), 254 (15), 247 (13), 209 (39), 208 (13), 152 (11), 127 (10), 126 (100), 102 (18), 96 (11), 82 (18), 68 (16), 58 (58), 55 (14), 46 (16), 44 (25). Anal. Calcd for $C_{12}H_{20}N_4O_6 \cdot H_2O$: C, 45.57; H, 6.73; N, 17.71. Found: C, 45.49; H, 6.36; N, 17.71.

***N*-[(5-Bromo-6-uracilyl)acetyl]-D,L-threonine *N*-Ethylamide (2).** *N*-(6-Uracilylacetyl)-D,L-threonine *N*-ethylamide (**5**) was brominated with pyridinium bromide perbromide exactly as described for the bromination of **4**. Product **5** was collected in 59% yield by suction filtration as a spectroscopically pure white solid with the following physical properties: mp 224–225 °C (dec); 250-MHz ^{1}H NMR (Me_2SO-d_6) δ 0.99 (t, J = 7 Hz, 3 H), 1.02 (d, J = 6, 3 H), 3.08 (d of q, J = 6, 7, 2 H), 3.59 (d, J = 14, 1 H), 3.65 (d, J = 14, 1 H), 3.95 (m, 1 H), 4.11 (d of d, J = 4, 9, 1 H), 4.84 (d, J = 4, 1 H), 7.73 (t, J = 6, 1 H), 8.11 (d, J = 9, 1 H), 11.28 (s, 1 H), 11.51 (s, 1 H); MS (70 eV), m/z (rel intensity) 360 (5), 358 (5), 280 (9), 209 (7), 208 (12), 207 (15), 126 (10), 102 (10), 81 (44), 80 (18), 79 (41), 78 (16), 74 (20), 71 (14), 64 (15), 58 (13), 56 (23), 44 (100). Anal. Calcd for $C_{12}H_{17}N_4O_5 \cdot H_2O$: C, 36.47; H, 4.85; N, 14.18. Found: C, 36.66; H, 4.85; N, 14.27.

Irradiations. A Lambda Physik EMG-101 excimer laser was used for laser irradiations and was operated at 308 nm by charging with 60 mbar of xenon and 80 mbar of 5% hydrogen chloride in helium and filled to 2500 mbar with helium. The laser output was focused with a quartz lens having a 10-cm focal length onto either a 3-cm diameter × 5 cm or a 5-cm diameter × 8-cm cell with quartz windows. Laser power output was measured with a Scientech 36-0001 disc calorimeter power meter. Continuous irradiations were performed with either a Osram 200-W high pressure mercury lamp in a Schoeffel lamp housing filtered with a Bausch and Lomb 250-mm monochromator equipped with a UV–vis grating (dispersion of 66 Å/mm) or with a Rayonet Reactor equipped with low pressure mercury lamps emitting at 254 nm. For all irradiations the solutions were buffered with Metrepak pHydrion pH 7 buffer, a potassium–sodium phosphate buffer, and degassed with nitrogen for 15 min prior to irradiation and continuously throughout the irradiation.

Laser Irradiation of 5-Bromouracil in 2-Propanol-*d* Solvent. 5-Bromouracil (4.8 mg, 0.025 mmol) was dissolved in 5 mL of 2-propanol-*d* solvent. The solution was placed in a quartz tube, degassed by 4 freeze (196 °C)–pump (5×10^{-6} torr)–thaw cycles and sealed. The sample was irradiaed with the unfocused beam of XeCl excimer laser (308 nm) for 45 min. The laser power output was 1.25 W at a repetition rate of 25 Hz. The exact amount of light absorbed by the sample could not be determined since the area of the laser beam was larger than the diameter of the sample tube. After the irradiation, a sample of the solution was diluted by a factor of 50, and a UV spectrum was recorded. Comparison

of the UV spectrum of the reaction mixture to spectra of authentic samples of 5-bromouracil and uracil showed that the mixture was approximately 39% uracil and 61% 5-bromouracil. Reverse phase HPLC analysis of the reaction mixture using the column described in the General Remarks section, eluting with 15% methanol/85% water (v/v) at 1.5 mL/min monitoring at 258 nm, showed only uracil and bromouracil. The identification of the peaks was confirmed by co-injection of authentic samples. The 250-MHz ^{1}H NMR spectrum (Me_2SO-d_6) showed peaks at δ 7.92, 11.27, and 11.55 ppm due to 5-bromouracil and at δ 5.46, 7.40, 10.84, and 11.04 ppm due to uracil. Integration of the peaks at δ 5.46 and 7.40 ppm indicated that the uracil had approximately 77% deuterium incorporated in the 5-position.

Laser Irradiation of (5-Bromo-6-uracilyl)-*N*-ethylacetamide (1). An Erlenmeyer flask equipped with a magnetic stirrer was charged with 47.5 mg (0.17 mmol) of **1**, 19 mg of pH 7 phosphate buffer, and 150 mL of HPLC grade water. The solution, 1.14×10^{-3} M in **1**, was stirred for 1 h at ambient temperature to complete dissolution. The 5 × 8 cm cell was charged with 125 mL of the solution, and the sample was irradiated for 2 h with the xenon chloride excimer laser at an average power of 1.5 W. The progress of the reaction was monitored by reverse phase analytical HPLC eluting with 25% methanol/75% water (v/v) at 1.0 mL/min; the retention times for **1**, **3**, and **4** using the column and detection conditions described in the General Remarks section were 3.14, 8.98, and 2.40 min, respectively. The reaction could not be carried to completion because the product absorbed radiation at 308 nm (Figure 2) and appeared to be somewhat photolabile. At 0.6 Wh 32% of **1** had been destroyed, and the product composition consisted of 7% 6-uracilyl-*N*-ethylacetamide (**4**) and 93% of 4,6-diaza-5,7-dioxo-2-(ethylamino)-4,5,6,7-tetrahydrobenzofuran (**3**); maximum furan was formed at 3 Wh and 52% destruction of **1**. Solvent was removed by high vacuum (0.1 torr) rotary evaporation, and the resulting sample was maintained at 0 °C to minimize product decomposition. The furan **3** was isolated by reverse phase preparative HPLC again eluting with 25% methanol/75% water at 1.0 mL/min and characterized from the following spectral data: 250-MHz ^{1}H NMR (Me_2SO-d_6) δ 1.11 (t, J = 7 Hz, 3 H), 3.08 (d of q, J = 5, 7, 2 H), 4.92 (s, 1 H), 7.55 (t, J = 5, 1 H), 10.35 (s, 1 H), 10.92 (s, 1 H); FAB positive ion mass spectrum m/z for M + 1 = 196.0794, theoretical M + 1 = 196.0722; UV (25% methanol/75% water v/v) λ_{max} 308 nm. Insufficient material could be obtained for elemental analysis.

Irradation of (5-Bromo-6-uracilyl)-*N*-ethylacetamide (1) at 310 ± 10 nm with Mercury Lamp Emission Filtered with a Monochromator. To 50 mL of HPLC grade water, 14.7 mg (0.053 mmol) of **1** and 12 mg of pH 7 phosphate buffer were added, and the mixture was stirred for 1 h at ambient temperature. A Pyrex cuvette was charged with 3.0 mL of the solution, and the solution was degassed with argon. The sample was irradiated with a mercury lamp filtered with a monochromator set at 310 ± 10 nm for 3 h. Reverse phase HPLC analysis as described in the preceding section showed 22% destruction of **1**. Of the starting material destroyed, 93% was converted to furan **3** and 7% to debrominated starting material **4**.

Irradiation of (5-Bromo-6-uracilyl)-*N*-ethylacetamide (1) at 254 nm. A large quartz tube was charged with 100 mL of an aqueous solution 0.010 M in **1** buffered to pH 7.0 with phosphate buffer. The solution was oxygen degassed by bubbling with nitrogen and irradiated for 3.7 h in a Rayonet Photochemical Reactor equipped with 8 low pressure mercury lamps with principal emission at 254 nm. HPLC analysis indicated that 35% of starting **1** had been destroyed with formation of 6-uracilyl-*N*-ethylacetamide (**4**) in 90% yield; none of the furan **3** was observed. The identity of **4** was confirmed by ^{1}H NMR spectroscopy.

Laser Irradiation of *N*-[(5-Bromo-6-uracilyl)acetyl]-D,L-threonine *N*-Ethylamide (2). A 70-mL sample of **2** (1.31×10^{-3} M) in pH 7 buffered water was prepared as described above for **1** and irradiated for 2.2 h with the XeCl excimer laser at an average power of 1.6 W. This resulted in 35% destruction of **2** with no formation of *N*-(6-uracilylacetyl)-D,L-threonine *N*-ethylamide (**5**) and 100% yield of *N*-(4,6-diaza-5,7-dioxo-4,5,6,7-tetrahydrobenzofuran-2-yl)-D,L-threonine *N*-ethylamide (**6**) based upon starting material destroyed. The analysis of the reaction mixture was performed by HPLC as described in the preceding section, and the retention times of **2**, **5**, and **6** were 2.76, 2.65, and 4.41 min, respectively. The water was removed by high vacuum (0.1 torr) rotary evaporation, and the product was isolated by preparative HPLC also as described in the preceding section. The furan structure **6** was assigned from the spectral data and analogy with **3**; 250-MHz ^{1}H NMR (Me_2SO-d_6) δ 0.099 (t, J = 7 Hz, 3 H), 1.10 (d, J = 7, 3 H), 3.08 (d of q, J = 5, 7, 2 H), 3.73 (d of q, J = 5, 9, 1 H), 3.91 (m, 1 H), 4.95 (d, J = 5, 1 H), 5.03 (s, 1 H), 7.50 (d, J = 9, 1 H), 8.01 (t, J = 5, 1 H), 10.37 (s, 1 H), 10.89 (s, 1 H); FAB MS, M + 1 m/z 297; UV (25% methanol/75% water v/v) λ_{max} 308 nm. Insufficient material could be obtained for elemental analysis.

Quantum Yield Measurements. Quantum yields were determined with the Lambda Physik EMG 101 XeCl excimer laser as the light source, and actinometry was performed with a Scientech Model 362 power energy meter connected to a calibrated Scientech 36-0001 disc calorimeter. The irradiation cell was cylindrical, 3-cm in diameter, with a path length of 5 cm, and equipped with quartz windows. The cell was placed 1.3 cm behind a spherical lense with a 10.16-cm focal length. The cell was charged with 30 mL of irradiation solution which was in the range of 2×10^{-3} M in bromouracil or bromouracil derivative, and the solution was oxygen degassed by bubbling with argon for 15 min prior to and during the irradiations. All irradiations were performed at ambient temperature with laser output in the range of 75 mJ/pulse at 20 Hz. Product and starting material analyses were accomplished with a Hewlett Packard 1090 HPLC equipped with a diode array detector, multichannel integrator, and 25 × 0.46 cm C-18 RSIL 10-μm reverse phase column. The column was eluted with 75% water/25% methanol (v/v) at 1.5 mL/min. Constant volume injections with detection at the λ_{max} of starting materials and products was employed. Detector response was calibrated with standard solutions of bromouracil, bromouracil derivatives, uracil, and uracil derivatives. Furan products were assumed to account for the balance of starting material destroyed. Irradiation times were selected to achieve 10–15% destruction of starting material.

Acknowledgment. Support of this research by PHS Grants GM-24965 and CA-24665 (DHHS), the Army Research Office (DOD), PHS's Biomedical Research Support Grant to the University of Colorado at Boulder (DHHS), and the University of Colorado Clinical Mass Spectrometry Resource, PHS Grant RR-01152 (DHHS) is gratefully acknowledged. We also thank Robert Murphy and Lee Miller for assistance with the FAB mass spectrometry and Christopher Kutzer for help with the initial steps in the synthesis of **1** and **2**.

Supplementary Material Available: The complete experimental section for the quantum yield measurements described in our earlier communication on the photoreduction of 5-bromouracil[12] is reported (13 pages). Ordering information is given on any current masthead page.