Mechanistic Studies of an Antibody-Catalyzed Elimination Reaction

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Abstract: Antibodies elicited against the ammonium ion containing hapten, *N*-methyl-*N*-(4-nitrobenzyl)-δ-aminovaleric acid, **3**, are capable of catalyzing HF elimination from (4*R*,4*S*)-fluoro-4-(4′-nitrophenyl)butan-2-one, **1**. The ammonium ion in **3** is responsible for generating a complementary negatively charged carboxylate ion in the antibody combining site which serves as a general base to abstract a proton from C-3 of **1**. The base responsible for catalysis in one antibody was identified by affinity labeling with [4-^3H]-(*E*)-3,4-epoxy-4-(4′-nitrophenyl)-butan-2-one. Peptide mapping of the derivatized heavy chain identified glutamate 46 as the carboxylate group responsible for proton abstraction. A primary kinetic isotope effect of $k_H/k_D = 2.35$ for the antibody catalyzed reaction ruled out an E1 elimination mechanism but does not differentiate between an E2 or E1cB mechanism. The stereochemistry of proton abstraction was assessed by use of the four possible C–3 monodeuterated diastereomers of substrate **1**. Product analysis demonstrated that 43D4-3D12 is capable of abstracting either the proR or proS proton at C-3 of **1**. These mechanistic studies validate the use of hapten complementarity as a rational design strategy for introducing precisely positioned catalytic groups in antibody combining sites.

Introduction

A number of strategies have been developed for exploiting the tremendous diversity of the immune system for the production of highly selective catalysts. These include the use of antibodies to selectively stabilize rate-determining transition states, the use of antibodies as "entropy traps", and the generation of antibodies that contain catalytic groups in their combining sites.[1] One approach to generating a combining site that contains a general acid or general base takes advantage of the notion of antibody–hapten charge complementarity.[2] Binding studies[3] and crystallographic data[4] on antibodies have shown that the structural features of the hapten induce complementary structural features in the antibody combining site, e.g., charged groups induce oppositely charged amino acids and π systems induce aromatic amino acids. Consequently, one should be able to design haptens that select for antibodies with, for example, acidic or basic residues appropriately positioned to abstract or deliver a proton to a desired site in the corresponding substrate.

One of the first examples of the use of antibody–hapten complementarity to induce an active site catalytic group was the antibody-catalyzed elimination of HF from 4-fluoro-4-(4′-nitrophenyl)butan-2-one (**1**) to give α,β-unsaturated ketone **2**.[2a] Antibodies were generated to hapten **3**, which resembles β-fluoroketone substrate **1**, with the exception that an ammonium group replaces the abstractable α-proton of the substrate. The positively charged alkyl ammonium ion was expected to induce a complementary negatively charged carboxylate residue in the antibody combining site, positioned to function as a general base for β-elimination of hydrogen fluoride. From a panel of six monoclonal antibodies, four were able to catalyze the elimination reaction; one antibody, 43D4-3D12, catalyzed the reaction with a rate acceleration of approximately 10^5 over that of the corresponding background reaction.

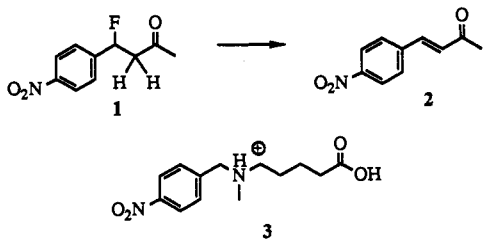

Figure 1. Elimination reaction catalyzed by the antibody 43D4-3D12 and hapten structure.

In order to gain further insight into the nature of this antibody catalyzed elimination reaction we have carried out a series of kinetic, stereochemical, and structural studies of antibody 43D4-3D12. These studies are consistent with an E2 or E1cB elimination mechanism in which the side chain of Glu 46 of the heavy chain abstracts either prochiral hydrogen of substrate **1** in the rate-determining step. The ability to rationally generate antibody combining sites containing basic or acidic residues should facilitate the generation of antibodies which catalyze reactions such as aldol condensations, isomerizations, acyl-transfer processes, and others.

Experimental Section

General Methods. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Pyridine, methylene chloride, and dimethylformamide (DMF) were dried by distilling from CaH_2. Dioxane and tetrahydrofuran were distilled from sodium with benzophenone as an indicator. All aqueous solutions were prepared from deionized distilled water. All oyxgen or moisture-sensitive reactions were carried out in oven-dried glassware under a positive pressure of nitrogen or argon. Analytical thin-layer chromatography was performed on precoated silica gel plates (Merck 60-254) which were

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visualized by UV light. Flash silica gel chromatography was performed[5] using Keiselgel 60 (230–400 mesh) silica gel. Gas chromatography–mass spectra were recorded using a HP 5890 series II gas chromatograph with a 20 m Carbowax 25 m × 0.2 mm × 0.2 μm film thickness column and an HP 5970 series mass selective detector controlled by a HP 9133 computer controller. For peptide mapping, reverse-phase high-pressure liquid chromatography (HPLC) was performed at ambient temperature on HP 1090 liquid chromatograph using a Rainin C-8 Microsorb column (300 Å, 5 μm, 4.6 mm × 15 cm) with a Waters 994 programmable photodiode array detector and a Gilson Model 203 fraction collector.

Melting points were determined using a Mel-Temp melting point apparatus and are uncorrected. Optical rotations were determined at 589 nm (sodium D line) with a Perkin-Elmer-Model 241 polarimeter and a 10-cm pathlength cell. Rotations are reported as standard degree values ($[\alpha]_D$ = ° obs[path length in dm][concentration in g·mL^{-1}] followed by the concentration (c, g·mL^{-1}) and the solvent. IR spectra were recorded on either a Matteson Polaris Fourier-Transform spectrophotometer using NaCl plates or KBr pellets. UV spectra were recorded in 10 mm quartz cells with a Varian Cary Model 2200 grating spectrophotometer equipped with a constant temperature cell.

^{1}H NMR spectra were recorded on an AM-400 (400 MHz) Fourier-transform NMR spectrometers at the University of California, Berkeley NMR facility. ^{1}H resonances are reported in units of ppm downfield from tetramethylsilane (TMS). ^{13}C NMR spectra were recorded on the AM-400 (100.6 MHz) spectrometer; all spectra are proton-decoupled. ^{13}C resonances are reported in units of ppm downfield from TMS. ^{19}F NMR spectra were recorded on the AM-400 (376.5 MHz) spectrometer using freon (∂ 0.0 ppm) as an internal standard. All spectra are proton-decoupled, and ^{19}F resonances are reported in units of ppm downfield from freon. Positive-ion and negative-ion fast atom bombardment mass spectra (FAB^+ and FAB^-, respectively) were recorded by the staff of the Mass Spectroscopy Laboratory at the University of California, Berkeley on a VG Zab2EQ or a Kratos MS50 (xenon beam, 7 V) mass spectrometer. Elemental analyses were performed by the Microanalytical Laboratory operated by the college of Chemistry at the University of California, Berkeley.

(4*R*,4*S*)-Hydroxy-4-(4′-nitrophenyl)butan-2-one (5).[6] To a solution of 4-nitrobenzaldehyde (10.0 g, 66.2 mmol) in 120 mL of acetone was added 12.0 mL of a 1% (w/v) aqueous NaOH solution at 0 °C. Stirring was continued at 0 °C for 15 min. The solution was then neutralized by addition of 0.50 N aqueous HCl and concentrated in vacuo. The black residue was dissolved in 100 mL of water and extracted with ether (3 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography on a 4.5 × 12 cm silica column (40% [v/v] hexanes in ethyl acetate; R_f = 0.33), affording 9.10 g (66%) of a yellow solid: mp 59–61 °C; IR (KBr) 3447, 3118, 1713, 1517, 1341 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.23 (s, 3H), 2.88 (d, 2H, J = 6.2), 3.90 (d, 1H, J = 3.4), 5.27 (m, 1H, 15 Hz), 7.54 (d, 2H, J = 8.7), 8.16 (d, 2H, J = 8.7); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 208.5, 150.0, 147.2, 126.3, 123.7, 123.6, 68.9, 68.7, 51.4, 30.7, 30.6; mass spectrum (EI) m/e 209 (M^+), 191 (M − H_2O). Anal. Calcd for $C_{10}H_{11}NO_4$: C, 57.44; H, 5.26; N, 6.69. Found: C, 57.35; H, 5.18; N, 6.66.

(4*R*,4*S*)-Hydroxy-4-(3′-nitrophenyl)butan-2-one (19). This compound was prepared as above using 3-nitrobenzaldehyde (5.00 g, 33.1 mmol), 60 mL of acetone, and 6.0 mL of a 1% (w/v) aqueous NaOH solution. The crude product was purified by flash chromatography on a 7.5 × 8 cm silica column (75% [v/v] hexanes in ethyl acetate; R_f 0.08), affording 3.40 g (49%) of a viscous yellow oil: IR (film) 3450, 3100, 2910, 1700, 1520, 1475, 1404, 1340, 1083, 1054, 890, 800, 733, 686 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.24 (s, 3H), 2.89 (d, 2H, J = 6.8), 3.69 (d, 1H, J = 3.4), 7.56 (5, 1H, J = 8.0), 8.13 (d, 1H, J = 8.8), 8.24 (s, 1H), 8.26 (m, 1H, 9 Hz); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 208.6, 148.3, 144.7, 131.7, 129.4, 122.5, 120.7, 120.6, 68.8, 68.6, 51.4, 30.7, 30.6; mass spectrum (EI) m/e 209 (M^+), 191 (M − H_2O), 151 (base). Anal. Calcd for $C_{10}H_{11}NO_4$: C, 57.42; H, 5.26; N, 6.70. Found: C, 57.42; H, 5.26; N, 6.73.

(4*R*,4*S*)-Fluoro-4-(4′-nitrophenyl)butan-2-one (1). To a solution of **5** (0.50 g, 2.39 mmol) in 10 mL of methylene chloride at −78 °C was added 0.32 mL (2.39 mmol) of diethylaminosulfur trifluoride. The solution was allowed to stir for 10 min at −78 °C and then poured into 20 mL of water. The phases were separated, and the aqueous phase was extracted with methylene chloride (2 × 10 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 0.46 g (91%) of product as an orange oil: IR (film) 3100, 3062, 2990, 2910, 1718, 1603, 1514, 1340, 1150, 1080, 1036, 850, 720, 690 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.24 (s, 3H), 2.87 (ddd, 1H, J = 29.2, 17.2, 4.4), 3.22 (ddd, 1H, J = 17.0, 16.0, 8.1), 6.08 (ddd, 1H, J = 46.4, 7.8, 4.5), 7.54 (d, 2H, J = 8.7), 8.25 (d, 2H, J = 8.6); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 203.7, 147.8, 146.3, 146.1, 126.1, 123.9, 89.6, 87.9, 50.3, 50.1, 30.6; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −144.35 (m, 110 Hz); mass spectrum (EI) m/e 211 (M^+), 176 (base). Anal. Calcd for $C_{10}H_{10}FNO_3$: C, 56.87; H, 4.74; N, 6.61. Found: C, 56.74; H, 4.83; N, 6.54.

(4*R*,4*S*)-Fluoro-4-(3′-nitrophenyl)butan-2-one (4). Compound **4** was prepared as above using 1.41 g (6.75 mmol) of **19**, 30 mL of methylene chloride, and 0.89 mL (6.75 mmol) of diethylaminosulfur trifluoride to afford 1.01 g (71%) of product as an orange oil: IR (film) 2900, 1716, 1542, 1366, 1163, 1051, 809, 737, 687 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) d 2.25 (s, 3H), 2.92 (ddd, 1H, J = 29.3, 17.2, 4.5), 3.26 (ddd, 1H, J = 17.2, 15.5, 8.1), 6.07 (ddd, 1H, J = 46.3, 8.1, 4.5), 7.59 (t, 1H, J = 7.9), 7.71 (d, 1H, J = 7.5), 8.25 (m, 2H, J = 15.0); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 203.7, 148.3, 144.8, 141.3, 141.1, 131.6, 129.7, 123.5, 120.4, 89.6, 87.0, 50.3, 50.0, 30.8, 30.7; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −146.469 (m, 97 Hz); mass spectrum (EI) m/e 211 (M^+), 191 (M − HF), 151 (base). Anal. Calcd for $C_{10}H_{10}FNO_3$: C, 56.87; H, 4.74; N, 6.61. Found: C, 56.69; H, 4.80; N, 6.71.

(*E*)-4-(4′-Nitrophenyl)-2-oxo-3-butene (2). Compound **5** was treated with 14.0 mL of a 1 N aqueous solution of sulfuric acid and heated at reflux overnight. The dark orange reaction mixture was then cooled to 25 °C and extracted with chloroform (2 × 30 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography on a 2.5 × 25 cm silica column (67% [v/v] hexanes in ethyl acetate: R_f 0.23), affording 1.38 g (58%) of the product as a yellow powder: mp 106–107.5 °C; IR (KBr) 3114, 1680, 1670, 1600, 1580, 1513, 1344 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.43 (s, 3H), 6.82 (d, 1H, J = 16.3), 7.54 (d, 1H, J = 16.3), 7.70 (d, 2H, J = 8.8), 8.27 (d, 2H, J = 8.8); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 197.4, 148.5, 140.6, 139.5, 130.3, 128.7, 124.1, 27.9; mass spectrum (EI) m/e 191 (M^+), 176 (M − CH_3). Anal. Calcd for $C_{10}H_9NO_3$: C, 62.83; H, 4.71; N, 7.33. Found: C, 62.75; H, 4.59; N, 7.22.

***N*-(4′-Nitrobenzyl)-δ-aminovaleric Acid.** δ-Aminovaleric acid potassium salt (1.55 g, 10.0 mmol) was dissolved in 40 mL of methanol, and the pH was adjusted to 6 with concentrated methanolic HCl. The precipitated potassium chloride was filtered off and washed with 10 mL of methanol. To the filtrate was added 1.51 g (10.0 mmol) of *p*-nitrobenzaldehyde and 437 mg (7.00 mmol) of sodium cyanoborohydride. The mixture was stirred for 16 h at 25 °C. The reaction was quenched by adding 5 mL of concentrated HCl. The mixture was concentrated in vacuo, redissolved in 100 mL of water, and washed with dichloromethane (2 × 50 mL). The aqueous phase was concentrated, and the residue was purified by anion exchange chromatography on a 3 × 30 cm column containing DEAE-sephadex (conditioned with 0.5 M triethylammonium bicarbonate, pH 8.2, and washed exhaustively with water). The product was eluted with a linear gradient = 5–50% of 0.5 M triethylammonium bicarbonate, pH 8.2, in water (500 mL). The fractions were monitored by TLC (*n*-BuOH/acetic acid/water, 4:1:1). The fractions containing pure product were combined, concentrated in vacuo, and applied to a 1.5 × 10 cm column containing Dowex 50X2-200 (H^+ form). After washing 100 mL of water, the adsorbed product was eluted with a solution of aqueous 1 M ammonium hydroxide (100 mL). The fractions containing product were combined and concentrated in vacuo and crystallized from water/acetone to afford 799 mg (32%) of the desired product: mp 123–125 °C; ^{1}H NMR (200 MHz, D_2O) δ 1.51 (m, 4H), 2.05 (t, 2H, J = 8.8), 2.97 (m, 2H), 4.19 (s, 2H), 7.52 (d, 2H, J = 8.8), 8.11 (d, 2H, J = 8.8); ^{13}C NMR (50 MHz, D_2O) δ 181.6, 175.7, 147.2, 137.2, 129.9, 123.3, 49.1, 46.4, 35.8, 24.4, 21.7; mass spectrum (FAB^+) m/e 253 (M + H); UV/vis (H_2O) λ_{max} 263 (10,100). Anal. Calcd for $C_{12}H_{16}N_2O_4$: C, 57.13; H, 6.39; N, 11.10. Found: C, 56.93; H, 6.46; N, 10.99.

***N*-Methyl-*N*-(4-nitrobenzyl)-δ-aminovaleric Acid (3).** To a solution of 300 mg (1.19 mmol) of *N*-(4-nitrobenzyl)-δ-aminovaleric acid in 6 mL of water were added 450 μL of a 37% aqueous formaldehyde solution (6.10 mmol) and 123 mg (1.96 mmol) of sodium cyanoborohydride. This mixture was stirred for 45 min at 25 °C. The reaction was quenched by the addition of aqueous 1 M HCl (6 mL) and was directly applied to a 2 × 10 cm column of Dowex 50X2-200 (H^+ form). The column was washed with 150 mL of water, and the product was eluted with a solution of aqueous 0.7 M ammonium hydroxide (50 mL). Concentration of the

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fractions containing product afforded a brown oil which was further purified by flash chromatography on a 1 × 15 cm silica column containing (50% [v/v] methanol in dichloromethane; R_f 0.22) affording 270 mg (85%) of **3** as a colorless oil: ^{1}H NMR (200 MHz, D_2O) δ 1.44 (m, 4H), 2.03 (m, 2H), 2.28 (s, 3H), 2.59 (m, 2H), 3.83 (s, 2H), 7.46 (d, 2H, J = 8.7), 8.10 (d, 2H, J = 8.7); ^{13}C NMR (50 MHz, DMSO-d_6) δ 175.3, 147.8, 146.4, 129.4, 123.3, 60.6, 56.6, 41.8, 34.1, 26.3, 22.5; mass spectrum (FAB$^+$) m/e 267 (M + H). Anal. Calcd for $C_{13}H_{18}N_2O_4 \cdot H_2O$: C, 55.26; H, 7.06; N, 9.91. Found: C, 55.34; H, 6.88; N, 9.89.

Bovine Serum Albumin (BSA) and Keyhole Limpet Hemocyanin (KLH) Conjugates of 3. A solution of **3** (27.0 mg, 0.10 mmol) in water (1 mL) was adjusted to pH 2 by addition of 6 drops of aqueous 1 M HCl. This solution was subsequently lyophilized, and the residue dissolved in DMF (1.5 mL) and treated with *N*-hydroxysuccinimide (62.0 mg, 0.540 mmol) and 1,3-dicyclohexylcarbodiimide (166 mg, 0.806 mmol). The solution was stirred for 20 h at 25 °C and then centrifuged. The supernatant was added to a solution of 30 mg of BSA or 20 mg of KLH in aqueous 75 mM sodium carbonate pH 9.3 (4 mL). The pH was periodically controlled and adjusted to 9.3 by addition of aqueous 0.1 M sodium hydroxide. After stirring for 20 h at 25 °C the solution was exhaustively dialyzed against 10 mM sodium phosphate, 150 mM NaCl pH 7.4 (PBS). The protein concentration of the final solution was determined according to the method of Lowry.[7] The level of derivatization of the protein by **3** was determined by the indirect method of Habeeb.[8] An epitope density of 14 haptens per protein monomer was determined by this method for the BSA conjugate and 18 for the KLH conjugate.

(*E*)-3,4-Epoxy-4-(4′-nitrophenyl)butan-2-one (7).[9] A solution of **2** (250 mg, 1.3 mmol) in 10 mL of toluene was treated with 0.51 mL (1.5 mmol) of a 3.0 M solution of *tert*-butyl hydroperoxide solution in 2,2,4-trimethylpentane containing 17 μL (0.042 mmol) of a 40% solution of Triton B in methanol at 0 °C. The solution was allowed to warm to 25 °C and was stirred overnight. The reaction mixture was diluted with 20 mL of water, and the two phases separated. The aqueous layer was washed with ether (3 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 2 × 8 cm silica column (methylene chloride; R_f 0.23), affording 91.0 mg (32%) of product as a yellow solid: mp 77–79 °C; IR (KBr) 1716, 1599, 1521, 1367, 1250, 1176, 1109, 848, 756 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.23 (s, 3H), 3.49 (d, 1H, J = 1.8), 4.15 (d, 1H, J = 1.7), 7.48 (d, 2H, J = 8.8), 8.23 (d, 2H, J = 8.8); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 202.9, 148.2, 142.3, 126.4, 123.9, 63.2, 56.4, 24.8; mass spectrum (EI) m/e 207 (M$^+$), 165 (base). Anal. Calcd for $C_{10}H_9NO_4$: C, 57.97; H, 4.35; N, 6.76. Found: C, 57.29; H, 4.40; N, 6.43.

4-(4′-Nitrophenyl)butan-2-one.[10] To benzylacetone (4.00 g, 27.0 mmol) and copper(II) nitrate hydrate (5.79 g, 27.0 mmol) were added trifluoroacetic anhydride (25 mL) and chloroform (25 mL) at 25 °C. The mixture was stirred for 2.5 h after which time no solid remained. The mixture was poured in a steady stream into 500 mL of water (0 °C). The water was extracted with ether (3 × 200 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 3 × 15 cm silica column (67% [v/v] hexanes in ethyl acetate; R_f 0.26), affording 0.133 g (3%) of a brown oil: IR (film) 3045, 2900, 1705, 1602, 1506, 1400, 1335, 1152, 1100, 1000, 852, 824, 742, 690 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.16 (s, 3H), 2.82 (t, 2H, J = 7.3), 3.00 (t, 2H, J = 7.3), 7.35 (d, 2H, J = 8.8), 8.13 (d, 2H, J = 8.7); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 206.6, 148.9, 146.4, 129.2, 123.6, 44.1, 29.9, 29.2; mass spectrum (EI) m/e 193 (M$^+$); exact mass calcd for $C_{10}H_{11}NO_3$ 193.0738, found 193.0737.

[4-^{3}H]-(*E*)-3,4-Epoxy-4-(4′-nitrophenyl)butan-2-one (8). To an ampule containing sodium borotritide (25 mCi, 350 mCi/mmol, 71.4 μmol) was added an aqueous 0.1 N solution of sodium hydroxide (100 μL). This solution was transferred to 4-nitrobenzaldehyde (36.0 mg, 0.238 mmol) in THF (3 mL) at 25 °C. The solution was stirred for 20 min and quenched by the addition of water (5 mL) and ethyl acetate (10 mL). The phases were separated, and the aqueous phase was extracted with ethyl acetate (3 × 5 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated by distillation at atmospheric pressure. The *p*-nitrobenzyl alcohol product was analyzed by silica gel TLC (75% [v/v] hexanes in ethyl acetate; R_f 0.30) indicating 70% conversion of the starting material to the desired product, with no other products visible by short wave UV light. To the residue from this reaction was added THF (2 mL) and manganese(IV) oxide (300 mg, 3.45 mmol). The mixture was stirred for 25 °C for 2 h, and additional manganese(IV) oxide (200 mg, 2.30 mmol) was added. After stirring for 12 h the solid was removed by gravity filtration. The solid was washed with THF (15 mL), and the combined filtrate was concentrated by distillation at atmospheric pressure. The *p*-nitrobenzaldehyde product was analyzed by silica gel TLC and comparison with authentic nonradioactive material (75% [v/v] hexanes in ethyl acetate; R_f 0.28) indicating 90% completion of the oxidation reaction with no other products visible by short wave UV. To the residue from this reaction was added chloroacetone (16.0 μL, 0.181 mmol), absolute ethanol (2 mL), and sodium ethoxide (12.6 mg, 0.185 mmol) at 0 °C. The ice bath was removed, and the mixture stirred for 25 °C for 15 h. The reaction was quenched by the addition of water (3 mL), and the phases separated. The aqueous phase was extracted with ether (3 × 10 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated by distillation at atmospheric pressure. The residue was purified on a 2 mm × 20 cm × 20 cm silica gel TLC plate (75% [v/v] hexanes in ethyl acetate; R_f 0.35). The ethyl acetate used to deadsorb the product from the silica gel was removed by distillation at atmospheric pressure. The residue was dissolved in ethanol (1 mL). The product was identified by UV/vis spectrophotometry, λ_{max} = 276 nm (7: λ_{max} = 276 nm [7784]). The specific activity was determined to be 13.4 mCi/mmol, corresponding to an overall chemical yield of <1%.

(4*R*,4*S*)-Hydroxy-4-(4′-nitrophenyl)butan-2-one-1,1,1,3,3-d_5 (20). Compound **20** was prepared as above using 4-nitrobenzaldehyde (1.00 g, 6.62 mmol), acetone-d_6 100 atom% D (12.0 mL), and 1.2 mL of a 1% (w/v) aqueous NaOD solution to afford 0.76 g (54%) of a yellow solid: mp 58–60 °C; IR (KBr) 3422, 3111, 1706, 1599, 1520, 1341, 1264, 1071 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.77 (s, 1H), 7.49 (d, 2H, J = 8.5), 8.12 (d, 2H, J = 8.6); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 208.0, 149.9, 147.2, 126.3, 123.7, 68.7; mass spectrum (EI) m/e 214 (M$^+$), 151 (base). Anal. Calcd for $C_{10}H_6D_5NO_4$: C, 56.07; H, 5.14; N, 6.54. Found: C, 56.14,; H, 5.14; N, 6.56.

(4*R*,4*S*)-Fluoro-4-(4′-nitrophenyl)butan-2-one-1,1,1,3,3-d_5 (9). Compound **9** was prepared as above using **20** (22.0 mg, 0.103 mmol) in 1 mL of methylene chloride and 13.6 μL (0.103 mmol) of diethylaminosulfur trifluoride to afford 21.4 mg (96%) of the desired product as an orange oil: IR (film) 3100, 3050, 3000, 2950, 2880, 1710, 1605, 1520, 1340, 1280, 1241, 1100, 1022, 850, 740, 690 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.07 (d, 1H, J = 46.4), 7.54 (d, 2H, J = 8.7), 8.25 (d, 2H, J = 8.3); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 147.8, 146.3, 146.1, 126.0, 124.1, 89.6, 87.8; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −143.92 (d, J = 45.8); mass spectrum (EI) m/e 216 (M$^+$); exact mass calcd for $C_{10}H_5D_5NO_3$ 216.0967, found 216.0968.

(2*R*,2*S*)-4-Phenyl-3-butyn-2-ol (21).[11] To a solution of phenylacetylene (44.0 mL, 0.40 mol) in 200 mL of THF at 0 °C was added 250 mL (0.40 mol) of 1.6 M *n*-butyllithium in hexanes. The solution was stirred for 15 min at 0 °C and treated with 22.4 mL (0.40 mol) of acetaldehyde. The solution was allowed to warm to 25 °C and was stirred for 1 h. The solution was concentrated, and the residue taken up in 100 mL of ether, which was washed with 50 mL of water, dried over $MgSO_4$, filtered, and concentrated. The residue was distilled at 84–87 °C (0.30 mm) to afford 25.1 g (43%) of the desired product: IR (film) 3350, 3000, 2205, 1664, 1598, 1489, 1410, 1371 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.55 (d 3H, J = 6.6 Hz, 1.63 (m 1H, J = 13 Hz), 4.76 (dq, 1H, J = 6.4, 5.6 Hz), 7.31 (m, 3H, J = 6.0 Hz), 7.43 (m, 2H, J = 20.0 Hz); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 145.0, 133.0, 131.6, 128.2, 122.5, 90.8, 83.9, 58.7, 24.2; mass spectrum (EI) m/e 146 (M$^+$), 131 (M − CH_3). Anal. Calcd for $C_{10}H_{10}O$: C, 82.19; H, 6.85. Found: C, 82.03; H, 6.85.

(*E*)-(2*R*,2*S*)-4-Phenyl-3-buten-2-ol-3-*d* (14).[11] To a solution of 25.1 g (0.17 mol) of **21** in 800 mL of THF at 0 °C was added 7.14 g (0.17 mol) of lithium aluminum deuteride. The resulting intermediate was hydrolyzed by adding 250 mL of ethyl acetate followed by the 1:1:3 method and worked up in the usual manner[12] (7 mL of water added cautiously, then 7 mL of 15% aqueous NaOH, and finally 21 mL of water), affording 24.8 g (98%) of the desired product as a slightly yellow oil: IR (film) 3300, 2950, 2232, 1601, 1494, 1447, 1369, 1139, 1059 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37 (d, 3H, J = 6.4 Hz), 4.48 (q, 1H, J = 6.3 Hz), 6.55 (s, 1H), 7.23 (t, 1H, J = 7.3 Hz), 7.31 (t, 2H, J = 7.4 Hz), 7.37 (d, 2H, J = 7.9 Hz); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 136.6, 133.1, 129.2, 128, 127.5, 126.4, 68.8, 23.3; mass spectrum (EI) m/e 149 (M$^+$), 134 (M − CH_3). Anal. Calcd for $C_{10}H_{11}DO$: C, 80.54; H, 8.72. Found: C, 80.61; H, 8.44.

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(*E*)-(2*R*,3*R*)-3,4-Epoxy-4-phenyl-2-butanol-3-*d* (15).[13] To a solution of **14** (10.0 g, 72.5 mmol) in 400 mL of methylene chloride (dried over activated 3 Å molecular sieve pellets) were added D-(−)-diisopropyl tartrate (2.29 mL, 10.9 mmol), powdered and activated 3 Å molecular sieves (5 g), and 2.9 mL of *n*-decane as an internal standard at 25 °C. The mixture was cooled to between −10 and −20 °C, treated with 2.16 mL (7.25 mmol) of Ti(O-*i*-Pr)$_4$, and allowed to stir for 30 min at −20 °C. The reaction was then treated with 10.0 mL (55.0 mmol) of a 5.5 M solution of *tert*-butyl hydroperoxide in 2,2,4-trimethylpentane (5.5 M, dried with freshly activated 3 Å molecular sieve pellets for 30 min prior to addition). The reaction was stirred at −20 °C and monitored by GC. The reaction was quenched prior to 50% conversion by the addition of 100 mL of a solution of aqueous ferrous sulfate (33 g) and citric acid trisodium salt dihydrate (17 g) and stirred vigorously without cooling for 30 min. The resulting emulsion was filtered through Celite. The phases were separated, and the aqueous phase was extracted with three 100-mL portions of ether. The combined organic layers were treated with 10 mL of a precooled (0 °C) solution of 30% NaOH (w/v) in saturated brine. The two-phase mixture was stirred vigorously for 1 h at 0 °C. To the two-phase mixture was added 50 mL of water, the phases were separated, and the aqueous layer was extracted with ether (3 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 7 × 13 cm silica column (80% [v/v] hexanes in ethyl acetate; R_f 0.11), affording 3.9 g (65%) of a slightly yellow oil: $[\alpha]^{25}_D$ + 19.0° (*c* 0.007, $CHCl_3$); IR (film) 1717, 1559, 1457, 1374, 1280, 1074, 757, 700 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.31 (d, 3H, *J* = 6.5 Hz), 2.13 (br s, 1H), 3.95 (s, 1H), 4.10 (q, 1H, *J* = 6.43 Hz), 7.32 (m, 5H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 136.8, 128.4, 128.2, 125.6, 64.6, 54.4, 18.6; mass spectrum (EI) *m/e* 165 (M^+). exact mass calcd for $C_{10}H_{11}DO_2$ 165.0900, found 165.0901.

(*E*)-(2*S*,3*S*)-3,4-Epoxy-4-phenyl-2-butanol-3-*d* (16). Compound **16** was prepared as above using **14** (9.62 g, 65.5 mmol), Ti(O-*i*-Pr)$_4$ (1.95 mL, 6.55 mmol), L-(+)-diisopropyl tartrate (2.08 mL, 9.82 mmol), 5.5 M *tert*-butyl hydroperoxide solution in 2,2,4-trimethylpentane (17.6 mL, 98.2 mmol), in 450 mL of methylene chloride to afford 3.38 g (63%) of product having the same physical properties as compound **25** with the exception of optical rotation: $[\alpha]^{25}_D$-19.8° (*c* 0.006, $CHCl_3$).

(2*R*,3*R*,4*S*)-4-Phenylbutan-2,4-diol-3-*d* (17). To a solution of **15** (4.2 g, 25.4 mmol) in 150 mL of THF was added 7.50 mL (25.4 mmol) of a 3.4 M solution of sodium bis(2-methoxyethoxy)aluminum hydride in toluene at 25 °C. The intermediate was hydrolyzed by the 1:1:3 method,[12] the aluminum hydroxide precipitate was filtered and washed with ether, the combined organic layers were dried over $MgSO_4$, and concentrated. To the residue in 50 mL of benzene was added 6.69 g (15.0 mmol) of lead tetraacetate, and the mixture was stirred for 10 min at 25 °C. The reaction was quenched by the addition of 0.90 g (15 mmol) of ethylene glycol. To the mixture was added 50 mL of water, the phases were separated, and the aqueous layer was extracted with benzene (2 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 3 × 7 cm silica column (50% [v/v] hexanes in ethyl acetate; R_f 0.21), affording 1.5 g (35%) of a clear oil: $[\alpha]^{25}_D$-66.0° (*c* 0.0030, $CHCl_3$); IR (film) 2726, 1559, 1201, 1117, 1050, 767, 701 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.23 (d, 3H, *J* = 6.3 Hz), 1.83 (m, 1H, 9 Hz), 1.39 (dq, 1H, *J* = 8.4, 6.3 Hz), 5.05 (d, 1H, *J* = 3.1 Hz), 7.28 (m, 1H), 7.36 (m, 4H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 144.3, 128.4, 127.3, 71.7, 65.3, 23.4; mass spectrum (FAB^+) *m/e* 174.1 (M + Li). Anal. Calcd for $C_{10}H_{13}DO_2$: C, 71.85; H, 8.98. Found: C, 71.47; H, 9.13.

(2*S*,3*S*,4*R*)-4-Phenylbutan-2,4-diol-3-*d* (18). Compound **18** was prepared as above using **16** (2.88 g, 17.5 mmol) and a 3.4 M solution of sodium bis(2-methoxyethoxy)aluminum hydride in toluene (5.15 mL, 17.5 mmol) in 100 mL of THF to afford 1.65 g (56%) of product having the same physical properties as compound **17** with the exception of optical rotation: $[\alpha]^{25}_D$ +67.3° (*c* 0.0048, $CHCl_3$).

(2*R*,3*R*,4*S*)-4-Fluoro-4-phenylbutan-2-ol-3-*d* (21) and (2*R*,3*R*,4*R*)-4-fluoro-4-phenylbutan-2-ol-3-*d* (22). To a solution of **17** (1.60 g, 9.6 mmol) in 1.6 mL of methylene chloride at −78 °C was added 1.28 mL (9.58 mmol) of diethylaminosulfur trifluoride dropwise over 30 min. The solution was allowed to warm to 25 °C. To the solution was added 200 mL of water, the phases were separated, and the aqueous layer was extracted with 75 mL of methylene chloride. The combined organic layers were back extracted with 100 mL of brine, and the phases were separated. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 3 × 15 cm silica column (80% [v/v] hexanes in ethyl acetate; R_f 0.11), affording 0.20 g (12%) of a clear oil: IR (film) 2069, 1208, 1109, 1046, 950, 762, 700 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 (d, 1.3H, *J* = 6.1), 1.29 (d, 1.7H, *J* = 6.2), 1.83 (m, 0.5H, 50 Hz), 2.23 (m, 0.5H, 31 Hz), 3.99 (dq, 0.45H, *J* = 7.3, 6.3), 4.17 (m, 0.55H, 28 Hz), 5.68 (dd, 0.45H, *J* = 8.5, 48.1), 5.75 (d, 0.55H, *J* = 48.4), 7.4 (m, 5H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 140.4, 140.2, 139.8, 139.6, 128.4, 128.2, 125.6, 125.5, 125.3, 94.7, 93.0, 92.4, 90.8, 65.7, 65.6, 64.0, 46.4, 46.2, 46.0, 45.8, 45.7, 45.4, 45.2, 23.9, 23.4, 14.1; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −145.5 (dd, *J* = 46.5, 38.4), −149.87 (d, *J* = 48.2); mass spectrum (EI^+) *m/e* 169 (M^+), 151 (M − CH_3); exact mass calcd for $C_{10}H_{12}DFO$ 169.1014, found 169.1014.

(2*S*,3*S*,4*R*)-4-Fluoro-4-phenylbutan-2-ol-3-*d* (23) and (2*S*,3*S*,4*S*)-4-fluoro-4-phenylbutan-2-ol-3-*d* (24). Compounds **23** and **24** were prepared as above using **18** (1.52 g, 9.10 mmol) and diethylaminosulfur trifluoride (1.20 mL, 9.10 mmol) in 1.6 mL of methylene chloride to afford 0.33 g (21%) of product: IR (film) 2069, 1208, 1109, 1046, 950, 762, 700 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.26 (d, 0.5H, *J* = 6.2), 1.26 (d, 0.5H, *J* = 6.3), 2.21 (m, 0.5H, 30 Hz), 3.97 (dq, 0.5H, *J* = 7.4, 6.4), 4.15 (m, 0.5H, 29 Hz), 5.64 (dd, 0.5H, *J* = 48.1, 8.5), 5.73 (d, 0.5H, 46 Hz), 7.36 (m, 5H, 48 Hz); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 140.4, 140.2, 139.8, 139.6, 128.5, 128.4, 12.2, 125.6, 125.3, 94.6, 92.9, 92.4, 90.8, 65.6, 65.5, 63.9, 46.4, 46.2, 46.0, 45.9, 45.7, 45.4, 45.3, 36.6, 24.0, 23.4, 13.8; ^{19}F NMR (376.5 MHz, $CDCl_3$) d −145.4 (dd, *J* = 46.7, 37.7), −149.8 (d, *J* = 47.8); mass spectrum (EI) *m/e* 169 (M^+), 154 (M-CH_3); exact mass calcd for $C_{10}H_{12}DFO$ 169.1014, found 169.1012.

(2*R*,3*R*,4*S*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-ol-3-*d* (26) and (2*R*,3*R*,4*R*)-4-fluoro-4-(4′-nitrophenyl)butan-2-ol-3-*d* (25).[10] To **21** and **22** (34.0 mg, 0.201 mmol) and copper(II) nitrate hydrate (24.0 mg, 0.101 mmol) were added trifluoroacetic anhydride (0.30 mL) and chloroform (2 mL) sequentially at 25 °C. The solution was stirred at 25 °C for 3 h and concentrated. The trifluoroacetyl protected forms of **25** and **26** were separated on a 20 × 20 cm × 1 mm silica TLC plate (88% [v/v] hexanes in ethyl acetate). The purified products were dissolved in 2 mL of methanol and treated with 10 mg each of anhydrous potassium carbonate at 25 °C for 12 h, in separate flasks. The mixtures were concentrated, and to the residues were added 2 mL each of ethyl acetate and water. The phases were separated, and the aqueous layer was extracted with ethyl acetate (2 × 2 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residues were purified on separate 20 × 20 cm × 1 mm silica TLC plates (75% [v/v] hexanes in ethyl acetate; R_f 0.18 for both products), affording clear oils 6.1 mg (23%) of **26** and 5.5 mg (21%) of **25**. **26**: $[\alpha]^{25}_D$ −12.0° (*c* 0.0025, $CHCl_3$); IR (film) 3071, 2802, 1607, 1560, 1525, 1357, 1265, 1124 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.3 (dd, 3H, *J* = 6.6, 6.2), 2.21 (m, 1H, 24 Hz), 3.95 (dq, 1H, *J* = 7.9, 6.3), 5.78 (dd, 1H, *J* = 47.7, 8.2), 7.54 (d, 2H, *J* = 8.8), 8.25 (d, 2H, *J* = 8.2); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 126.2, 126.1, 123.7, 92.8, 91.4, 65.2, 65.1, 23.6; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −144.55 (d, *J* = 49.6 Hz); mass spectrum (EI) *m/e* 215 (M^+); exact mass calcd for $C_{10}H_{11}DFNO_3$ 215.0987, found 215.0939.

25: $[\alpha]^{25}_D$ = −15.2 (*c* 0.00125, $CHCl_3$); IR (film) 3071, 2802, 1607, 1560, 1525, 1357, 1265, 1124 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.30 (d, 3H, *J* = 6.2), 1.83 (m, 1H, 60 Hz), 4.21 (m, 1H, 17 Hz), 5.86 (d, 1H, *J* = 48.2), 7.51 (d, 2H, *J* = 8.9), 8.25 (d, H, *J* = 8.2); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 126.2, 126.1, 123.7, 92.8, 91.4, 65.2, 65.1, 23.6; ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −139.9 (dd, *J* = 40.3, 43.7); mass spectrum (EI) *m/e* 215 (M^+); exact mass calcd for $C_{10}H_{11}DFNO_3$ 215.0987, found 215.0937.

(2*S*,3*S*,4*R*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-ol-3-*d* (28) and (2*S*,3*S*,4*S*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-ol-3-*d* (27). Compounds **27** and **28** were prepared as above using **23** and **24** (36.4 mg, 0.216 mmol), copper(II) nitrate hydrate (26.0 mg, 0.108 mmol), trifluoroacetic anhydride (0.40 mL), and chloroform (2 mL) to afford 6.0 mg (26%) of **28** and 6.0 mg (26%) of **27**. **28**: $[\alpha]^{25}_D$ = +6.0° (*c* 0.0005, $CHCl_3$); **27**: $[\alpha]^{25}_D$ = +9.4° (*c* 0.0016, $CHCl_3$).

(3*R*,4*R*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-one-3-*d* (10).[14] To a solution of chromium trioxide (1.50 g) in aqueous 6 N HCl (2.75 mL) was added pyridine (1.19 g) at 40 °C. The mixture was cooled on ice until a yellow orange solid formed. The solid was then reheated to 40 °C affording a dark orange solution. Alumina (12 g) was then added to the solution. After concentration the free flowing orange solid was dried for 2 h at 0.5 mmHg. The reagent was stored in the dark under

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vacuum. To a solution of **25** (6.0 mg, 28 μmoles) in benzene (1 mL) was added 250 mg of the above PCC/alumina reagent. The mixture was stirred at 25 °C for 12 h. The resulting brown solid was filtered and washed with 10 mL of benzene. The filtrate was concentrated affording 4.5 mg (76%) of a yellow oil: $[\alpha]^{25}_D$ = +6.1° (*c* 0.0015, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, 2H, *J* = 7.8), 7.54 (d, 2H, *J* = 8.2), 6.07 (dd, 1H, *J* = 46.3, 7.4), 3.2 (m, 1H, 40 Hz), 2.24 (s, 3H); ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −144.1 (dd, *J* = 42.0, 16.0); exact mass calcd for $C_{10}H_9DFO_3$ 212.0735, found 212.0705.

(3*R*,4*S*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-one-3-*d* (11). Compound **11** was prepared as above using **26** (6.0 mg, 28 μmol) and the PCC/alumina reagent (300 mg) in 1 mL of benzene to afford 5.0 mg (84%) of product **9**: $[\alpha]^{25}_D$ = −10.0° (*c* 0.0014, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, 2H, *J* = 8.3), 7.52 (d, 2H, *J* = 8.3), 6.05 (dd, 1H, *J* = 3.9, 46.6), 2.82 (d, 1H, *J* = 29.5), 2.22 (s, 3H); ^{19}F NMR (376.5 MHz, $CDCl_3$) δ −144.20 (dt, *J* = 25.6, 21.5); exact mass calcd for $C_{10}H_9DFO_3$ 212.0735, found 212.0703.

(3*S*,4*S*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-one-3-*d* (13). Compound **13** was prepared as above using **27** (6.0 mg, 28 μmol) and the PCC/alumina reagent (300 mg) in 1 mL of benzene to afford 4.0 mg (67%) of product **12**: $[\alpha]^{25}_D$ = −6.4° (*c* 0.0014, $CHCl_3$).

(3*S*,4*R*)-4-Fluoro-4-(4′-nitrophenyl)butan-2-one-3-*d* (12). Compound **12** was prepared as above using **28** (5.0 mg, 23.3 μmol) and the PCC/alumina reagent (0.30 g) in 1 mL of benzene to afford 4.0 mg (81%) of product **11**: $[\alpha]^{25}_D$ = +8.4° (*c* 0.0011, $CHCl_3$).

Antibody Purification: Protein-A Chromatography. This procedure was adapted from that of Fagerstam *et al.*[15] 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 glass wool (5 mm × 5 mm). The solution was then applied (flow rate = 0.5 mL/min) to a 10-mL (settled volume) column of Affinica Protein A gel (Schleicher and Schuell) previously equilibrated with binding buffer. The column was then washed until the absorbance of the eluent was <0.05 absorbance units (at 280 nm). Elution buffer (aqueous 0.1 M sodium citrate, pH 3.0) was applied to the column, and 2.5-mL fractions were collected with stirring into test tubes containing 0.8 mL of collection buffer (aqueous 1 M Tris·HCl, pH 9.0). The fractions containing protein determined by absorbance at 280 nm were pooled and dialyzed against PBS.

Gel Electrophoresis. All protein gel electrophoresis was performed using a Pharmacia Phast system. Either 12.5% homogeneous or gradient 10–15% denaturing polyacrylamide gels with SDS buffer strips were used. Samples were prepared by denaturation in loading buffer[16] at 90 °C for 1 min. One μL aliquots were typically applied to the gel, electrophoresed, and developed with Coomasie blue using procedures described in the Phast system manual.

Protein assays were performed by the method of Lowry *et al.*[7] or by the method of Bradford[17] using the commercially available dye (Bio-Rad Protein Assay Kit I, no. 500-0001, Bio-Rad, Richmond, CA). Concentrations were determined by comparison to standard curves using bovine serum albumin (fraction V).

Antibody Kinetics and Product Analysis. UV kinetic measurements were determined using a Varian Cary Model 2200 grating spectrophotometer equipped with a constant temperature cell held at 37 °C throughout all measurements. Velocities were determined spectrophotometrically by measuring the initial absorbance increase (<1% substrate depletion) at 330 nm: **1**, λ_{max} = 282 nm (3.99); **2**, λ_{max} = 312 nm (4.43); $\Delta\epsilon$ **2**–**1** (330 nm) = 16 820 cm^{-1} M^{-1}. The concentration of 43D4-3D12 was 2.00 μM as determined by absorbance at 280 nm using ε (1 cm, 0.1%) = 1.37 and M_W = 150 000 for IgG. 43D4-3D12 was preincubated at 37 °C in 10 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (bis-Tris), 100 mM NaCl, pH 6.0. The reaction was initiated by adding 10 μL of a stock solution of **1** in CH_3CN to give a final CH_3CN concentration of 2%. The uncatalyzed reaction rate was measured under the same conditions. Inhibition experiments were carried out with 2 μM antibody and at two concentrations of **1**. Rate constants (k_{obs}) were obtained by dividing by the concentration of antibody combining sites (2 × [IgG]). Background rates (k_{uncat}) were determined in the absence of antibody under otherwise identical conditions. The Michaelis–Menten parameters k_{cat} and K_M were obtained from either Eadie–Hofstee plots[18] of v_{obs}–v_{uncat} (ordinate) *vs* (v_{obs}–v_{uncat})/[substrate] (abscissa), where k_{cat} is the *y*-intercept and $-K_M$ is the slope or from a Lineweaver–Burk double reciprocal plots of $1/v_{obs}$–v_{uncat} (ordinate) *vs* 1/[substrate] (abscissa) where the *y*-intercept is $1/V_{max}$ and the *x*-intercept is $-1/K_M$. Inhibition constants (K_i) were obtained from Dixon[19] plot analyses of $1/v_{obs}$–v_{uncat} (ordinate) *vs* total inhibitor concentration (abscissa) where $-K_i$ is obtained from the intercept of a vector drawn from $1/V_{max}$ or $1/k_{cat}$. Reaction products were analyzed by extracting reactions with ethyl acetate (50 μL). Two μL aliquots were applied to a DB-1 25 m capillary GC column equilibrated at 120 °C. The *trans*-alkene, **2**, eluted at t_r = 11.4 min using a linear gradient of 120–280 °C at 15°/min. The pH dependence of the antibody-catalyzed reaction was measured in 10 mM bis-Tris, 100 mM NaCl. Antibody was assayed for cis–trans isomerization activity by incubating *cis*-**2** (1 mM) at 37 °C at pH 6.5 in 10 mM bis-Tris, 95 mM NaCl, with or without antibody (10 μM). After 21.5 h, a 100-μL aliquot of the reaction solution was applied to reverse-phase HPLC (Microsorb 5 μm C18, 4.6 mm i.d. × 25 cm long). Peaks were eluted with a linear gradient of 35–45% CH_3CN in aqueous triethylammonium acetate, pH 7.5, over 20 min. Retention times for *cis*- and *trans*-enone **2** were 9.5 and 8.6 min, respectively. Both reactions gave the cis–trans ratio of approximately 73:27 (peak area ratio at 300 nm, no correction was made).

Affinity Labeling/Peptide Mapping of 43D4-3D12. A 4 mg/mL solution of 43D4-3D12 in 10 mM sodium phosphate and 100 mM sodium chloride, pH 7.5, was treated with 33 μL of an 18.3 mM stock solution of **8** affording a final concentration of 604 μM. Incubation at 37 °C for 24 h was followed by removal of unreacted epoxide by gel filtration using a Nap-10 column (Pharmacia) equilibrated with 5 M guanidinium chloride 0.1 M Tris pH 7.0. The specific activity of the excluded protein fractions was measured to be 25.3 mCi/mmol. This material was treated with 20 mM dithiothreitol containing 5 mM ethylenediaminetetraacetic acid for 2 h at 25 °C followed by addition of 1.7 μL of freshly distilled 4-vinylpyridine and incubation for 30 min at 25 °C.

The denatured, reduced, and vinyl pyridine blocked protein was then dialyzed overnight against a solution of 5 M guanidinium hydrochloride and 1 M propionic acid. The heavy and light chains were separated using an FPLC Superose 12 10 mm × 30 cm column equilibrated with 5 M guanidinium hydrochloride and 1 M propionic acid. One hundred and fifty μL aliquots of the labeled protein were applied to the column and eluted at a flow rate of 0.4 mL/min. The radioactive fractions were pooled and concentrated by vacuum dialysis against 10 mM bis-Tris and 100 mM NaCl, pH 6.0, affording 1 mL of protein solution with a protein concentration of 0.47 mg/mL. The specific activity of the labeled heavy chain was calculated to be 7.6 mCi/mmol. The protein was then dialyzed against water, and 100 μL aliquots were lyophilized to afford a fluffy white material which could be stored for long periods at −80 °C. The lyophilized protein was dissolved in 90 μL of 0.1 M Tris 5 mM calcium chloride and 2 M urea and treated with 20 μg of sequencing grade trypsin (Boehringer–Mannheim) in 10 μL of aqueous 0.01% trifluoroacetic acid and incubated at 37 °C for 2 h. The digested protein (100 μL) was applied to a Microsorb 5 μm 300 Å C8, 25 cm bed, 4.6 mm i.d. column equilibrated with ddH_2O containing 0.1% TFA and eluted with a linear gradient of 0–37.5% acetonitrile with 0.08% TFA in ddH_2O with 0.1% TFA over 60 min followed by 37.5–60% acetonitrile with 0.08% TFA over 30 min at a flow rate of 0.5 mL/min. The eluent was monitored at 216 and 280 nm and 500-μL fractions were collected, Figure 5A. Ten μL aliquots were removed for scintillation counting, and the fractions containing radioactivity were pooled, Figure 5B.

A 100-μL aliquot of the pooled radioactive fragments was treated with 13 μL of 1 M NH_4OAc, pH 9.0, for 2 h, at 37 °C. The reaction solution was applied to the same C8 column used above equilibrated with 5% acetonitrile containing 0.08% TFA in ddH_2O containing 0.1% TFA eluting with a linear gradient of 5–35% acetonitrile with 0.08% TFA over 40 min at a flow rate of 0.5 mL/min, Figure 6A. The new peak at t_r = 33 min was not radioactive and had the expected mobility properties associated with loss of the hydrophobic nitrophenyl radioactive label. The fractions containing this material were pooled and concentrated to a final volume of 10 μL on a Savant speed-vac. The residue was then applied to Applied Biosystems Model 473A Protein Sequencer.

Chemical Modification by Diazoacetamide. Diazoacetamide (DAA) was prepared according to the procedure described by Wilcox.[20] Inactivation of antibody 43D4-3D12 by treatment with DAA was

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(20) Wilcox, P. E. *Methods Enzymol.* **1976**, *100*, 605.

performed according to the procedure of Grossberg and Pressman.[2] Antibody 43D4-3D12 (500 μL at 15.6 μM) in 0.15 M $NaClO_4$ and 20 mM borate at pH 8 was treated with two drops of *n*-octanol, 25.0 mg of DAA, and 13 μL of 0.15 M $HClO_4$ affording a pH of 5 in the presence and absence of 1 mM *p*-nitrobenzyl acetone (used to block the combining site). In a third vial a control containing 500 μL (15.6 μM) of 43D4-3D12 in 0.15 M $NaClO_4$ and 20 mM borate at pH 8, two drops of *n*-octanol, and 13 μL of 0.15 M $HClO_4$ (final pH 5) was prepared. The solution was stirred 25 °C for 1.5 h and centrifuged to remove precipitated protein. The supernatant was filtered through a 0.22 μm filter and applied to a Pharmacia fast desalting 10/10 column equilibrated with 10 mM bis-Tris 100 mM NaCl, pH 6.0, at a flow rate of 1 mL/min. The eluent was monitored at 280 nm, and the excluded protein peak was collected. The three reaction solutions were then assayed for catalytic activity according to the procedures described above. The control lacking DAA was used to define 100% activity which was measured to be 3.41×10^{-7} M/min (*E*)-4-(4′-nitrophenyl)-2-oxo-3-butene formation at a concentration of 1.5 μM 43D4-3D12 (determined by A_{280}) and 750 μM (4*R*,4*S*)-fluoro-4-(4′-nitrophenyl)butan-2-one at 37 °C. The catalytic activities of the antibody solutions lacking the inhibitor *p*-nitrobenzyl acetone and containing the inhibitor measured under the same conditions were 7.97×10^{-8} M/min (23%) and 2.79×10^{-7} M/min (82%), respectively.

Results and Discussion

Synthesis and Hybridoma Production. Hapten **3** was designed to induce an antibody combining site with an active site carboxylate side chain positioned to catalyze the elimination of HF by acting as a general base. In order to insure that antibodies generated against positively charged hapten **3** would have reasonable affinity for substrate **1**, the nitrophenyl group was introduced as a common recognition element. By conjugating hapten **3** through the aliphatic side chain it was anticipated that the carbonyl group of substrate **1** would be solvent exposed and relatively free to rotate into conjugation with the incipient Cα carbanion. The change in hybridization in the α and β carbons during the elimination reaction was expected to minimize product inhibition.

Synthesis of hapten **3** was carried out using two successive reductive aminations. Treatment of *p*-nitrobenzaldehyde with δ-aminovaleric acid and sodium cyanoborohydride afforded *N*-(*p*-nitrobenzyl)-δ-aminovaleric acid which was then treated with formaldehyde and sodium cyanoborohydride to yield hapten **3**. Substrate **1** was synthesized by treatment of *p*-nitrobenzaldehyde with a catalytic amount of 1% aqueous sodium hydroxide in acetone at 0 °C to give (*R*,*S*)-4-hydroxy-4-*p*-nitrophenylbutan-2-one, **1**. Treatment of this alcohol with diethylaminosulfur trifluoride in methylene chloride at −78 °C and aqueous extraction afforded the racemic substrate.

Small non-peptidyl molecules such as hapten **3** are not immunogenic unless they are first coupled to a carrier protein. A commonly used carrier is keyhole limpet hemocyanin (KLH). Hapten **3** was coupled to KLH and bovine serum albumin (BSA) via amide linkages with the ε-amino groups of surface lysine residues using the activated *N*-hydroxysuccinimide ester. The tertiary nitrogen of the hapten obviates competing lactam formation during the coupling reaction. The BSA conjugate of **3** was used in the screening of antibodies in enzyme linked immunosorbant assays (ELISAs). The ratio of hapten/carrier monomer (epitope density) has been found to be important for generating a robust immune response. Epitope densities of between 4 and 15 are considered optimal for most haptens.[21] The epitope densities of KLH and BSA conjugates were 18 and 14, respectively, as determined by measuring the number of free lysine residues remaining following conjugation.[8]

Monoclonal antibodies were elicited against hapten **3** using standard hybridoma technology.[22] Briefly, Balb/c mice were injected intraperitoneally with the hapten carrier conjugate, KLH-**3**, emulsified in complete Freund's adjuvant. Several rounds of immunization using incomplete Freund's adjuvant were performed to increase the immune response and enrich for high affinity antibody production. Mice were hyperimmunized immediately prior to fusion by intravenous injection of KLH-**3** in the absence of adjuvant. Spleen cells were fused with an immortalized nonantibody producing myeloma cell line (SP2/O cells) in a solution of 50% polyethylene glycol (PEG). Although typical PEG fusions (using 5×10^8 spleen cells from a hyperimmunized mouse) generally result in 20–50 specific hybridomas, a total of only six antibody secreting hybridomas were isolated specific for hapten **3**. Antibodies elicited against KLH-**3** were purified by affinity chromatography on protein A-coupled Sepharose 4B.[15] Antibodies were determined to be ≥95% homogeneous by 10–15% SDS polyacrylamide gell electrophoresis with Coomasie blue staining.

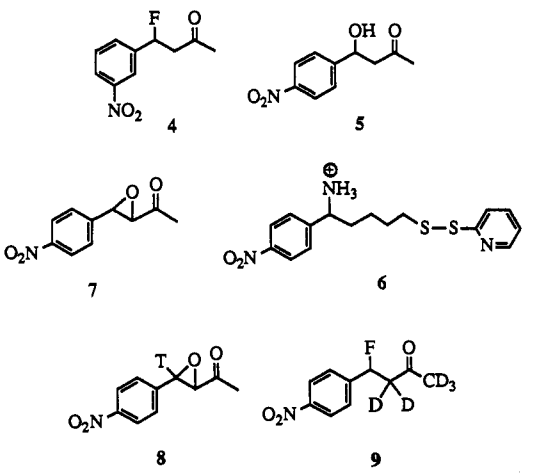

Figure 2. Structures of alternative substrates, hapten, inhibitors, and affinity labels.

Kinetics, Inhibition, Specificity, and pH Dependence of Antibody Catalysis. From a panel of six monoclonal antibodies specific for hapten **3**, four were found to catalyze the elimination of HF from substrate **1** as determined spectrophotometrically by an increase in absorbance at 330 nm. Of these four catalytic IgGs one, 43D4-3D12, possessed significantly greater catalytic activity and was characterized in greater detail. The reaction catalyzed by 43D4-3D12 was shown to obey classical Michaelis–Menten kinetics: a k_{cat} of 0.193 min^{-1} and a K_M of 182 μM for substrate **1** were measured at pH 6.0. The rate constant for acetate-catalyzed conversion of **1** to **2**, k_{OAc^-}, is 1.13×10^{-2} M^{-1} min^{-1} at 37 °C in 100 mM NaCl. The k_{cat}/K_M value of the antibody-catalyzed reaction at pH 6 is 9.95×10^2 M^{-1} min^{-1}.

The catalyzed reaction is competitively inhibited by hapten **3** (K_I = 290 nM) demonstrating that the catalytic activity is associated with binding in the antibody combining site. An unreactive substrate analog, *p*-nitrobenzylacetone, is also a competitive inhibitor of the catalyzed reaction (K_I = 280 μM). As expected, the antibody-catalyzed reaction is substrate specific, in accordance with the characteristic specificity of antibodies for their ligands. The K_M and k_{cat} values for 4-fluoro-4-*m*-nitrophenylbutan-2-one, **4**, are 571 μM and 0.079 min^{-1}, respectively ($k_{cat}/K_M = 1.38 \times 10^2$ M^{-1} min^{-1}), at pH 6.50, compared with a k_{cat} of 0.304 min^{-1} and K_M of 214 μM ($k_{cat}/K_M = 1.42 \times 10^3$ M^{-1} min^{-1}) for **1** at this pH. These values suggest that the antibody discriminates between the corresponding transition states for the elimination reactions of **1** and **4** more so than the ground states.

Antibody 43D4-3D12 does not catalyze the corresponding dehydration reaction of **5**, presumably due to its lack of a general acid to protonate the OH-leaving group. This lack of catalytic activity is not merely due to poor binding because substrate **5** does bind to 43D4-3D12 as measured by fluorescence quenching with a K_D of 4.7 mM. However, it has been shown that an antibody generated against hapten **6**, which mimics the oxonium ionlike

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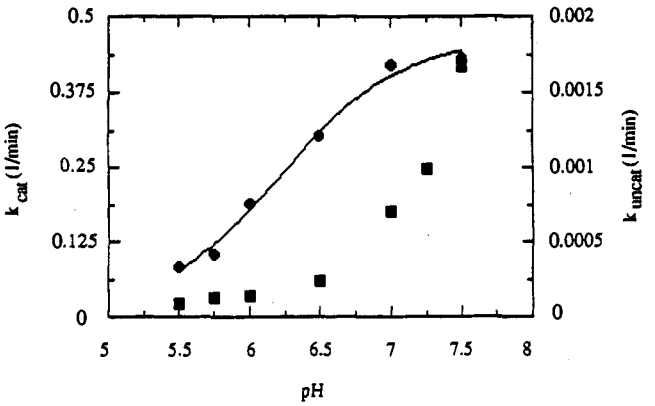

Figure 3. The k_{cat} versus pH profile for the conversion of **1** to **2** catalyzed by antibody 43D4-3D12: (circles) k_{cat} and (squares) k_{uncat}. At each pH the k_{cat} value was obtained from a Lineweaver–Burke plot. The buffer used was 10 mM bis-Tris, in the presence of 100 mM NaCl for both the antibody catalyzed and background reactions.

transition state formed in the acid-catalyzed elimination reaction, catalyzes the elimination of H_2O from substrate **5**.[2e] Consequently, a hapten which combines the features of both haptens **3** and **6** might elicit an antibody containing an active site catalytic dyad.

The pH dependence of k_{cat} shows the classical profile for catalysis attributable to a single titratable group (Figure 3). The pK_a for the active site residue responsible for catalysis was determined by fitting the experimental data to the following equation: $k_{app} = k_0(10^{pK_{EH}-pH} + 1)$ where k_{app} is the apparent k_{cat} at a given pH, k_0 is the maximum k_{cat} achieved by the antibody when the catalytic base is fully deprotonated, pK_{EH} is the pK_a of the catalytic base, and pH is the $-\log[H^+]$. The assumption is made that when the catalytic base is fully protonated $k_{cat} = 0$. This assumption is reasonable since the antibody-catalyzed reaction is essentially unmeasurable below pH 5.5. The experimental data have a correlation coefficient of $R = 0.996$ with $k_0 = 0.47$ min^{-1} and $pK_{EH} = 6.20$. This elevated pK_a value for a carboxylate group is consistent with the loss of a salt-bridge interaction on substitution of the positively charged hapten by the neutral substrate, demonstrating that hapten design may also be used to modulate basicity. This pK_a is very close to the pK_a for the active-site Glu 135 in carboxypeptidase A ($pK_a = 6.5$), which is known to be responsible for the catalysis of a similar β-elimination reaction (not the physiologically relevant reaction for this enzyme).[23] The value is also very similar to that found for the residue responsible for catalysis of an ester hydrolysis reaction ($pK_a = 6.26$) elicited against a pyridinium containing hapten.[2b] The pH dependence of k_{uncat} is also shown in Figure 3. The exponential increase in the rate of the uncatalyzed reaction above pH 6.5, in contrast to the antibody-catalyzed reaction, suggests the major contributor to k_{uncat} in this pH range is k_{OH^-}.

Chemical Modification and Peptide Mapping. The initial chemical modification experiments of 43D4-3D12 with diazoacetamide (DAA) suggested the importance of a combining site carboxylate in catalysis.[25] Modification of the antibody with DAA in the absence of inhibitor led to a 77% loss of catalytic activity; 82% activity was retained in the presence of inhibitor. Diazoacetamide nonspecifically modifies solvent accessible carboxylates by forming glycolamide esters. In order to identify the specific carboxylate group involved in proton abstraction the antibody was treated with an affinity labeling reagent, epoxide **7**. Ring opening of epoxides by asparatate and glutamate affords a base labile ester, whereas attack by cysteine affords a thiol ether. Epoxide **7** was synthesized by treatment of enone **2** with anhydrous *tert*-butyl hydroperoxide and a catalytic amount of Triton B in toluene.[9]

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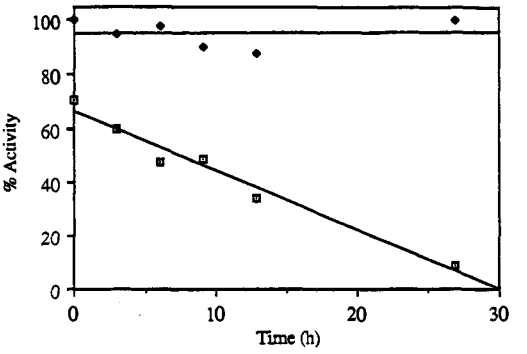

Figure 4. The percent activity versus time for inactivation of 43D4-3D12 by epoxide **7**: (closed diamonds) control without **7** and (open squares) 2 mM **7**. Activity is defined as $(v_{sample} - v_{background})/v_{unmodified}$ 43D4-3D12.

Treatment of antibody 43D4-3D12 with 1 mM *trans*-epoxide **7** (10 mM, bis-Tris, pH 6.5, 100 mM NaCl) led to a time dependent loss of activity (Figure 4). In the presence of the competitive inhibitor, *p*-nitrobenzylacetone, no labeling occurred, confirming that the modified residue is in the antibody combining site. After exhaustive dialysis following treatment with the affinity label, no catalytic activity was recovered, indicating irreversible covalent modification of the antibody. In order to distinguish between thiol ether and ester formation the inactivated antibody was dialyzed against an aqueous 0.5 M ammonium hydroxide solution at pH 9.0. Following exhaustive dialysis to remove any noncovalently bound affinity label, all catalytic activity was recovered. The recovery of activity under mildly basic conditions strongly suggests a carboxylate group was modified in the antibody combining site.

In order to precisely identify the carboxylate residue responsible for catalysis by 43D4-3D12 a peptide mapping experiment was performed. Radiolabeled epoxide **8** was synthesized by reduction of *p*-nitrobenzaldehyde with sodium borotritide followed by oxidation with MnO_2 to afford tritated *p*-nitrobenzaldehyde. Base-catalyzed condensation with chloroacetone in ethanol afforded the desired tritiated affinity label with a specific activity of 13.4 mCi/mmol. Treatment of antibody 43D4-3D12 with the tritiated epoxide followed by removal of unreacted label by gel filtration afforded labeled antibody with a specific activity of 25.3 mCi/mmol, corresponding to two affinity labels per antibody molecule. Since each antibody molecule contains two combining sites, this result is consistent with the modification of one active site residue. The antibody was then reduced with dithiothreitol, alkylated with vinylpyridine, and the heavy and light chains were separated by gel filtration. All of the radioactivity eluted in the peak corresponding to the heavy chain, consistent with one specific residue being covalently modified by tratment with affinity label **8**.

The heavy chain was subjected to trypsin digestion, and radiolabeled peptides were purified by reverse-phase HPLC. Attempts to microsequence the radioactive fractions ($t_r = 74$ min) directly from the trypsin digest were unsuccessful due to the presence of several peptides which were inseparable under a wide variety of HPLC conditions. However, hydrolysis of the ester linkage to the radioactive label resulted in separation of the labeled peptide from the contaminating peptide fragments (Figure 6). The peak at $t_r = 33$ min had the expected mobility properties associated with loss of the hydrophobic nitrophenyl radioactive label. The fractions containing this material were pooled, concentrated, and microsequenced (Table 1). The peptide sequence has one ambiguity at position 50. The phenylthiohydantoin derivative of this residue could not be unambiguously assigned. However, its retention time using a C_8 reverse phase silica column ruled out it being a highly polar residue. The peptide contains only one acidic residue, glutamate at position 46 in the

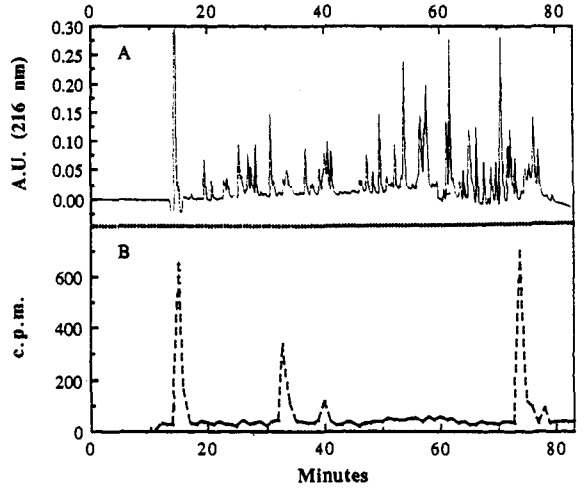

Figure 5. (A) HPLC analysis of tryptic digest of 43D4-3D12 heavy chain. The tryptic digest was applied to a Microsorb 5 μm 300 Å C8, 25 cm bed, 4.6 mm i.d. column equilibrated with H_2O containing 0.1% TFA and eluted with a linear gradient of 0–37.5% acetonitrile with 0.08% TFA over 60 min followed by 37.5–60% acetonitrile with 0.08% TFA over 30 min at 0.5 mL/min. The eluent was collected over 30 min at 0.5 mL/min. The eluent was collected in 250-μL fractions and monitored at 216 nm. (B) A 10-μL aliquot of each fraction was added to 5 mL of scintillation fluid (Ecolume TM, ICN Biomedical) and counted using an LKB Luminometer Model no. 1209–405.

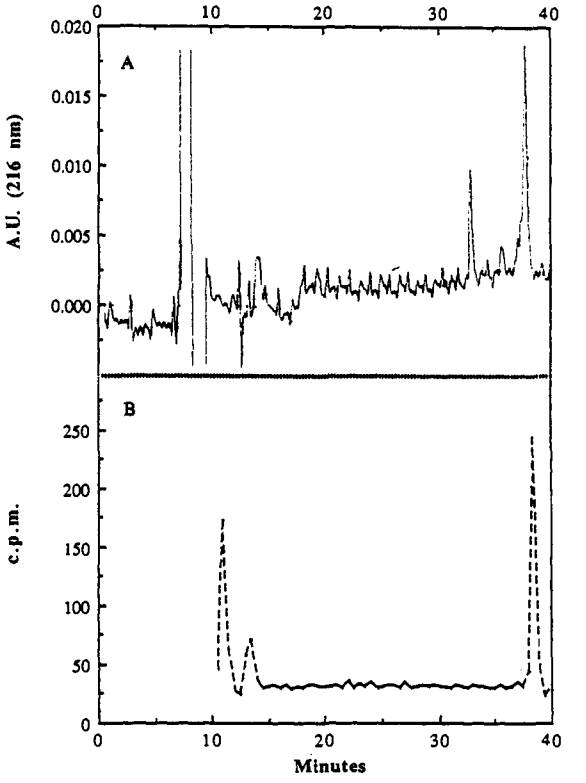

Figure 6. (A) HPLC analysis of base treated tryptic fragment isolated in fractions 74 and 75 (shown in Figure 5) of total heavy chain tryptic digest. A 100-μL aliquot of pooled fractions 74 and 75 was treated with 13 μL of 1 M NH_4OAc, pH 9.0, for 2 h at 25 °C. This solution was applied to the same C8 column equilibrated with 5% acetonitrile containing 0.08% TFA in ddH_2O containing 0.1% TFA eluting with a linear gradient of 5–35% acetonitrile with 0.08% TFA over 40 min at a flow rate of 0.5 mL/min. All other conditions were identical to those in Figure 5A. (B) Scintillation counting as described in Figure 5B, except 0.25-mL fractions were collected.

framework region. This residue can be assigned to be the catalytic residue responsible for proton abstraction in the β-elimination reaction of **1** → **2**. Interestingly, the residues adjacent to this glutamate are quite hydrophobic, three isoleucines and one leucine, perhaps explaining the high pK_a value (6.20) measured for the putative catalytic residue. Analysis of known antibody heavy chain sequences revealed that the other antibodies specific for para-substituted phenyl containing haptens share almost exactly the same framework 2 region and two additional identical residues in the complementary determining region 2 (Ile 51 and Pro 53) with 43D4-3D12.[24] Structural studies of antibodies which bind steroids indicate that residues in framework 2 of the heavy chain do contact the bound ligand.[25]

Kinetic Isotope Effect Studies. Many enzymes catalyze elimination reactions which involve removal of a proton α to a carbonyl carbon. A recent comprehensive survey suggests that in general these enzymes proceed via stepwise E1cB mechanisms involving an enol intermediate.[26] In an attempt to compare the antibody-catalyzed elimination reaction to its enzymatic counterparts, kinetic isotope effect studies were carried out using the deuterated substrate **9**. A Lineweaver–Burke plot of the 43D4-3D12 catalyzed elimination of **1** and **9** shows no isotope effect on K_M but rather an effect on k_{cat} as expected (Figure 7). The kinetic isotope effect on k_{cat} for the antibody-catalyzed reaction is $k_{catH}/k_{catD} = 2.35$. For the acetate-catalyzed background reaction the k_H/k_D is 3.7. The value of $k_{catH}/k_{catD} = 2.35$ for the antibody-catalyzed reaction is consistent with either an E2 or an E1cB transition state. Due to the instability of substrate **1**, we were unable to analyze unreacted substrate for Cα proton exchange when the reaction was carried out in D_2O. *p*-Nitrobenzylacetone, which lacks the β fluoride binds to antibody 43D4-3D12 with a K_D of 1.1 mM. When this substrate was incubated with 43D4-3D12 in D_2O for several days, no deuterium was incorporated into the α-methylene carbon.

Stereoselectivity. The chiral nature of the antibody combining site might be expected to result in some degree of stereoselectivity in the antibody-catalyzed elimination reaction.[27] To test whether the elimination of HF occurs in a stereoselective fashion, the four C3 monodeuterated diastereomeric analogs of substrate **1** were synthesized. Analysis of the antibody-catalyzed elimination of substrates **10–13**, Figure 8, with respect to product stereochemistry and deuterium content should reveal the syn/anti preference of the antibody as well as stereochemistry of proton abstraction and fluoride elimination. The monodeuterated substrates were prepared in seven steps (Figure 9). Alkylation of lithium phenyl acetylide with acetaldehyde afforded the corresponding propargyl alcohol which was reduced with lithium aluminum deuteride to give the *trans*-olefin **14** specifically deuterated at the C3 position.[28] Kinetic resolution of **14** by catalytic asymmetric epoxidation according to the method of Sharpless afforded epoxy alcohol **15**[13] and its enantiomer **16**. The enantiomeric purity of the epoxy alcohols was determined to be >98% by formation of the corresponding Mosher's esters and analysis by ^{19}F NMR. Epoxy alcohol **15** was then treated with Red-Al, affording an inseparable mixture of the 2,3- and 2,4-diols. Similarly, treatment of the enantiomeric epoxy alcohol **16** with Red-Al afforded 2,3- and 2,4-diols. The undesired 2,3-isomer in each case was removed by treatment with $Pb(OAc)_4$ in benzene. Each of the two enantiomeric diols **17** and **18** was then treated with diethylaminosulfur trifluoride in methylene chloride at −78 °C to afford an inseparable mixture of the corresponding diasteriomeric fluoro alcohols. The two mixtures were nitrated by treatment with cupric nitrate in trifluoroacetic anhydride.[10] The diasteriomeric trifluoroacetate products were separable by preparative thin-layer

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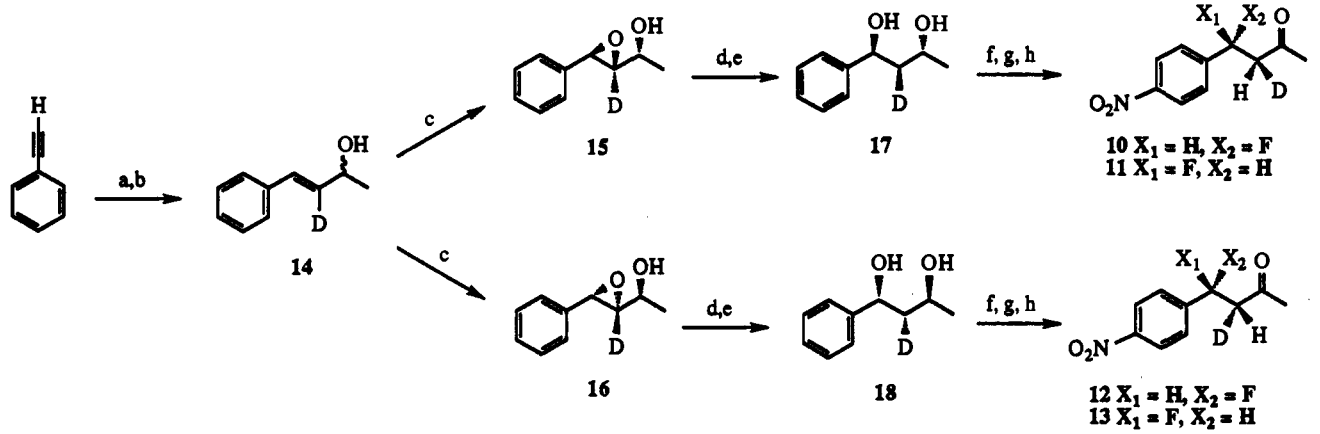
Table 1. Comparison of Peptides Sequences from 43D4-3D12, 48G7 (*anti-p*-Nitrophenyl Phosphonate), 20G9 (*anti*-Phenylphosphonate), and Consensus *anti-p*-Azophenyl Arsonate[a]

	FR2											CDR2				
	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
43D4-3D12	Q	R	P	G	H	I	L	E	I	I	G	X	I	L	P	R
anti-p azo	–	–	–	–	Q	G	–	–	W	–	–	Y	–	Q	–	–
48G7	–	–	–	K	–	–	–	–	–	–	–	R	–	D	–	A
20G9	–	–	–	–	Q	G	–	–	W	–	–	R	–	H	–	S

[a] The region sequenced contains sequences derived from framework 2 (FR2) and complementary determining region 2 (CDR2) of the heavy chain (dashes indicate the occurrence of identical residues).

Scheme 1[a]



[a] (a) *n*-BuLi, CH_3CHO, THF, 0 °C, 43%; (b) $LiAlD_4$, THF, 0 °C, 98%; (c) D-(−)-DIPT (synthesis of **15**) *or* L-(+)-DIPT (synthesis of **16**), 3 Å powdered molecular sieves, CH_2Cl_2, $Ti(O\text{-}i\text{-}Pr)_4$, *tert*-butyl hydroperoxide, CH_2Cl_2, 65% (**15**), 63% (**16**); (d) Red-Al, THF, 25 °C; (e) $Pb(OAc)_4$, PhH; 35% (**17**) for two steps, 56% (**18**) for two steps; (f) DAST, CH_2Cl_2, −78 °C, 12% (**21** and **22**), 21% (**23** and **24**); (g) $Cu(NO_3)_2$, TFAA, $CHCl_3$, 25 °C, K_2CO_3, CH_3OH, 21% (**25**), 23% (**26**), 26% (**27**), 26% (**28**); (h) PCC-alumina, PhH, 25 °C, 84% (**10**), 76% (**11**), 81% (**12**), 67% (**13**).

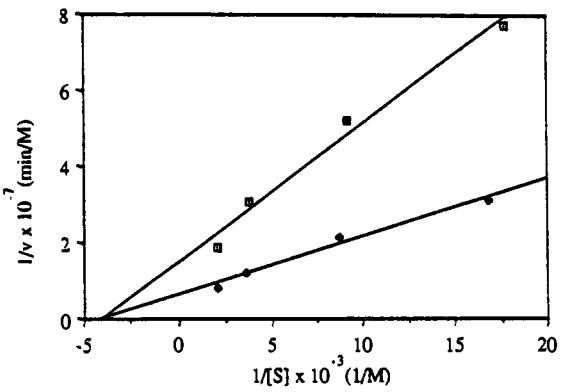


Figure 7. Lineweaver–Burke plot of 43D4-3D12 catalyzed HF elimination of substrate **1** (closed diamonds) and deuterated substrate **9** (squares).

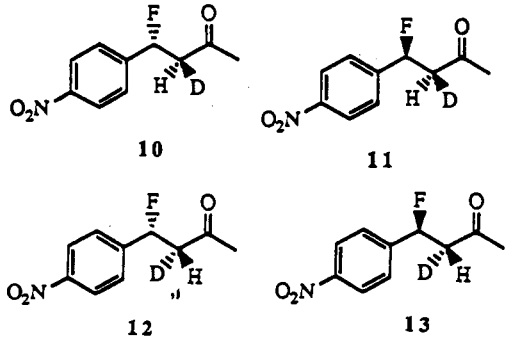


Figure 8. Monodeuterated substrates **10–13**.

chromatography on silica. The trifluoroacetate esters were hydrolyzed by treatment with methanol and potassium carbonate. Oxidation by treatment with pyridinium chlorochromate (PCC) adsorbed on alumina afforded substrates **10–13** with no contaminating starting materials or undesired side products.

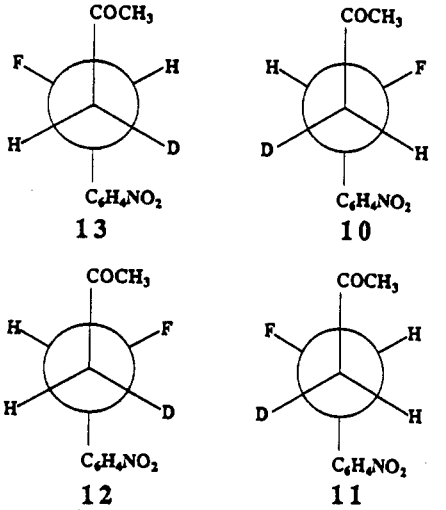


Figure 9. Neuman projections of substrates **10–13**.

Each of the four isomeric substrates **10–13** (1.2 mM) was incubated both with and without antibody 43D4-3D12, and the reaction products were extracted into ethyl acetate and analyzed by GC–MS. Product analysis indicated that no *cis*-alkene product was formed in any of the reactions. Controls showed that antibody 43D4-3D12 does not catalyze the cis–trans isomerization of enone **2**. The deuterium content of the products was then determined by mass spectrometric analysis comparing the ratio of the *m/e* 176 and *m/e* 177 fragments which are generated by loss of the terminal methyl group from enone **2**. The measured ratios were corrected for the isotope effect of antibody-catalyzed proton abstraction using a correction factor developed by Cooke and Coke in their study of Hofmann eliminations.[29] In this case the correction method becomes $k_D/(k_{catH}/k_{catD}) + k_H = 100$, where k_D represents the rate constant for removal of the deuteron, k_H

(29) Cooke, M. P.; Coke, J. C. *J. Am. Chem. Soc.* **1968**, *90*, 5556–5561.

Table 2. GC–MS Product Analysis of Background and Antibody 43D4-3D12 (3 mg/mL) Catalyzed β-Elimination Reaction in 10 mM bis-Tris, 100 mM NaCl pH 6.0

	m/e %176:%177	
substrate	background	43D4-3D12 catalyzed
10	32%:68%	55%:45% (99%:1%)[a] (anti:syn)
13	33%:66%	43%:57% (99%:1%)[a] (anti:syn
11	22%:78%	20%:80% (47%:53%)[a] (syn:anti)
12	24%:76%	13%:87% (30%:70%)[a] (syn:anti)

[a] Calculated syn:anti ratios corrected for k_{catH}/k_{catD}.[29]

represents the rate constant for removal of the proton, and k_{cat}/k_{catD} represents the primary kinetic isotope effect on k_{cat}. The correction factor (k_{catH}/k_{catD}) assumes that the deuterium isotope effects on the syn and anti elimination processes are similar. Since in acyclic systems one or the other process predominates, this assumption is difficult to experimentally confirm. The corrected ratios of products from the antibody-catalyzed reaction of substrates **10–13** are given in Table 2.

Antibody 43D4-3D12 does not catalyze cis/trans isomerization of **2**. In addition, no *cis*-olefin is formed in the antibody-catalyzed or the background eliniation of **1**. These results indicate that the antibody-catalyzed β elimination occurs from the conformers depicted in Figure 9. From the corrected ratios of syn:anti elimination processes shown in Table 2 it is clear that the antibody favors the anti elimination process with each of the four possible monodeuterated diastereomer substrates. This stereochemical course for the antibody-catalyzed reaction sets its mechanism apart from that of the naturally occurring enzymes which catalyze β elimination reactions of aldehydes, ketones, and esters. These enzyme catalyzed transformations all proceed via the syn stereochemical course.[26] They involve a single catalytic general base that both abstracts a proton and then delivers the same proton to the leaving group on the same side of the C–C bond, resulting in a syn elimination. In the 43D4-3D12 catalyzed elimination, protonation of F^- is not required and thus syn elimination would not be expected to be favored.

With respect to the preference of antibody 43D4-3D12 for either the proR or proS hydrogen, it appears that the antibody possesses little stereofacial selectivity. The fact that the catalytic Glu-46H is able to abstract either prochiral hydrogen from the substrate is consistent with the alkyl side chain being relatively free to rotate in the antibody combining site as expected from the site of conjugation of hapten **3** to KLH. Alternatively, the substrate alkyl chain could be rigidly held in the combining site, and the catalytic carboxylate could be in a position where it bridges between the proR and the proS C-α hydrogens.

Rate Enhancement. Chemical derivatization and site-directed mutagenesis have also been used to introduce catalytic groups into antibody combining sites. Specifically, these techniques were used to introduce a nucleophilic thiol[30] and catalytic imidazole group[31] into the combining site of antibody MOPC315 to generate antibodies that hydrolyze activated esters. Comparison of the ratio of k_{cat}/k_{base} gives the effective molarity (the concentration of an external base required to give the same first-order rate constant) for the respective base in the combining site of the antibody. The effective molarities for the His mutant (generated by site directed mutagenesis) and thiol-containing derivative (generated semisynthetically) of MOPC315 are 0.2 and 0.073 M, respectively. These values compare with an effective molarity for the β-elimination reaction catalyzed by 43D4-3D12 of 16.8 M. This difference suggests that charge complementarity is a more effective strategy for introducing catalytic side chains to antibody combining sites. Presumably semisynthetic and mutagenesis approaches may not lead to proper positioning of the base in the active site or in the former case side-chain entropy reduces catalytic efficiency.

Summary

Antibody catalysis of reactions in which the appropriate transition-state analog is not accessible or those which require the involvement of a specific catalytic group have been achieved by exploitation of the principle of hapten-charge complementarity.[2] This strategy was first applied to the generation of an efficient catalyst of HF elimination from **1**.[2a] From the pH rate profile, chemical modification studies, and affinity labeling/peptide mapping studies we have validated our original design strategy by identifying glutamate 46H of 43D4-3D12 as the induced catalytic base. One challenge for the generation of new antibody catalysts is to incorporate several design strategies into one hapten such as transition-state stabilization and antibody-hapten charge complementarity.

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(30) Pollack, S. J.; Nakayama, G. R.; Schultz, P. G. *Science* **1988**, *242*, 1038–40.
(31) Baldwin, E.; Schultz, P. G. *Science* **1989**, *245*, 1104–07.