Mechanistic Studies of Antibody-Catalyzed Pyrimidine Dimer Photocleavage

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Abstract: An antibody elicited against the *trans, syn* uracil cyclobutane dimer hapten **1** catalyzes the light-dependent cleavage of uracil dimers **1** and **2** to the corresponding monomers **3** and **4**. Kinetic analysis of the antibody-catalyzed reaction affords a value of $k_{cat}/K_M = 1.7 \times 10^3\ M^{-1}\ min^{-1}$ for substrate **2**, and comparison to the uncatalyzed reaction gives a rate acceleration of $k_{cat}/k_{uncat} = 380$. The wavelength dependence of the reaction and fluorescence quenching behavior suggest that a tryptophan residue is acting as a photosensitizer. The reaction mechanism was probed by measurement of secondary deuterium isotope effects. Substrates with selective deuterium substitutions in the cyclobutane ring were prepared, and isotope effects were measured by the method of internal competition using electrospray-ionization mass spectrometry to quantify the products. Kinetic isotope effects of $^{\alpha\text{-}D}(V/K) = 1.11$, 1.14, and 1.20 were observed for the 5,5′-, 6,6′-, and 5,5′,6,6′-labeled substrates, respectively. These results are comparable to those observed in a similar study on the *E. coli* enzyme DNA photolyase[1] and suggest that the reaction may proceed via a radical anion intermediate with concerted breakage of the 5,5′ and 6,6′ bonds. Alternatively the reaction may proceed via a mechanism in which the first bond is cleaved in a reversible fashion.

Introduction

Pyrimidine dimerization is the major form of damage to DNA produced by UV light.[2] The enzyme DNA photolyase directly repairs these lesions by catalyzing dimer cleavage in the presence of near UV-visible light (300−500 nm).[3] The unusual nature of the reaction has generated considerable interest in the enzymatic reaction mechanism.[4] Although many notions have been put forth to explain enzymatic catalysis involving substrates in their ground states (e.g., proximity effects, transition state stabilization, general acid and general base catalysis, etc.), very little is known about how specific binding energy can be effectively used to control physical and chemical processes of excited-state molecules. In order to gain greater insight into the mechanisms whereby enzymes mediate excited state processes, we have begun to generate and characterize antibodies that catalyze photochemical reactions.

Earlier work demonstrated that an antibody could be generated which is capable of accelerating the light-dependent cleavage of a thymine cyclobutane dimer.[5] We have now extended these earlier studies to a cyclobutane dimer of uracil, **1**, and have isolated an antibody (UD4C3.5) that catalyzes the corresponding cleavage reaction of uracil dimers (Figure 1). This antibody has made possible a series of kinetic studies, including the measurement of secondary deuterium kinetic isotope effects of uracil dimers containing deuterium substituents at the 5,5′ and 6,6′ centers. The observed isotope effects indicate that both bond-breaking steps are partially rate-determining. These experiments suggest that a photoexcited tryptophan residue in the antibody transfers an electron to the dimer, resulting in either concerted cleavage of the cyclobutane ring or stepwise cleavage in which the breaking of the first bond is reversible.

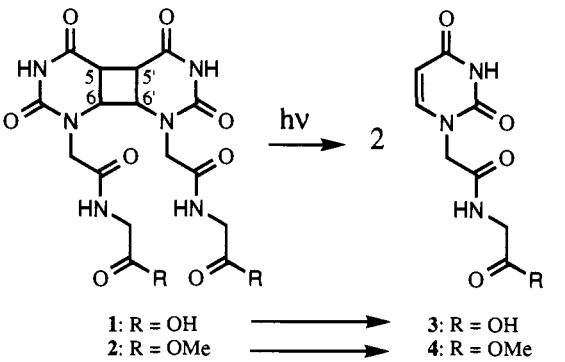

Figure 1. Uracil dimer cleavage reactions catalyzed by antibody UD4C3.5. Antibodies were raised against compound **1**.

Experimental Section

General Methods. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. *N,N'*-Dimethylformamide (DMF) was purchased as anhydrous grade and stored over molecular sieves. All aqueous solutions were prepared from deionized distilled water. Filtration of aqueous reaction mixtures was achieved by forcing solutions through Millipore 0.45 μm syringe tip filters (no. SJHV004NS) using a syringe under positive pressure. All moisture sensitive reactions were carried out in oven-dried glassware under a positive pressure of nitrogen. Analytical thin-layer chromatography was performed on precoated silica gel plates (Merck 60-F254); compounds were visualized using ninhydrin stain. Flash chromatography[6] was performed using silica gel (Merck Kieselgel 60, 230−400 mesh). Removal of solvent *in vacuo* refers to distillation using a Büchi

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rotary evaporator at approximately 3 mmHg. Products obtained as solids were dried under vacuum (*ca.* 1 mmHg) at elevated temperature (50−100 °C), with P_2O_5 as a desiccant. Products which precipitated from aqueous reactions were isolated by centrifugation according the following procedure: the reaction mixture was transferred to microcentrifuge tubes and spun at 14 000 rpm for 4 min in a microcentrifuge. The supernatant was decanted and discarded. The product was twice washed by resuspension in water, centrifugation (14 000 rpm × 4 min), and removal of the supernatant.

Melting points were determined using a Mel-Temp melting point apparatus and are uncorrected. IR spectra were recorded on a Matteson Polaris Fourier transform spectrophotometer using KBr pellets (KBr) or NaCl plates (film). UV spectra were recorded in 10 mm quartz cells with a Kontron Uvikon 930 spectrophotometer. ^{1}H NMR spectra were recorded on an AM-400 (400 MHz) or AM-500 (500 MHz) Fourier-transform NMR spectrometer at the University of California, Berkeley NMR facility. Proton resonances are reported in units of ppm downfield from tetramethylsilane (TMS). For spectra obtained in DMSO-d_6, the resonance from residual protons (2.49 ppm) was used as the reference. ^{13}C NMR spectra were recorded on AM-400 (101 MHz) or AM-500 (126 MHz) spectrometers; all spectra are proton decoupled. ^{13}C resonances are reported in units of ppm downfield from TMS. For spectra obtained in DMSO-d_6, the central solvent peak (39.5 ppm) was used as the reference. Positive and negative ion fast atom bombardment mass spectra (FAB^+ and FAB^-) and electron impact (EI^+) spectra were recorded by the staff of the Mass Spectroscopy Laboratory at the University of California, Berkeley on a VG-70 SE (Fison Instruments) or VG ZAB2-EQ (Fison Instruments), (FAB^+, FAB^-), or a VG Prospec (Fison Instruments), (EI^+). Elemental analyses were performed by the Microanalytical Laboratory operated by the College of Chemistry at the University of California, Berkeley.

1-Carboxymethyluracil (5).[7,8] Uracil (10.1 g, 89.7 mmol), chloroacetic acid (15.1 g, 160 mmol), and potassium hydroxide (22.1 g, 394 mmol) were added to 200 mL water, and the mixture was heated to reflux. After 1 h, the resulting solution was cooled to 25 °C and acidified to pH 2 with concentrated aqueous HCl. The solution was cooled to 4 °C, and after 12 h the product was isolated by vacuum filtration. The product was dried to yield 10.4 g (68%) of **5** as a white crystalline solid: mp 284−287 °C (dec) (lit.[8] mp 287 °C); IR (KBr) 3104, 1707, 1475, 1404, 1277, 1204 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.39 (s, 2H), 5.58 (dd, 1H, J = 2.2, 7.9 Hz), 7.60 (d, 1H, J = 7.8 Hz), 11.33 (s, 1H), 13.11 (br s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.7, 100.9, 146.1, 151.1, 163.9, 169.6; mass spectrum (EI^+) *m/e* 170 (M^+). Anal. Calcd for $C_6H_6N_2O_4$: C, 42.36; H, 3.55; N, 16.47. Found: C, 42.33; H, 3.39; N, 16.39.

Bis(1-carboxymethyluracil) Ethylene Glycol Ester (6). Compound **5** (4.53 g, 26.6 mmol) was dissolved in 100 mL of DMF with stirring at 50 °C. The resulting solution was cooled to 0 °C, and *N,N'*-carbonyldiimidazole (CDI) (4.61 g, 28.4 mmol) was added. The reaction was warmed to 25 °C and stirred for 1.5 h after which time gas evolution was no longer apparent. Ethylene glycol (0.740 mL, 13.3 mmol) was added, and the reaction was stirred at 25 °C for 12 h. The solvent was removed *in vacuo*, and the resulting oil was diluted with 30 mL of water. The solution was reduced *in vacuo* to afford a white solid which was suspended in 50 mL of water and isolated by vacuum filtration. The product was washed with additional water and dried to yield 3.60 g (74%) of **6** as a white powder: mp 262−264 °C (dec); IR (KBr) 3162, 3044, 1745, 1717, 1696, 1684, 1669, 1465, 1391, 1201 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.33 (s, 4H), 4.53 (s, 4H), 5.62 (d, 2H, J = 7.8), 7.60 (d, 2H, J = 7.9), 11.42 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.5, 62.8, 101.2, 145.9, 151.0, 163.8, 168.1; mass spectrum (FAB^+) *m/e* 367 (MH^+). Anal. Calcd for $C_{14}H_{14}N_4O_8$: C, 45.91; H, 3.85; N, 15.30. Found: C, 46.00; H, 3.91; N, 15.34.

1-Carboxymethyluracil *trans, syn*-Cyclobutane Dimer, Ethylene Glycol Ester (7). Ester **6** (1.01 g, 2.76 mmol) was dissolved in 600 mL of 50% [v/v] aqueous acetone. The solution was transferred to a 600 mL photolysis reaction vessel and deoxygenated by bubbling N_2 through the solution for 30 min. The solution was irradiated for 5 h (25 °C) with a 450-W Hanovia medium pressure Hg immersion lamp equipped with a Pyrex filter. The volume was reduced to 50 mL *in vacuo* and stored at 4 °C for 5 h. The product was isolated by vacuum filtration and dried to yield 552 mg (55%) of **7** as a white powder: mp 345−388 °C (dec); IR (KBr) 3190, 3063, 2864, 1734, 1684, 1486, 1361, 1238 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.39 (d, 2H, J = 8.1 Hz), 3.51 (d, 2H, J = 18.0 Hz), 4.03 (d, 2H, J = 10.4 Hz), 4.30 (d, 2H, J = 8.0 Hz), 4.51 (d, 2H, J = 18.0 Hz), 4.86 (d, 2H, J = 10.4 Hz), 10.72 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.8, 49.0, 60.2, 63.6, 151.7, 169.9, 170.3; mass spectrum (FAB^+) *m/e* 367 (weak) (MH^+). Anal. Calcd for $C_{14}H_{14}N_4O_8$: C, 45.91; H, 3.85; N, 15.30. Found: C, 45.85; H, 3.98; N, 15.06.

1-Carboxymethyluracil *trans, syn*-Cyclobutane Dimer (8). Compound **7** (1.21 g, 3.30 mmol) was dissolved in 25.9 mL of 0.50 N sodium hydroxide and stirred at room temperature for 1 h. The solution was acidified to pH 1.6 with concentrated hydrochloric acid and stored at 4 °C for 48 h. The crystalline product was filtered, washed with cold water, and dried to yield 235 mg (21%) of **8** as colorless crystals: mp 346−350 °C (dec); IR (KBr) 3172, 3064, 2860, 1745, 1684, 1486, 1303, 1189 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.42 (d, 2H, J = 8.2 Hz), 3.89 (d, 2H, J = 7.5 Hz), 3.98 (d, 2H, J = 17.5 Hz), 4.30 (d, 2H, J = 8.1 Hz), 10.64 (s, 2H), 12.84 (br s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.4, 48.5, 59.5, 151.5, 169.8, 170.7; mass spectrum (FAB^+) *m/e* 341 (MH^+); HRMS calcd for $C_{12}H_{12}N_4O_8$ 341.0733, found 341.0735.

1-(Carboxymethyluracil, glycine amide) *trans, syn*-Cyclobutane Dimer (1). Dimer **8** (150 mg, 441 μmol) was crushed to a fine powder and dissolved in 1.76 mL of 0.50 N aqueous NaOH. *tert*-Butyl glycinate hydrochloride (230 mg, 1.37 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (348 mg, 1.81 mmol) were added, and the resulting solution was filtered and acidified to pH 5.3 with 1.0 N HCl. The reaction was stirred at 25 °C for 12 h, and the precipitate was isolated by centrifugation. The crude *tert*-butyl ester was dried over P_2O_5 and then dissolved in 8.6 mL of trifluoroacetic acid (TFA). The solution was stirred for 1.5 h and then concentrated *in vacuo*. The residue was dissolved in acetone (3 mL) and reconcentrated to give a white solid which was recrystallized from water to yield 133 mg (66%) of **1** as colorless plates: mp 246−250 °C (dec); IR (KBr) 3024, 2854, 1718, 1695, 1669, 1653, 1559, 1481, 1285, 1220 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.38 (d, 2H, J = 8.1 Hz), 3.75 (d, 4H, J = 5.7 Hz), 3.94 (d, 2H, J = 30.3 Hz), 3.97 (d, 2H, J = 30.3 Hz), 4.26 (d, 2H, J = 8.2 Hz), 8.24 (t, 2H, J = 5.7 Hz), 10.56 (s, 2H), 12.59 (br s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.4, 40.6, 49.0, 59.2, 151.6, 168.4, 170.0, 171.1; mass spectrum (FAB^+) *m/e* 455 (MH^+). Anal. Calcd for $C_{16}H_{18}N_6O_{10}\cdot 2H_2O$: C, 39.19 H, 4.52; N, 17.14. Found: C, 39.29; H, 4.59; N, 17.25.

Bovine Serum Albumin (BSA) and Keyhole Limpet Hemocyanin (KLH) Conjugates of 1 (BSA-1) and (KLH-1). To a solution of **1** (10.4 mg, 22.9 μmol) in 1.1 mL of DMF was added *N*-hydroxysuccinimide (13.1 mg, 114 μmol) and EDC (27.2 mg, 142 μmol), and the resulting solution was stirred at 25 °C for 10 h. BSA (35.4 mg, Fraction V, Sigma) was dissolved in 3.5 mL of water, and KLH (30.9 mg, Sigma) was dissolved in 5.0 mL of water. Each protein solution was adjusted to pH 9.0 by dropwise addition of 1 M sodium carbonate and filtered. To each protein solution was added 0.5 mL of the DMF solution by dropwise addition with stirring. The solutions were stirred for 12 h during which time the pH was periodically adjusted back to 9.0 by dropwise addition of 0.1 N NaOH. The protein conjugates were exhaustively dialyzed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.5. Protein concentrations of the final solutions were determined by the method of Bradford.[9] The level of coupling was determined by the indirect method of Habeeb.[10] An epitope density of 14 haptens per protein BSA monomer was determined by this method; the epitope density of the KLH conjugate was 8.

1-(Carboxymethyluracil, methyl glycinate amide) *trans, syn*-Cyclobutane Dimer (2). Dimer **8** (29.7 mg, 87.3 μmol) was crushed to a fine powder and dissolved in 350 μL of 0.50 N aqueous NaOH.

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The solution was diluted with 1.5 mL of water, and methyl glycinate hydrochloride (33.0 mg, 263 μmol) and EDC (67.0 mg, 349 μmol) were added. The resulting solution was filtered, acidified to pH 5.3 with 1.0 N HCl, and stirred at 25 °C for 12 h. The precipitate was isolated by centrifugation and dried to yield 19.1 mg (45%) of **2** as a white powder: mp 282−284 °C (dec); IR (KBr) 3398, 3186, 3082, 1751, 1696, 1676, 1546, 1474, 1375, 1284, 1220 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.38 (d, 2H, J = 8.2 Hz), 3.62 (s, 6H), 3.84 (d, 4H, J = 5.8 Hz), 3.94 (d, 2H, J = 24.3 Hz), 3.98 (d, 2H, J = 24.2 Hz), 4.26 (d, 2H, J = 8.0 Hz), 8.37 (t, 2H, J = 5.8 Hz), 10.57 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.4, 40.5, 49.1, 51.7, 59.3, 151.6, 168.6, 170.0, 170.1; mass spectrum (FAB$^+$) *m/e* 483 (MH$^+$). Anal. Calcd for $C_{18}H_{22}N_6O_{10}$: C, 44.82; H, 4.60; N, 17.42. Found: C, 44.66; H, 4.73; N, 17.08.

Methyl-d_3 Glycinate Hydrochloride (27). To a solution of *tert*-butyloxycarbonylglycine (1.31 g, 7.48 mmol) in 20 mL of ethyl acetate was added methanol-d_4 (99.8 atom% D, Aldrich) (460 μL, 11.3 mmol) and 1,3-dicyclohexylcarbodiimide, and the reaction was stirred at room temperature for 24 h. Precipitate was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was purified by flash chromatography on a 4 × 15 cm silica column (20% [v/v] ethyl acetate in hexanes; R_f 0.23), affording 1.27 g of the ester as a colorless oil. The ester was dissolved in 60 mL of diethyl ether, and anhydrous HCl was bubbled through the solution for 20 min. The precipitate was isolated by filtration and dried to afford 363 mg (38%) of **27** as a white powder: mp 175−176 °C (dec); IR (KBr) 2944, 2635, 1751, 1583, 1498, 1413, 1277 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.74 (s, 2H), 8.62 (br s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 39.4, 168.0; mass spectrum (EI$^+$) *m/e* 92 (M$^+$). Anal. Calcd for $C_3H_4D_3NO_2$·HCl: C, 28.03; H, 6.27; N, 10.90. Found: C, 27.87; H, 6.17; N, 10.53.

1-(Carboxymethyluracil, methyl-d_3 glycinate amide) *trans, syn*-Cyclobutane Dimer (9). Compound **9** was prepared by the procedure described for the preparation of compound **2** using **8** (30.8 mg, 90.5 μmol), 0.50 N NaOH (350 μL), water (1.0 mL), **27** (33.3 mg, 259 μmol), and EDC (72.7 mg, 379 μmol) to yield 32.8 mg (74%) of **9** as a white powder: mp 278−282 °C (dec); IR (KBr) 3352, 3212, 3187, 3065, 1750, 1700, 1676, 1546, 1474, 1378, 1284, 1201 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.38 (d, 2H, J = 8.3 Hz), 3.84 (d, 4H, J = 5.9 Hz), 3.94 (d, 2H, J = 24.1 Hz), 3.98 (d, 2H, J = 24.2 Hz), 4.26 (d, 2H, J = 8.2 Hz), 8.37 (t, 2H, J = 5.8 Hz), 10.57 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.4, 40.5, 49.0, 59.2, 151.6, 168.6, 170.0, 170.1; mass spectrum (FAB$^+$) *m/e* 489 (MH$^+$). Anal. Calcd for $C_{18}H_{16}D_6N_6O_{10}$: C, 44.27; H, 4.54; N, 17.21. Found: C, 44.35; H, 4.62; N, 17.31.

Propiolic Acid-3-*d* (13).[11] Acetylene dicarboxylic acid monopotassium salt (34.11 g, 224 mmol) was added to 100 mL of D_2O (99.9% atom D, MSD isotopes), and the mixture was gradually heated to reflux over 2 h. After an additional 1 h, the solution was cooled to 0 °C, acidified with DCl (20% [w/w], 40.4 g), and extracted with three 120 mL portions of diethyl ether. The ether layers were pooled, dried over $MgSO_4$, and concentrated. The residue was distilled to afford 8.74 g (54%) of **13**: bp 89−92 °C (100 mm); IR (film) 1975, 1700, 1364, 1258 cm^{-1}; ^{13}C NMR (126 MHz, DMSO-d_6) δ 75.6, 77.3 (t, J = 39.2 Hz), 153.5; mass spectrum (EI$^+$) *m/e* 71 (M$^+$); HRMS calcd for C_3HDO_2 71.0118, found 71.0118.

Uracil-6-*d* (14).[12] Urea (3.51 g, 58.4 mmol), **13** (4.26 g, 59.1 mmol), and polyphosphoric acid (105 g) were combined and heated to 90 °C for 4.5 h. The mixture was cooled to −15 °C and 200 mL of ice water was added. A mechanical stirrer was employed to stir the viscous mixture for 12 h at 4 °C after which time the product had precipitated. Product was collected by filtration, recrystallized from water, and dried to yield 3.25 g (49%) of **14** as a white powder: mp 326−328 °C (dec); IR (KBr) 3481, 3114, 2964, 2917, 1718, 1654, 1559, 1418 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 5.43 (s, 1H), 10.79 (s, 1H), 10.98 (s, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 100.0, 151.5, 164.3; mass spectrum (EI$^+$) *m/e* 113 (M$^+$). Anal. Calcd for $C_4H_3DN_2O_2$: C, 42.49; H, 3.57; N, 24.77. Found: C, 42.65; H, 3.51; N, 25.05.

1-Carboxymethyluracil-6-*d* (15). Compound **15** was prepared by the procedure described for the preparation of compound **5** using **14** (3.34 g, 29.5 mmol), chloroacetic acid (5.05 g, 53.4 mmol), and potassium hydroxide (7.37 g, 131 mmol) in 70 mL of water to yield 2.14 g (42%) of **15** as a white solid: mp 265−274 °C (dec); IR (KBr) 3114, 1700, 1654, 1559, 1465, 1405, 1376, 1276 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 4.40 (s, 2H), 5.57 (s, 1H), 11.33 (s, 1H), 13.02 (br s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.6, 100.7, 151.0, 163.8, 169.6; mass spectrum (EI$^+$) *m/e* 171 (M$^+$). Anal. Calcd for $C_6H_5DN_2O_4$: C, 42.12; H, 3.53; N, 16.37. Found: C, 41.74; H, 3.58; N, 16.38.

1-Carboxymethyluracil-5,6-d_2 (16). This procedure was adapted from that of Hill.[12] To 75 mL of D_2O was added 3.67 g (21.4 mmol) of **15**. The mixture was stirred, and NaOD was added dropwise until all solid material had dissolved. The pH was reduced to 5.0 by dropwise addition of DCl, and the solution was lyophilized to a powder. To the powder was added 100 mL of DCl (20% [w/w] in D_2O), and the mixture was heated to reflux for 5 h. The resulting solution was cooled to 4 °C. After 12 h, the crystalline product was collected by filtration and dried to afford 2.79 g (75%) of **16** as a white solid: mp 288−290 °C (dec); IR (KBr) 1718, 1696, 1684, 1654, 1457, 1359, 1321 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 4.41 (s, 2H), 11.33 (s, 0.1H), 13.12 (br s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.5, 150.9, 163.7, 169.5; mass spectrum (EI$^+$) *m/e* 174 (M$^+$). Anal. Calcd for $C_6H_2D_4N_2O_4$: C, 41.40; H, 3.47; N, 16.09. Found: C, 41.34; H, 3.53; N, 16.00.

1-Carboxymethyluracil-5-*d* (17). Compound **17** was prepared by the procedure described for the preparation of **16** using **5** (3.04 g, 17.9 mmol) and appropriate amounts of the deuterated reagents to yield 2.80 g (91%) of **17** as a white crystalline solid: mp 287−289 °C (dec); IR (KBr) 1695, 1685, 1617, 1559, 1458, 1367, 1325, 1217 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 4.41 (s, 2H), 7.60 (s, 1H), 11.32 (s, 0.1H), 13.11 (br s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.6, 146.0, 151.0, 163.7, 169.5; mass spectrum (EI$^+$) *m/e* 172, 173, 174 (M$^+$). Anal. Calcd for $C_6H_3D_3N_2O_4$: C, 41.63; H, 3.49; N, 16.18. Found: C, 41.42; H, 3.60; N, 16.10.

Bis(1-carboxymethyluracil-5-*d*) Ethylene Glycol Ester (18). Compound **18** was prepared by the method described for the preparation of **6** from **17** (2.71 g, 15.7 mmol), DMF (60 mL), CDI (2.73 g, 16.8 mmol), and ethylene glycol (0.435 mL, 7.80 mmol) to yield 2.21 g (77%) of **18** as a white powder: mp 265−267 °C (dec); IR (KBr) 1745, 1691, 1675, 1654, 1458, 1208 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 4.33 (s, 4H), 4.53 (s, 2H), 7.60 (s, 2H), 11.40 (s, 0.2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.5, 62.8, 145.7, 151.0, 163.7, 168.0; mass spectrum (FAB$^+$) *m/e* 369, 370, 371, 372 (MH$^+$). Anal. Calcd for $C_{14}H_{10}D_4N_4O_8$: C, 45.42; H, 3.81; N, 15.13. Found: C, 45.24; H, 4.02; N, 15.04.

Bis(1-carboxymethyluracil-6-*d*) Ethylene Glycol Ester (19). Compound **19** was prepared by the method described for the preparation of **6** from **15** (2.18 g, 12.7 mmol), DMF (40 mL), CDI (2.25 g, 13.9 mmol), and ethylene glycol (0.355 mL, 6.37 mmol) to yield 1.61 g (69%) of **19** as a white powder: mp 261−264 °C (dec); IR (KBr) 3461, 3010, 1745, 1701, 1676, 1457, 1375, 1210 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 4.33 (s, 4H), 4.53 (s, 4H), 5.62 (s, 2H), 11.39 (s, 0.2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.4, 62.8, 101.0, 150.9, 163.7, 168.0; mass spectrum (FAB$^+$) *m/e* 369 (MH$^+$). Anal. Calcd for $C_{14}H_{12}D_2N_4O_8$: C, 45.66; H, 3.83; N, 15.21. Found: C, 45.40; H, 4.05; N, 15.09.

Bis(1-carboxymethyluracil-5,6-d_2) Ethylene Glycol Ester (20). Compound **20** was prepared by the method described for the preparation of **6** from **16** (2.59 g, 14.9 mmol), DMF (60 mL), CDI (2.67 g, 16.5 mmol), and ethylene glycol (0.410 mL, 7.35 mmol) to yield 2.30 g (84%) of **20** as a white powder: mp 266−267 °C (dec); IR (KBr) 3421, 1745, 1700, 1654, 1457, 1207 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 4.33 (s, 4H), 4.53 (s, 2H), 11.40 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.5, 62.8, 151.0, 163.7, 168.1; mass spectrum (FAB$^+$) *m/e* 371, 372, 373, 374, 375 (MH$^+$). Anal. Calcd for $C_{14}H_8D_6N_4O_8$: C, 45.17; H, 3.79; N, 15.05. Found: C, 44.94; H, 3.96; N, 14.95.

1-Carboxymethyluracil-5-*d trans,syn*-Cyclobutane Dimer, Ethylene Glycol Ester (21). A solution of **18** (2.04 g, 5.54 mmol) in 1.20 L of 1:1 [v/v] aqueous acetone was divided into two 600 mL portions, and each portion was subjected to the photolysis and purification conditions described for the preparation of **7**. The products from the two portions were combined to afford 1.31 g (55%) of **21** as

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a white powder: mp 345−388 °C (dec); IR (KBr) 3462, 3065, 2856, 1732, 1684, 1477, 1360, 1262 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.49−3.53 (m, 1H), 4.03 (d, 2H, J = 10.1 Hz), 4.29 (s, 2H), 4.49−4.53 (m, 1H), 4.86 (d, 2H, J = 10.3 Hz), 10.72 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.9, 60.1, 63.6, 151.7, 169.9, 170.3; mass spectrum (FAB$^+$) *m/e* 371 (weak) (MH$^+$). Anal. Calcd for $C_{14}H_{10}D_4N_4O_8$: C, 45.42; H, 3.81; N, 15.13. Found: C, 45.21; H, 4.09; N, 14.95.

1-Carboxymethyluracil-6-*d* *trans,syn*-Cyclobutane Dimer, Ethylene Glycol Ester (22). Compound **22** was prepared from a solution of 1.59 g (4.32 mmol) of **19** in 1.20 L of 1:1 [v/v] aqueous acetone according to the procedure described for the preparation of **21** to yield 840 mg (53%) of **22** as a white powder: mp 345−390 °C (dec); IR (KBr) 3420, 3066, 1735, 1684, 1458, 1373, 1344, 1219 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.38 (s, 2H), 3.51 (d, 2H, J = 17.9 Hz), 4.02 (d, 2H, J = 9.8 Hz), 4.50 (d, 2H, J = 18.0 Hz), 4.86 (d, 2H, J = 9.7 Hz), 10.71 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.6, 48.9, 63.5, 151.6, 169.9, 170.3; mass spectrum (FAB$^+$) *m/e* 369 (weak) (MH$^+$). Anal. Calcd for $C_{14}H_{12}D_2N_4O_8$: C, 45.66; H, 3.83; N, 15.21. Found: C, 45.20; H, 4.00; N, 15.00.

1-Carboxymethyluracil-5,6-d_2 *trans,syn*-Cyclobutane Dimer, Ethylene Glycol Ester (23). Compound **23** was prepared from 2.01 g (5.40 mmol) of **20** in 1.20 L of 1:1 [v/v] aqueous acetone according to the procedure described for the preparation of **21** to yield 1.20 g (60%) of **23** as a white powder: mp 345−390 °C (dec); IR (KBr) 3478, 3066, 2861, 1735, 1676, 1458, 1306, 1228 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.49−3.54 (m, 1H), 4.03 (d, 2H, J = 10.3 Hz), 4.49−4.53 (m, 1H), 4.87 (d, 2H, J = 10.5 Hz), 10.69 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.9, 63.5, 151.7, 169.9, 170.3; mass spectrum (FAB$^+$) *m/e* 373 (weak) (MH$^+$). Anal. Calcd for $C_{14}H_8D_6N_4O_8$: C, 45.17; H, 3.79; N, 15.05. Found: C, 44.81; H, 3.98; N, 14.67.

1-Carboxymethyluracil-5-*d* *trans,syn*-Cyclobutane Dimer (24). Compound **21** (801 mg 2.16 mmol) was dissolved in 17.4 mL of NaOD (0.5 N in D_2O), and the solution was stirred at 25 °C for 1.0 h. The solution was then acidified to pH 1.6 with DCl (20% [w/w] in D_2O) and cooled to 4 °C. After 48 h, the crystalline product was isolated by filtration and dried to yield 196 mg (26%) of **24** as white crystals: mp 325−332 °C (dec); IR (KBr) 3157, 3076, 1748, 1691, 1648, 1482, 1390, 1329, 1180 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.87−4.01 (m, 2H), 4.29 (s, 2H), 10.63 (s, 2H), 12.82 (br s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.5, 59.4, 151.5, 169.7, 170.6; mass spectrum (FAB$^-$) *m/e* 341, 342, 343, 344, 345 ((M − H)$^-$). Anal. Calcd for $C_{12}H_8D_4N_4O_8$: C, 41.87; H, 3.51; N, 16.28. Found: C, 41.53; H, 3.52; N, 16.11.

1-Carboxymethyluracil-6-*d* *trans,syn*-Cyclobutane Dimer (25). Compound **22** (804 mg, 2.18 mmol) was dissolved in 17.5 mL of 0.5 N aqueous NaOH, and the solution was stirred at 25 °C for 1.0 h. The solution was then acidified to pH 1.6 with concentrated aqueous HCl and cooled to 4 °C. After 48 h, the crystalline product was isolated by filtration and dried to yield 206 mg (28%) of **25** as white crystals: mp 305−330 °C (dec); IR (KBr) 3173, 3065, 2873, 1745, 1685, 1654, 1465, 1355, 1339, 1194 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.41 (s, 2H), 3.91 (d, 2H, J = 36.3 Hz), 3.96 (d, 2H, J = 36.5 Hz), 10.63 (s, 2H),. 12.82 (br s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.3, 48.5, 151.6, 169.8, 170.7; mass spectrum (FAB$^-$) *m/e* 341 ((M − H)$^-$). Anal. Calcd for $C_{12}H_{10}D_2N_4O_8$: C, 42.12; H, 3.53; N, 16.37. Found: C, 41.86; H, 3.88; N, 16.06.

1-Carboxymethyluracil-5,6-d_2 *trans,syn*-Cyclobutane Dimer (26). Compound **26** was prepared from **23** (804 mg, 2.17 mmol) according to the procedure described for the preparation of compound **24** to yield 271 mg (36%) of **26** as white crystals: mp 327−332 °C (dec); IR (KBr) 3447, 3193, 2881, 1751, 1685, 1648, 1459, 1373, 1194 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.86−3.97 (m, 2H), 10.59 (s, 2H), 12.78 (br s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 48.5, 151.6, 169.8, 170.7; mass spectrum (FAB$^-$) *m/e* 343, 344, 345, 346, 347 ((M − H)$^-$). Anal. Calcd for $C_{12}H_6D_6N_4O_8$: C, 41.63; H, 3.49; N, 16.18. Found: C, 41.34; H, 3.54; N, 16.17.

1-(Carboxymethyluracil-5-*d* methyl-d_3 glycinate amide) *trans,syn*-Cyclobutane Dimer (10). Compound **10** was prepared by the procedure described for the preparation of compound **2** except that D_2O was used as solvent, and D_2O solutions of NaOD and DCl were used in place of aqueous NaOH and HCl, respectively. The precipitated product was washed with H_2O. The reaction mixture consisted of **24** (30.8 mg, 89.5 μmol), 0.5 N NaOD in D_2O (350 μL), D_2O (1.0 mL), **27** (34.2 mg, 266 μmol), and EDC (71.9 mg, 375 μmol) to yield 30.5 mg (69%) of **10** as a white powder: mp 282−285 °C (dec); IR (KBr) 3447, 1749, 1701, 1684, 1654, 1459, 1378, 1326, 1225 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.84 (d, 4H, J = 5.4 Hz), 3.90−4.01 (m, 2H), 4.26 (s, 2H), 8.37 (t, 2H, J = 5.5 Hz), 10.57 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 40.4, 49.0, 59.1, 151.6, 168.5, 170.0, 170.1; mass spectrum (FAB$^+$) *m/e* 491, 492, 493, 494, 495, 496, 497 (MH$^+$). Anal. Calcd for $C_{18}H_{12}D_{10}N_6O_{10}$: C, 43.91; H, 4.50; N, 17.07. Found: C, 43.53; H, 4.56; N, 16.83.

1-(Carboxymethyluracil-6-*d* methyl-d_3 glycinate amide) *trans,syn*-Cyclobutane Dimer (11). Compound **11** was prepared by the procedure described for the preparation of compound **2** using **25** (30.2 mg, 88.3 μmol), 0.5 N NaOH (350 μL), water (1.0 mL), **27** (34.6 mg, 269 μmol), and EDC (72.8 mg, 380 μmol) to yield 31.9 mg (74%) of **11** as a white powder: mp 282−285 °C (dec); IR (KBr) 3388, 3066, 2945, 1750, 1696, 1676, 1541, 1458, 1335, 1225 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.42 (s, 2H), 3.88 (d, 4H, J = 5.9 Hz), 3.98 (d, 2H, J = 29.2 Hz), 4.02 (d, 2H, J = 29.3 Hz), 8.41 (t, 2H, J = 5.9 Hz), 10.61 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 38.3, 40.5, 49.0, 151.6, 168.6, 170.0, 170.1; mass spectrum (FAB$^+$) *m/e* 491 (MH$^+$). Anal. Calcd for $C_{18}H_{14}D_8N_6O_{10}$: C, 44.09; H, 4.52; N, 17.14. Found: C, 43.85; H, 4.65; N, 16.76.

1-(Carboxymethyluracil-5,6-d_2 methyl-d_3 glycinate amide) *trans,syn*-Cyclobutane Dimer (12). Compound **12** was prepared by the procedure described for the preparation of compound **10** using **26** (30.9 mg, 89.3 μmol), 0.5 N NaOD (350 μL), D_2O (1.0 mL), **27** (34.8 mg, 271 μmol), and EDC (72.2 mg, 377 μmol) to yield 32.1 mg (73%) of **12** as a white powder: mp 282−285 °C (dec); IR (KBr) 3452, 3411, 1749, 1700, 1684, 1654, 1458, 1355, 1222 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.84 (d, 4H, J = 3.9 Hz), 3.90−4.01 (m, 2H), 8.37 (t, 2H, J = 5.8 Hz), 10.57 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 40.4, 48.8, 151.6, 168.5, 170.0, 170.1; mass spectrum (FAB$^+$) *m/e* 493, 494, 495, 496, 497, 498, 499 (MH$^+$). Anal. Calcd for $C_{18}H_{10}D_{12}N_6O_{10}$: C, 43.74; H, 4.49; N, 17.00. Found: C, 43.35; H, 4.70 N, 16.69.

1-Carboxymethyluracil Methyl Glycinate Amide (4). Compound **4** was prepared by the procedure described for the preparation of compound **2** using **5** (185 mg, 1.09 mmol), 0.5 N aqueous NaOH (2.2 mL), water (6.0 mL), methyl glycinate hydrochloride (205 mg, 1.63 mmol), and EDC (427 mg, 2.23 mmol) to yield 137 mg (52%) of **4** as a white powder: mp 189−191 °C (dec); IR (KBr) 3306, 3016, 2835, 1745, 1689, 1669, 1580, 1472, 1429, 1354, 1243, 1211 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.62 (s, 3H), 3.89 (d, 2H, J = 5.8 Hz), 4.39 (s, 2H), 5.55 (d, 1H, J = 7.8 Hz), 7.54 (d, 1H, J = 7.8 Hz), 8.63 (t, 1H, J = 5.7 Hz), 11.26 (s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 40.6, 49.2, 51.8, 100.6, 146.5, 151.0, 163.9, 167.5, 170.1; mass spectrum (EI) *m/e* 241 (M$^+$); UV/vis (H_2O) λ_{max} = 263 nm (9280 M^{-1} cm^{-1}). Anal. Calcd for $C_9H_{11}N_3O_5$: C, 44.82; H, 4.60; N, 17.42. Found: C, 44.84; H, 4.62; N, 17.38.

1-Carboxymethyluracil Glycine Amide (3). Compound **3** was prepared by the procedure described for the preparation of **1** using **5** (513 mg, 3.01 mmol), 0.5 N aqueous NaOH (6.0 mL), *tert*-butyl glycinate hydrochloride (561 mg, 3.35 mmol), and EDC (832 mg, 4.34 mmol). The crude ester was deprotected in 4.0 mL of TFA to yield 200 mg (29%) of **3** as a white powder: mp 255−256 °C (dec); IR (KBr) 3301, 3063, 2603, 2536, 1740, 1647, 1569, 1464, 1415, 1393, 1362, 1247, 1203 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.79 (d, 2H, J = 5.8 Hz), 4.38 (s, 2H), 5.54 (dd, 1H, J = 2.2, 7.8 Hz), 7.54 (d, 1H, J = 7.9 Hz), 8.50 (t, 1H, J = 5.7 Hz), 11.25 (s, 1H), 12.52 (br s, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 40.7, 49.2, 100.6, 146.6, 151.0, 163.9, 167.3, 171.0; mass spectrum (FAB$^+$) *m/e* 228 (MH$^+$); UV/vis (H_2O) λ_{max} = 263 nm (10,600 M^{-1} cm^{-1}). Anal. Calcd for $C_8H_9N_3O_5$: C, 42.30; H, 3.99; N, 18.50. Found: C, 42.18; H, 4.17; N, 18.56.

Crystallographic Study of 8. An X-ray crystallographic study of **8** was performed at the X-ray diffraction facility (CHEXRAY) at the University of California, Berkeley. Clear colorless needlelike crystals of **8** were obtained by crystallization from an aqueous hydrochloric acid solution at pH 1.6. A fragment cut from one of these crystals was mounted on a glass fiber using Paratone N hydrocarbon oil. The crystal was then transferred to an Enraf-Nonius CAD-4 diffractometer,

centered in the beam, and cooled to −105 °C. Automatic peak search and indexing procedures yielded a triclinic reduced primitive cell. Inspection of the Niggli values revealed a monoclinic C-centered cell.

The structure was solved by direct methods (SHELXS) in space group *C2/c* and refined via standard least-squares and Fourier techniques. In a difference Fourier map calculated following the refinement of all non-hydrogen atoms with anisotropic thermal parameters, peaks were found corresponding to the positions of most of the hydrogen atoms. Hydrogen atoms were assigned fixed thermal parameters based on their location in the molecule, and their positional parameters were refined.

The final residuals for 145 variables refined against the 777 accepted data for which $F^2 > 3\sigma(F^2)$ were $R = 8.10\%$, $R_w = 10.00\%$, and GOF = 3.06. The R value for all 1076 data was 10.28%.

Antibody Production and Purification. Monoclonal antibodies were elicited against hapten **1** using standard hybridoma technology.[13] Balb/c mice were injected intraperitoneally with the KLH conjugate of **1** (100 μg), emulsified in complete Freund's adjuvant. After 2 weeks the mice were boosted with KLH-**1** using incomplete Freund's adjuvant, and after an additional 2 weeks, hyperimmunized by intravenous injection of KLH-**1** in phosphate-buffered saline solution. Fusions were performed 3 days after hyperimmunization. Cells from a single spleen were fused with 10^8 P3 myeloma cells in a solution of 50% polyethylene glycol, plated into 40 96-well plates and grown on HAT media to select for hybridoma cells. Initial screening by enzyme-linked immunosorbent assay (ELISA) using BSA-**1** identified 18 cell lines which produced anti-**1** antibodies. Monoclonal cell lines were obtained by dilution to 0.1 cell/well. Colonies which grew from a single cell were rescreened by ELISA, and seven monoclonal lines were obtained. Ascites fluid was produced in nude mice for each cell line.

Antibodies were purified by protein A affinity chromatography using a procedure adapted from that of Fagerstam *et al.*[14] To a 10-mL sample of ascitic fluid was added 20 mL of binding buffer (aqueous 1.5 M glycine, 3.0 M NaCl, pH 8.9), and the solution was filtered through a plug of glass wool. The solution was then applied (0.5 mL/min flow rate) to a 10-mL (settled volume) column of Affinica Protein A-agarose (Schleicher and Schuell) which had been previously equilibrated in binding buffer. The column was then washed with binding buffer (1.0 mL/min flow rate) until the absorbance of the eluent was <0.05 absorbance units (280 nm). Elution buffer (100 mM sodium citrate, pH 3.0) was applied to the column and 3.0-mL fractions were collected into tubes containing 1.0 mL of collection buffer (1.0 M Tris, pH 9.0). Fractions with absorbance greater than 0.50 (280 nm) were pooled and dialyzed exhaustively into 20 mM phosphate, 40 mM NaCl, pH 7.5. Protein concentrations were determined by absorbance at 280 nm ($\epsilon = 1.37\sigma(0.1\%,1\text{ cm})$ with a molecular weight of 150 000 for immunoglobulin G). Antibodies were judged to be >95% pure by polyacrylamide gel electrophoresis. Samples were prepared by denaturation in loading buffer containing DTT and electrophoresed on 12% SDS polyacrylamide gels. Proteins were visualized by staining with Coomassie blue.

Antibody Kinetics and Product Analysis. Kinetic assays were performed using an Oriel Photomax photolysis apparatus equipped with a 200-W high pressure mercury lamp. An Oriel model 77200 monochrometer was used with 10 nm slits. Product formation was monitored by change in UV absorbance at 263 nm using a Kontron Uvikon 930 UV/vis spectrophotometer. Stock solutions of substrate **1** or **2** (100 mM in DMSO) were prepared fresh daily. Substrate was diluted into assay buffer (20 mM sodium phosphate, 40 mM NaCl, pH 7.5) containing 2.0 μM antibody to give a final reaction mixture of 2% acetonitrile and 1% DMSO in assay buffer. For each reaction, 80 μL of the mixture was placed in a microcuvette (2 mm × 2 mm window, 10 mm path length, Starna Cells 16.40-Q-10) such that the entire window was within the beam spot. Samples were irradiated for ten 30 s intervals (total conversion to product < 2% in all experiments), and the absorbance at 263 nm was measured after each interval. Product concentration was calculated from the appropriate extinction coefficient, and initial rates were determined by linear least squares fitting. For the determination of kinetic constants, initial rates were measured at 25 °C with irradiation at 300 nm in the presence of 2.0 μM antibody UD4C3.5. Background rates were determined in the presence of 2.0 μM antibody CPA23C3, which does not catalyze the cleavage reaction. Catalyzed rates were calculated by subtraction of the background rate from the rate observed in the presence of UD4C3.5. For substrate **2**, concentrations of 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 500, and 600 μM were assayed. All reactions were carried out under subsaturating light. The kinetic parameters V_{max} and K_M were determined by plotting catalyzed rate V as a function of [**2**] and fitting the data to the Michaelis−Menten equation $V = V_{max}[\mathbf{2}]/(K_M + [\mathbf{2}])$ using the Levenberg−Marquardt algorithm of the KaleidaGraph computer program (Abelbeck Software). Product formation was confirmed by reversed-phase high pressure liquid chromatography (RP-HPLC) as described below for measurement of isotope effects. For the determination of wavelength dependence, 500 μM substrate **1** was irradiated in the presence of 2.0 μM antibody with 290, 295, 300, 303, 305, and 310 nm light. Initial rates due to antibody catalysis were determined as for the kinetic analysis. Observed rates were corrected for differences in light intensity as measured by ferrioxalate actinometry[15] and are normalized to the rate observed at 300 nm.

Quenching of Antibody Fluorescence by Hapten Binding. Antibody fluorescence was monitored using a Hitachi F-4500 fluorescence spectrophotometer. A 10 × 10 mm fluorescence cuvette equipped with a stir bar was loaded with 2.00 mL of a 250 nM solution of antibody in assay buffer with 0.1% DMSO and 0.45% acetonitrile. The solution was excited at 280 nm, and fluorescence was observed at 348 nm. The solution was constantly stirred as it was titrated with increasing amounts of **1** (5−20 μL portions of a 1, 10, or 100 μM solution of **1** in assay buffer containing 0.1% DMSO and 0.45% acetonitrile) until fluorescence ceased to decrease with added **1**. Fluorescence measurements were corrected for changes in volume over the course of the titration as well as for nonspecific quenching at high concentrations of **1**. The fraction bound (R) was calculated based on the titration end points. Data corresponding to 20−80% saturation of the antibody binding sites was analyzed by Scatchard plot[16] to give the dissociation constant, K_D, for the Ig·**1** complex. An analogous titration was attempted with substrate **2**; however, only modest quenching was observed using the same concentrations of antibody and ligand described above.

Kinetic Isotope Effects. Secondary deuterium isotope effects were measured by the method of internal competition.[17] Substrates **2**, **9**, **10**, **11**, and **12** were dissolved in DMSO to form 100 mM stock solutions. Mixtures of **2** (protio) with each of the other substrates (deuterio) at ratios of approximately 1:1 were prepared. These were diluted to make reaction mixtures containing 600 μM total substrate, 2.5 μM antibody in assay buffer containing 0.6% DMSO, and 1.2% acetonitrile. For each experiment, a 3.0 mL reaction mixture was irradiated at 303 nm (25 °C) with constant stirring for 25 to 30 min. Product from each experiment was purified by RP-HPLC (Microsorb 5 μm C18 column, 4.6 mm i.d. × 25 cm, Rainin Instruments) using a linear gradient of 0−50% acetonitrile in 0.1% [v/v] aqueous TFA over 14.0 min at a flow rate of 1.0 mL/min. The reaction product was identified by coinjection with an authentic sample (**4**) (retention times: **2**, 13.9 min; **4**, 12.4 min). Reaction products were collected and reduced *in vacuo* to remove acetonitrile, and the aqueous residue were lyophilized to yield approximately 10 μg of product per reaction. Unreacted substrate for each experiment was purified by the same method from an aliquot that had not been irradiated. The ratios of deuterated to nondeuterated substrate and product were determined by electrospray-ionization mass spectrometry (ESI-MS). Lyophilized samples were dissolved in 4.0 μL of 1% acetic acid in 50% [v/v] aqueous *n*-propyl alcohol and analyzed by flow injection at 2 μL/min (Hewlett Packard 5989A mass spectrometer equipped with a model 59987A electrospray ion source). Signals (the sum of at least 100 scans) arising from the deuterated and nondeuterated products were separately integrated, and the quotient of the integrals (P_H/P_D) taken as the mole

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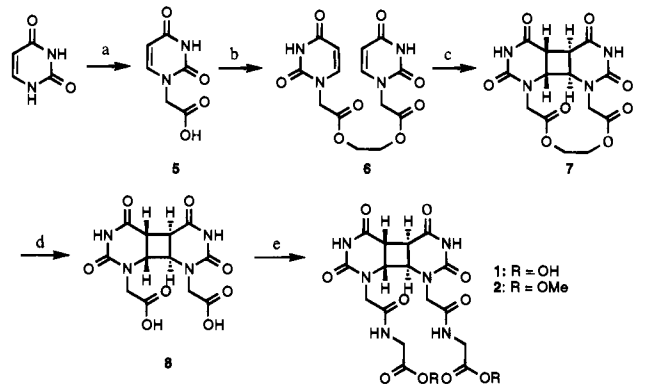
Scheme 1[a]



[a] (a) NaOH, chloroacetic acid, H_2O, 100 °C, 68%; (b) CDI, ethylene glycol, DMF 25 °C, 74%; (c) hν (300 nm), aqueous acetone, 25 °C, 55%; (d) 4.0 eq. NaOH, H_2O, 25 °C, 21%; (e) *tert*-butyl glycinate hydrochloride (synthesis of **1**) *or* methyl glycinate hydrochloride (synthesis of **2**), EDC, H_2O, 25 °C, followed by TFA (synthesis of **1** only), 25 °C , 66% (**1**), 45% (**2**).

ratio of protio to deuterio products. The ratio of protio to deuterio substrates was measured by the same method, and the ratio of substrates (S_H/S_D) compared to the product ratio to give the isotope effect $^{\alpha\text{-}D}(V/K)$ as $(P_H/P_D)/(S_H/S_D)$. Isotope effects were measured for substrates **10**, **11**, and **12** as well as the control compound **9**. Compound **9** contains the methyl-d_3 tag for mass separation but contains no deuterium substitution at positions expected to produce an isotope effect. The observed isotope effect for compound **9** is 1.08 ± 0.02 based on five determinations. This value results from an inherent bias in the mass spectrometric quantification of the products. It is therefore subtracted from the isotope effects measured for the other positions.

Results and Discussion

Synthesis and Hybridoma Production. The design and synthesis of uracil dimer hapten **1** was based on previously reported work on antibody-mediated thymine dimer cleavage.[5] It was expected that an antibody raised against a pyrimidine dimer would be likely to contain a binding-site tryptophan which could interact with the polarized π system of the dimer. Tryptophan was shown to act as a photosensitizer for the light-dependent cleavage reaction in model systems[18,19] as well as in the previously described catalytic antibody.[5]

The synthesis of hapten **1** is illustrated in Scheme 1. Uracil was alkylated with chloroacetic acid in aqueous potassium hydroxide to give carboxymethyluracil **5**. Subsequent treatment with *N,N*-carbonyldiimidazole followed by 0.5 equiv of ethylene glycol afforded bis-ester **6**, which was photodimerized by irradiation with 300 nm light in deoxygenated aqueous acetone. It was expected that the ethylene glycol bridge would effectively control the stereochemistry of dimerization; however, ^{1}H NMR analysis of aliquots of the reaction mixture indicated the formation of two products. Upon concentration of the reaction mixture, a single isomer, **7** precipitated.

Cleavage of the ethylene glycol bridge was carried out with 4.0 equiv of aqueous 0.5 N sodium hydroxide. Upon acidification, dimer **8** crystallized from solution. Attempts to assign the stereochemistry as the *cis, syn* adduct by alkylation with α,α′-dibromo-*o*-xylene[20] failed. The X-ray crystal structure of **8** showed the stereochemistry to be *trans, syn* (Figure 2). Crystallographic analysis of the corresponding thymine dimer[5] demonstrated that it also has a *trans, syn* configuration.[21] The

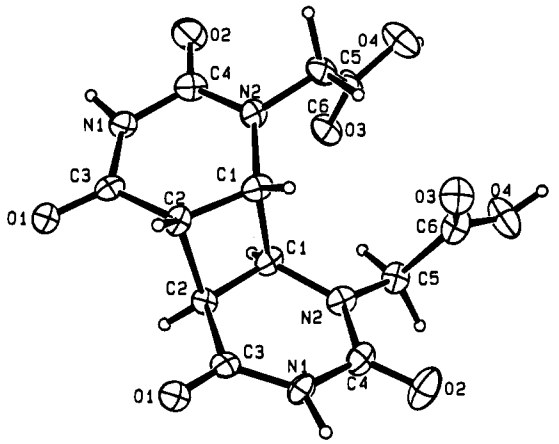


Figure 2. ORTEP structure of dimer **8**.

generation of high quality crystals of derivatives **1** and **2** proved difficult; however, the chemistry involved in elaboration of **8** to **1** and **2** is not expected to affect the core ring structure.

Dimer **8** was elaborated to hapten **1** by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) coupling to glycine *tert*-butyl ester followed by deprotection in trifluoroacetic acid. Substrate **2** was prepared by direct coupling of methyl glycinate to dimer **8**.

While the *cis, syn* isomer is the only detected thymine cyclobutane dimer in UV-damaged double-stranded DNA, the corresponding *trans, syn* dimer constitutes a significant fraction (13%) of the cyclobutane dimers found in damaged single-stranded DNA.[22,23] *E. coli* DNA photolyase repairs these lesions poorly due to its low affinity for *trans,syn* substrates,[24] and because repair by excision of damaged nucleotides is limited to double-stranded DNA, antibodies that cleave the *trans,syn* photoproduct would complement the repair pathways found in nature.

Small, non-peptidyl molecules such as hapten **1** are not immunogenic unless they are first coupled to a carrier protein. Hapten **1** was coupled to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) via the activated bis-*N*-hydroxysuccinimide ester to give epitope densities of 14 haptens per BSA monomer and 8 for KLH. The KLH conjugate was used to immunize mice, and seven monoclonal cell lines were generated using standard hybridoma technology.[13] Milligram quantities of each antibody were produced in ascites fluid and purified by protein-A affinity chromatography. Antibodies were judged ≥95% pure by SDS-polyacrylamide gel electrophoresis.

Kinetics, Specificity, and Wavelength Dependence of Antibody Catalysis. Of the seven antibodies complementary to hapten **1**, one (UD4C3.5) was found to catalyze the cleavage of **1** in the presence of 300 nm light. Other antibodies tested, including several which had been raised against other haptens, showed initial rates of reaction indistinguishable from the rate measured in the absence of antibody. An antibody (CPA23C3) raised against a phosphonate hapten was chosen for use as a control; all background rates were measured in the presence of this antibody.

Initial rates of reaction were measured at a variety of concentrations of **1**, and the rate showed minimal dependence on [**1**] down to the lowest concentrations at which product formation could be reliably quantified (8 μM), suggesting that

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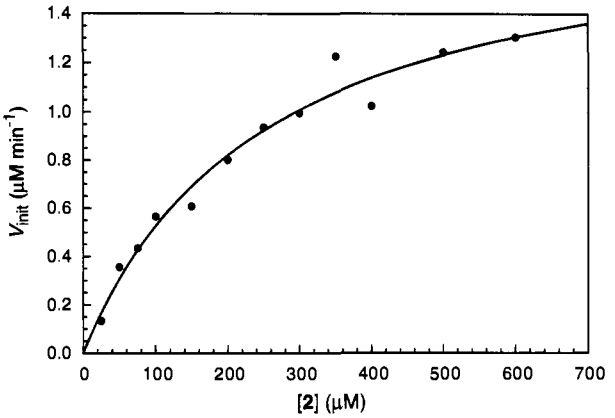

Figure 3. V vs [S] plot for UD4C3.5-catalyzed cleavage of **2**. Data were fit to the Michaelis–Menten equation: $V = V_{max}$ [**2**]/(K_M + [**2**]). Initial rates are corrected for the background reaction (measured in the presence of control antibody CPA23C3).

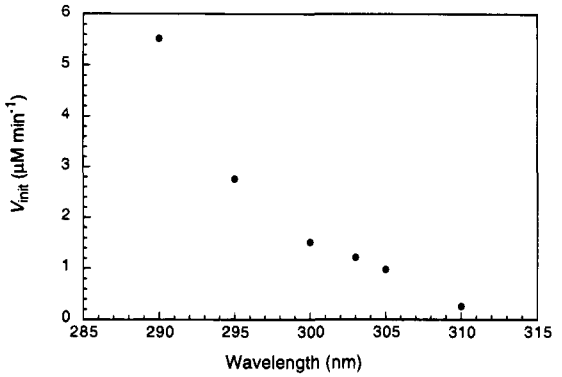

Figure 4. Wavelength dependence of the UD4C3.5-catalyzed cleavage of **1**. Initial rates are corrected for variation in light intensity.

K_M(**1**) ≪ 8 μM. Consequently, at substrate concentrations above 8 μM the antibody is operating under saturating conditions, allowing a rough estimate for V_{max} of 0.95 μM min^{-1}. Substrate **2**, in which the carboxylate groups have been converted to methyl esters, was investigated as an alternative substrate. Antibody UD4C3.5 catalyzes the cleavage of **2** with a comparable V_{max}, but binds this substrate much less tightly. Initial rates of reaction were measured at eight different concentrations of **2** ranging from 50 to 600 μM, and the data were fit to the Michaelis–Menten equation, $V = V_{max}$ [**2**]/(K_M + [**2**]) to give V_{max} = 1.9 μM min^{-1}, k_{cat} = 0.47 min^{-1} per combining site, and K_M = 280 μM (Figure 3). The rate constant for the uncatalyzed reaction, k_{uncat}, was measured to be 1.2 × 10^{-3} min^{-1}. Comparison of the catalyzed and uncatalyzed rates gives k_{cat}/k_{uncat} = 380, which is comparable to the value of 220 obtained for antibody-catalyzed thymine dimer cleavage.[5]

In order to determine the wavelength dependence of catalysis, the initial rate of cleavage of **1** was measured with irradiation at several wavelengths ranging from 290 to 310 nm. A plot of rate *vs* wavelength (Figure 4) reflects the absorbance spectrum of indole and is consistent with photosensitization by a tryptophan residue.

In order to provide additional evidence for an active site tryptophan residue, the binding of substrates **1** and **2** to antibody UD4C3.5 was assayed by fluorescence quenching. Titration of the antibody with increasing amounts of **1** resulted in a 40% decrease in antibody fluorescence (Figure 5a). This result is consistent with quenching of tryptophan fluorescence upon binding of the substrate. Scatchard analysis of the titration data gives a dissociation constant, K_D, for the antibody·**1** complex

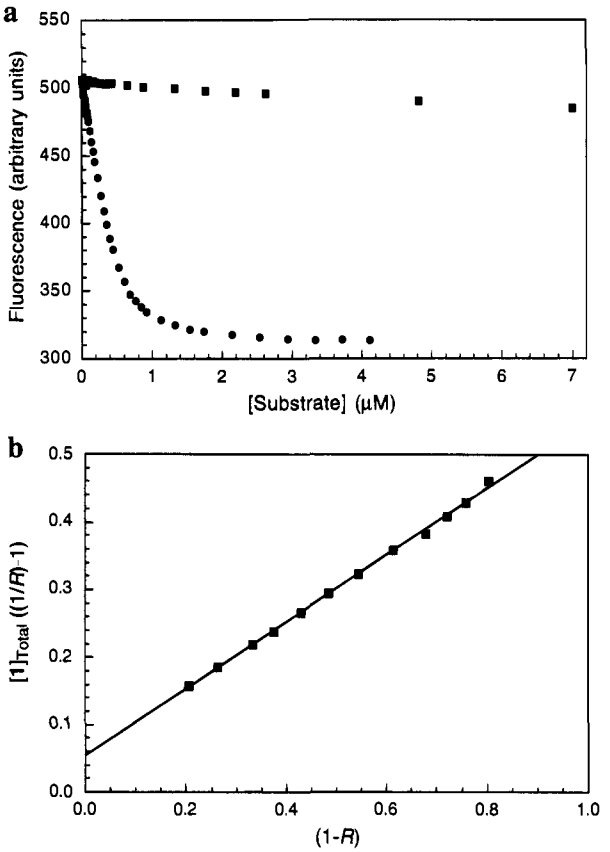

Figure 5. a. Fluorescence quenching of UD4C3.5 fluorescence by substrates **1** (circles) and **2** (squares). **b.** Scatchard plot of the titration data for **1**, where R is the fraction of sites which are bound.

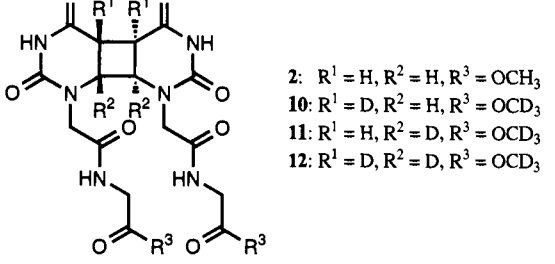

Figure 6. Substrates for measurement of kinetic isotope effects.

of 54 nM (Figure 5b). Similar titration with substrate **2** gives only very modest fluorescence quenching, consistent with the observation that K_M for **2** is much higher than K_M for **1**.

The wavelength dependence of catalysis and the fluorsesecence quenching behavior of antibody UD4C3.5 are both similar to those observed for DNA photolyase under conditions where the normal cofactors are inactive.[25] It was shown that under such conditions, a specific tryptophan residue in the active site of photolyase acts as the photosensitizer.[25]

Secondary Deuterium Isotope Effects on the Antibody Catalyzed Cleavage Reaction. In order to further study the mechanism of the cleavage reaction, we measured the kinetic isotope effects of deuterium substitutions to the cyclobutane ring protons of **2**. Selectively deuterated analogs of **2** were prepared containing deuterium at the 5,5′ positions (**10**), 6,6′ positions (**11**), and 5,5′,6,6′ positions (**12**) (Figure 6). In each case methyl-d_3 tags were incorporated as terminal esters. Due to its distance from the reaction center, this additional deuterium tag was not expected to result in an isotope effect but was

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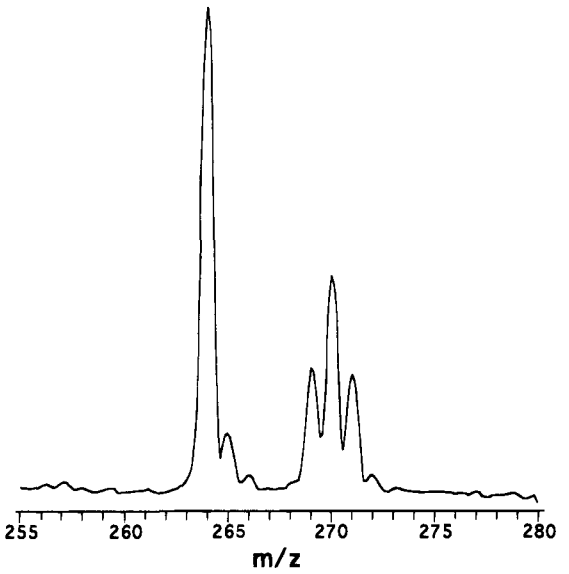


Figure 7. Typical mass spectrum of the reaction products from a mixture of **2** and **12**.

incorporated to increase the mass resolution between products, allowing facile determination of product ratios by mass spectrometry.

Uracil-6-*d* was prepared from propiolic acid-3-*d* and urea by the method of Hill[11,12] and elaborated to the carboxymethyl derivative **15**. Heating **15** to reflux in deuterochloric acid resulted in exchange of hydrogen at the C5 position for deuterium, giving the disubstituted compound **16**. The monosubstituted carboxymethyluracil-5-*d* (**17**) was similarly prepared from **5**. These were elaborated to the corresponding analogs of **8**. Alkaline hydrolysis of the ethylene glycol bridge in 5,5′- and 5,5′,6,6′-deuterated compounds **21**, and **23** was performed in NaOD/D_2O solutions to avoid exchange at the base-labile 5-position. This, however, introduced partial deuterium labeling at the methylene carbon α to N1 of uracil. While this generates a distribution of masses centered one dalton higher than the expected mass, it does not compromise the ability to measure product ratios, nor is it expected to contribute an additional isotope effect.

Isotope effects were measured by the method of internal competition.[17] Approximately equimolar concentrations of **2** and deuterated substrate **10**, **11**, or **12** were combined, and the mixture was irradiated in the presence of antibody UD4C3.5. Reaction products were purified by RP-HPLC and analyzed by electrospray-ionization mass spectrometry (Figure 7).[26] The signals resulting from **4**, and the deuterated product were separately integrated, and the ratio of integrals (P_H/P_D) was taken as the mole ratio of products. The mole ratio of substrates (S_H/S_D) was similarly determined. For the reaction conditions used (< 2% completion), substrate concentrations are effectively unchanged, and the isotope effect $^{\alpha\text{-D}}(V/K)$ can be calculated simply as $(P_H/P_D)/(S_H/S_D)$. Isotope effects were measured for substrates **10**, **11**, and **12**, and are reported in Table 1.

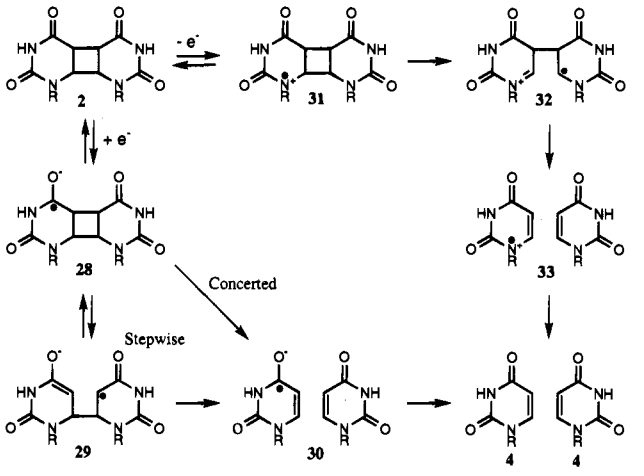
Mechanistic Implications. Studies on DNA photolyase and model systems have suggested at least three likely mechanistic possibilities for the photosensitized cleavage reaction; these are summarized in Scheme 2.[27,28] The initial step is thought to

Table 1. $^{\alpha\text{-D}}(V/K)$ Isotope Effects

substrates	positions substituted	$^{\alpha\text{-D}}$(V/K)
2 + **10**	5,5′	1.11 ± 0.03[a,b]
2 + **11**	6,6′	1.14 ± 0.03[a,c]
2 + **12**	5,5′,6,6′	1.20 ± 0.03[a,c]

[a] Confidence coefficient = 95%. All isotope effects were corrected as described in the experimental section. [b] Based on seven determinations. [c] Based on 13 determinations.

Scheme 2



involve electron transfer between the excited photosensitizer and the dimer to form a dimer radical cation or radical anion. The dimer radical cation **31** would then undergo sequential cleavage of the 6,6′ and 5,5′ bonds to form a pyrimidine monomer and monomer radical cation. Reduction by the photosensitizer radical anion would then complete the catalytic cycle. An analogous mechanism can be written for the cleavage of radical anion **28** with the 5,5′ bond breaking before the 6,6′ bond.

The cleavage reaction results in the conversion of four sp^3 centers to sp^2 centers. If the first bond cleavage is assumed to be irreversible, secondary deuterium isotope effects should distinguish between a radical cation and radical anion mechanism.[1] If a radical anion mechanism is operating, deuterium substitution at the 5,5′ positions should result in an isotope effect. Conversely, an isotope effect should be observed for the 6,6′ substituted isomer if the reaction proceeds via the radical cation mechanism. An alternative mechanism of direct conversion of **28** to **30** via concerted breakage of both 5,5′ and 6,6′ bonds has been proposed[28] based on calculations[29] as well as on the observed deuterium isotope effects on DNA photolyase[1] and model systems.[28,30] Such a mechanism would give isotope effects for the breakage of both bonds.[31]

The observation that significant isotope effects are associated with the breaking of both the 5,5′ and 6,6′ bonds in the antibody-catalyzed reaction prohibits unambiguous assignment of the mechanism as proceeding via the dimer radical cation or radical anion. While this pattern might arise from a stepwise mechanism and a significant β-isotope effect, this is unlikely since β-isotope effects are dependent on the cosine of the bond angle, and the C5−C6−H6 and C6−C5−H5 angles in dimer **8** are 106° and 125°, respectively. Our results are similar to those

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(31) Anthraquinone-mediated uracil dimer cleavage, which proceeds via the dimer radical cation, gives an isotope effect which is associated almost entirely with 6,6′-bond cleavage.[30]

obtained by Begley and co-workers for DNA photolyase[1] as well as for a model system consisting of a thymine dimer covalently tethered to an indole photosensitizer.[30] The excited state of the electron-rich indole ring acts as an electron donor, and formation of the dimer radical anion from indole photosensitization has been established spectroscopically.[32] Photosensitization by tryptophan is consistent with our fluorescence quenching and wavelength dependence data.[25] Moreover, in the case of antibody 15F1−3B1 which catalyzes thymine dimer repair, NMR studies of selectively deuterated antibodies and a dimer derivative containing a spin label have demonstrated that a tryptophan residue is within 5Å of the bound dimer.[33] However, attempts to spectroscopically observe dimer radical anion formation directly with the thymine dimer-cleaving antibody were unsuccessful.[34]

Based on model studies designed to trap intermediate **29,** Begley has proposed that the approximately equal distribution of isotope effects reflects a concerted mechanism.[28] Our results are consistent with such a mechanism. However, an equally attractive alternative is a mechanism in which the two transition states for 5,5′ and 6,6′ bond cleavages are energetically similar, with the initial 5,5′ bond cleavage being reversible. The assumption that the first bond-breaking step is irreversible[1] is based on the slow rates of closure measured for the pent-4-enyl radical.[35] With the substrate tightly bound in the antibody combining site, it seems possible that reformation of the cyclobutane ring would be favored relative to an unconstrained system. Spectroscopic evidence for the reversibility of the cleavage reaction in a tethered thymine dimer model system has recently been reported.[36] In either case, the antibody appears to catalyze this light-dependent reaction by binding the substrate in close proximity to the photosensitizer, insuring efficient electron transfer from excited state tryptophan to the uracil dimer. In addition, studies using an indole photosensitizer covalently linked to a pyrimidine dimer demonstrated a dramatic effect of solvent polarity on the quantum yield of cleavage.[37,38] These studies suggest that the antibody's hydrophobic binding pocket may also favor partitioning of the radical cation-radical anion pair to product *vs* back to the dimer substrate.

Summary

Antibody catalysis of a uracil dimer cleavage has been achieved by a design analogous to that used in a previous study of thymine dimer cleavage, thus demonstrating the generality of this strategy. The tight binding of the antibody to *trans,syn* substrate **1** makes the antibody an efficient catalyst at low concentrations of **1**. Wavelength dependence of the reaction as well as fluorescence quenching behavior of the antibody suggest that a binding site tryptophan catalyzes the reaction by acting as a photosensitizer. Secondary deuterium kinetic isotope effects do not provide unambiguous evidence for the expected radical anion intermediate but suggest either a concerted mechanism or a mechanism in which the first bond cleavage is reversible. The similarity of these results to those obtained for model systems supports the proposal that a radical anion mechanism is operative in the antibody-catalyzed reaction. We are currently attempting to generate antibodies that use an anthraquinone cofactor to catalyze cleavage via a radical cation mechanism.

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Supplementary Material Available: Full details of the X-ray crystal structure of **8** (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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