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Mechanistic Studies of an Antibody-Catalyzed Pericyclic Rearrangement

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Abstract: We report NMR and kinetic studies of antibody AZ-28, which was generated against the diaryl-substituted cyclohexanol derivative **3** and catalyzes the oxy-Cope rearrangement of the corresponding hexadiene **1** to aldehyde **2** (Braisted, A. C., Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2211−2212). Conformational studies of free substrate and the antibody−substrate complex using transferred-NOE experiments demonstrate that the antibody binds the substrate in a cyclic conformation, consistent with the chair-like geometry of **3**. In contrast, free substrate adopts an extended conformation in solution. In spite of the conformational restriction of the bound substrate revealed by NMR, the temperature dependence of the reaction indicates that the antibody functions primarily by lowering $\Delta H^{\ddagger}$, offsetting a decrease in $\Delta S^{\ddagger}$; the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are 15.4 ± 2.4 kcal/mol and −23 ± 8 cal/mol K, respectively. A secondary kinetic isotope effect (k_{cat}H/k_{cat}D) of 0.61 ± 0.1 was measured for substrate **23** in which both allylic termini are dideuterated, indicating that the chemical rearrangement step is wholly or partially rate limiting under saturating conditions. The magnitude of this secondary isotope effect is consistent with a significant degree of bond formation between C1 and C6 of the substrate in the transition state. These results, together with the recently reported three-dimensional crystal structure for the antibody−hapten **3** complex (Ulrich, H.; Mundroff, E.; Santarsiero, B. D.; Driggers, E. M.; Stevens, R. C.; Schultz, P. G. *Nature* **1997**, *389*, 271−275), provide a detailed mechanistic model for this antibody-catalyzed reaction.

Introduction

Despite the importance of pericylic transformations in synthetic and mechanistic organic chemistry, there are few examples of enzymes that catalyze this class of reactions. Of the few enzymes that have been identified, the best studied is chorismate mutase, which catalyzes the Claisen rearrangement of prephenate to chorismate in the shikimate pathway for aromatic amino acid biosynthesis.[3] More recently, a "Diels−Alderase" has been identified, broadening the scope of known enzyme catalyzed pericyclic reactions to include cycloaddition reactions.[4] In view of the small number of natural catalysts for study, the generation and characterization of catalytic antibodies provides an opportunity to investigate the mechanisms by which proteins can utilize binding energy to catalyze this important class of reactions. Indeed, examples of antibody-

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(1) Braisted, A. C.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2211−2212.
(2) Ulrich, H.; Mundroff, E.; Santarsiero, B. D.; Driggers, E. M.; Stevens, R. C.; Schultz, P. G. *Nature* **1997**, *389*, 271−275.
(3) Romero, R. M.; Roberts, M. F.; Phillipson, J. D. *Phytochemistry* **1996**, *40*, 1015−1025.
(4) Laschat, S. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 289−291.

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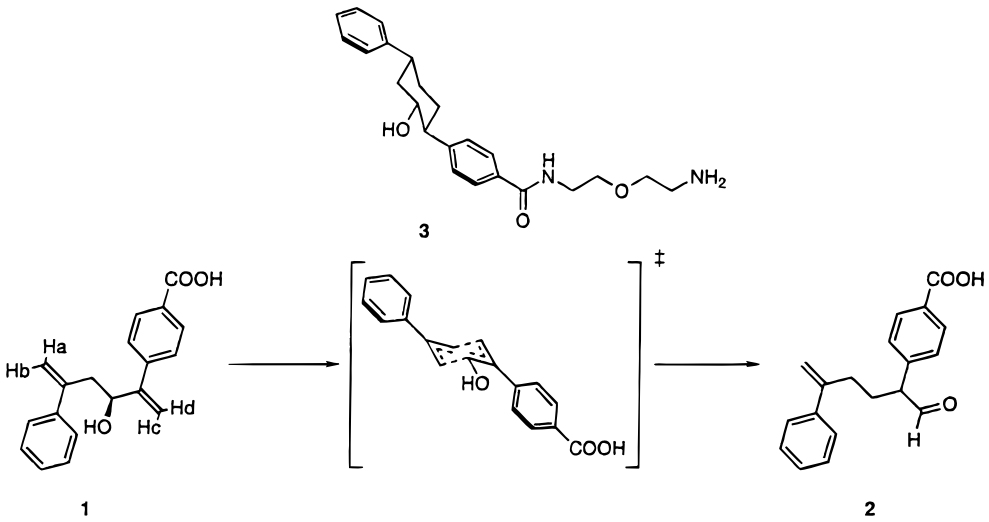


Figure 1. Antibody-catalyzed oxy-Cope rearrangement of substrate **1** to product **2**; structure **3** is the hapten.

catalyzed Diels−Alder reactions,[5,6] Claisen[7,8] and Cope rearrangements,[1] and a [2,3]-sigmatropic rearrangement[9] have been reported.

We have previously shown that antibody AZ-28, which was generated against hapten **3** (Figure 1), catalyzes the oxy-Cope rearrangement of the 2,5-diaryl hexadiene **1** to aldehyde **2** with a rate enhancement (k_{cat}/k_{uncat}) of 5300-fold over the uncatalyzed reaction.[1] More recently, a series of related antibodies and their germline precursors have been characterized, one of which catalyzes the rearrangement with a rate acceleration of 3.0×10^5.[2] In addition, the crystal structure of the AZ-28 Fab-hapten complex **3** has been determined at 2.6 Å resolution.[2] As part of a detailed investigation into the mechanism, structure, and evolution of this catalytic antibody, we now report the results of transferred-NOE (tr-NOE) experiments on the antibody−substrate complex, as well as temperature dependence and kinetic isotope effect measurements. Taken together, this information provides a detailed mechanistic picture of this antibody-catalyzed reaction, in which a combination of binding interactions programmed by hapten **3** control the stereoelectronic course of the rearrangement. Furthermore, insights from this study provide a basis for the design of second generation haptens and directed mutagenesis experiments to generate antibodies with enhanced catalytic properties.

Experimental Section

General Procedures and Methods. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Solvents were dried and distilled under nitrogen using standard procedures. All aqueous solutions were prepared from distilled H_2O purified on a Milli-Q purification system. Unless otherwise noted, all reagents were added to reactions vessels via syringe and stirred under an inert (N_2 or argon) atmosphere. Flash chromatography was performed on Merck Kieselgel 60, 230−400 mesh, and unless otherwise noted, all chromatographic solvent systems were chosen such that the compound of interest eluted with an R_f of approximately 0.25.

NMR spectra for compound characterization were recorded at either 400 or 500 MHz at the UC Berkeley Department of Chemistry NMR facility. Unless otherwise noted, spectra were determined in $CDCl_3$ with tetramethylsilane as an internal standard at 0.00 ppm. Infrared spectra were recorded on a Perkin-Elmer Paragon 500 FTIR or a Mattson Polaris FTIR. Mass spectra were recorded at the UCB mass spectroscopy facility on either a Kratos MS-50 (EI) or VG 70-SE (FAB) mass spectrometer. Elemental analyses were performed by the microanalytical lab at UC Berkeley.

Vinyl Triflate of 4-Phenylcyclohexanone (4). To a solution of 4-phenylcyclohexanone (2.2 g, 12.6 mmol) in 40 mL of tetrahydrofuran at −78 °C was added 27.5 mL of a 0.5 M solution of potassium hexamethyldisilazide in toluene over the course of 10 min. The solution was allowed to warm slowly to room temperature and then stirred for an additional hour. The reaction was then cooled to −78 °C, and a solution of *N*-phenyltriflimide (5.0 g, 14.0 mmol) in 20 mL of THF was added. The reaction was again allowed to warm slowly to room temperature and then stirred for 3 h at which time TLC analysis (10:1 hexanes/ ethyl acetate) showed the reaction to be complete. The solvent was removed in vacuo, and the residue was purified by two rounds of flash chromatography (4 × 20 cm; first, 3% ethyl acetate in hexanes; second, 3% diethyl ether in hexanes) to yield 2.75 g (71.4%) of the desired product **4** as a colorless oil. IR: 1414, 1211, 1142, 1026, cm^{-1}. ^1H NMR (400 MHz): δ 1.90−1.99 (m, 1H), 2.02−2.08 (m, 1H), 2.31−2.35 (m, 1H), 2.38−2.44 (m, 1H), 2.46−2.54 (m, 1H), 2.80−2.84 (m, 1H), 5.83 (dd, 1H, J = 2.0, 2.9 Hz), 7.18−7.24 (m, 3H), 7.31 (d, 2H, J = 5.4 Hz). ^{13}C NMR (101 MHz): δ 27.8, 29.6, 31.5, 41.0, 116.9, 118.0, 126.6, 128.6, 129.9, 144.5, 148.5. LRMS (EI^+): *m/z* 306 (M^+). HRMS: calcd for $C_{13}H_{13}O_3F_3S$ 306.0537, found 306.0535.

4-Trimethylstannane Methyl Benzoate (5). To a solution of methyl 4-bromobenzoate (3.0 g, 14.0 mmol) in toluene (50 mL) at 25 °C was added to 5.0 g (15.4 mmol) of hexamethylditin followed by 1.70 g (1.54 mmol) of tetrakis(triphenylphosphino)palladium(0). The resulting solution was heated at reflux for 5 h, by which time it had turned black. The reaction was allowed to cool to room temperature, and 25 mL of a saturated aqueous potassium fluoride solution was added, followed by 25 mL of hexanes, and the mixture was stirred vigorously at room temperature for 30 min. After filtration through Celite, the resulting pale yellow, biphasic solution was separated, and the organic portion was washed twice with 50 mL of saturated aqueous potassium fluoride and once with 50 mL of brine and then dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 5 × 20 cm silica column (3% ethyl acetate in hexanes) yielding 2.67 (64%) of the product **5** as a colorless oil. IR: 2983, 2950, 1726, 1591, 1434, 1279, 1114, 745 cm^{-1}. ^1H NMR (400 MHz): δ 0.31 (s, 9H), 3.90 (s, 3H), 7.57 (d, 2H, J = 6.8 Hz), 7.97 (d, 2H, J = 7.5 Hz). ^{13}C NMR (101 MHz): δ −9.6, 51.9, 128.4, 129.7, 135.7, 149.5, 167.3.

1-(4-Methyl benzoate)-4-phenyl-1-cyclohexene (6). To a mixture

(5) Braisted, A. C.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 7430−7431.
(6) Hilvert, D.; Hill, K. W.; Nared, K. D.; Auditor, M. T. M. *J. Am. Chem. Soc.* **1989**, *111*, 9261−9262.
(7) Jackson, D. Y.; Jacobs, J. W.; Sugasawara, R.; Reich, S. H.; Bartlett, P. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 4841.
(8) Hilvert, D.; Carpenter, S. H.; Nared, K. D.; Auditor, M. T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4953.
(9) Yoon, S. S.; Oei, Y.; Sweet, E.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 11686−11687.

of LiCl (2.50 g, 58.8 mmol) and tetrakis(triphenylphosphino)palladium-(0) (220 mg, 0.20 mmol) was added a solution of organostannane **5** (2.35 g, 7.84 mmol) in 25 mL of THF followed by a solution of vinyl triflate **4** (2.40 g, 7.84 mmol) in 25 mL of THF. The mixture was heated at reflux for 20 h. After cooling to room temperature, the reaction mixture was diluted with 75 mL of diethyl ether and washed with three 50 mL portions of aqueous 10% NH_4OH and once with 50 mL of brine. The combined aqueous washes were re-extracted once with 50 mL of diethyl ether, and the combined organic layers were then dried over $MgSO_4$, filtered, and concentrated. The crystalline residue was purified by flash chromatography on a 5 × 20 cm column of silica gel (30:1 hexanes/ethyl acetate) to provide 1.10 g (48%) of the desired product **6** as a white crystalline solid (mp 125−130 °C). IR: 1714, 1277, 1109, 766, 754, 700 cm^{-1}. ^1H NMR (400 MHz): δ 1.87−1.97 (m, 1H), 2.12−2.17 (m, 1H), 2.34−2.41 (m, 1H), 2.53−2.63 (m, 2H), 2.86−2.91 (m, 1H), 3.91 (s, 3H), 6.33−6.35 (m, 1H), 7.20−7.35 (m, 5H), 7.47 (d, 2H, J = 8.5 Hz), 7.99 (d, 2H, J = 8.5 Hz). ^{13}C NMR (101 MHz): δ 28.0, 30.2, 34.3, 39.8, 52.2, 125.0, 126.4, 126.8, 127.1, 128.5, 128.7, 130.0, 136.0, 146.7, 146.8, 167.3. LRMS (FAB^+): m/z 293 (MH^+). Anal. Calcd for $C_{20}H_{20}O_3$: C, 82.16; H, 6.90. Found: C, 82.03; H, 6.92.

(1*S*,2*S*,5*S*)-2-(4-Methyl benzoate)-5-phenyl-1-cyclohexanol (7). To a solution of olefin **6** (600 mg, 2.05 mmol) in 20 mL of tetrahydrofuran at 0 °C was added 6.15 mL of a 1 M solution of borane−tetrahydrofuran complex. The solution was allowed to warm to room temperature slowly, and after 14 h, TLC (5:1 hexanes/ethyl acetate) indicated that all of the olefin (R_f = 0.90) had been converted to baseline material. The reaction was cooled to 0 °C, and 3 mL of water was added cautiously, followed by 3 mL of 3 N NaOH and then 3 mL of 30% hydrogen peroxide. After 2 h the reaction mixture was diluted with 50 mL of diethyl ether, washed with 50 mL of water and 50 mL of brine, and then dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a 2.5 × 20 cm column of silica (3:1 hexanes/ethyl acetate) to provide 340 mg (53%) of the product as a mixture of diastereomers (mp 85−92 °C). The diastereomers were separated by preparative TLC: 40 mg of the mixture was applied to a 1 mm × 20 cm × 20 mm silica gel plate, and after repeated elution with 3:1 hexanes/ethyl acetate, two distinct bands were visible. The less polar of the two corresponded to the desired 5*S* diastereomer **7** and was isolated a white solid. IR: 3441, 2929, 2867, 1720, 1610, 1435, 1281, 1113, 757, 701 cm^{-1}. ^1H NMR (400 MHz): δ 1.52−1.76 (m, 4H), 1.98 (dd, 2H, J = 12.6, 3.2 Hz), 2.31 (dd, 1H, J = 12.8, 2.9 Hz), 2.61 (td, 1H, J = 11.0, 3.3 Hz), 2.79 (td, 1H, J = 12.4, 3.0 Hz), 3.90 (s, 3H), 7.20−7.34 (m, 5H), 7.36 (d, 2H, J = 8.3 Hz), 8.01 (d, 2H, J = 8.3 Hz). ^{13}C NMR (101 MHz): δ 32.8, 33.8, 41.8, 42.7, 52.0, 52.6, 74.0, 126.3, 126.7, 127.9, 128.5, 128.7, 130.0, 145.6, 148.6, 166.9. LRMS (FAB^+): m/z 311 (MH^+). HRMS: calcd for $C_{20}H_{23}O_3$ 311.1647, found 311.1645.

(1*S*,2*S*,5*S*)-2-((4-(*tert*-Butoxycarbamoyl)diethylene glycol)benzamide)-5-phenyl-1-cyclohexanol (9). To a solution of the carboxylic acid **7** (38 mg, 128 μmol) in 10 mL of CH_2Cl_2 was added the amine **8** (32 mg, 157 μmol) prepared by reaction of 2-(2-aminoethoxy)ethanol with 2-(((*tert*-butoxycarbonyl)oxy)imino)-2-phenylacetonitrile, followed by 21 mg (157 μmol) of 1-hydroxybenzotriazole and 32 mg (157 μmol) of dicyclohexylcarbodiimide. After 14 h, a thick precipitate had formed, and the reaction mixture was filtered through Celite and concentrated. The residue was purified by flash chromatography (2 × 20 cm, 2% MeOH in CH_2Cl_2) to yield 55 mg (89%) of **9** as a white foam. IR: 3347, 2924, 1690, 1638, 1541, 1507, 1281, 1170, 1120 cm^{-1}. ^1H NMR (400 MHz): δ 1.42 (s, 9H), 1.52−1.74 (m, 3H), 1.98 (br d, 2H, J = 12.4 Hz), 2.32 (br d, 1H, J = 12.7 Hz), 2.79 (t, 1H, J = 12.3 Hz), 3.29 (br d, 2H, J = 5.0 Hz), 3.51 (t, 2H, J = 5.1 Hz), 3.60 (br s, 4H), 3.87 (td, 1H, J = 10.1, J = 2.8 Hz), 5.02 (br s, 1H), 6.81 (br s, 1H), 7.20−7.34 (m, 5H), 7.75 (d, 2H, J = 8.1 Hz). ^{13}C NMR (101 MHz): δ 32.9, 33.8, 39.6, 40.3, 41.9, 42.7, 52.4, 69.6, 70.0, 73.9, 79.3, 126.3, 127.4, 127.9, 128.4, 132.9, 145.6, 147.0, 156.0, 167.4. LRMS (FAB^+): m/z 483 (MH^+). HRMS: calcd for $C_{29}H_{39}N_2O_5$: 483.2859, found 483.2861.

Isothiocyanate Derivative of 9 (10). A solution of **9** (10 mg, 20.7 μmol) in 5 mL of CH_2Cl_2 was stirred with 500 μL of trifluoroacetic acid at 0 °C for 1 h to remove the *tert*-butoxycarbonyl protecting group. The solvent was removed in vacuo, and the residue was purified by preparative TLC (90:10:1 acetone/methanol/ammonium hydroxide) to give 8 mg of the free amine as a colorless oil. The free amine was dissolved in CH_2Cl_2 (5 mL) with 10 μL of diisopropylethylamine. Thiophosgene (1.8 μL, 240 μmol) was added, and the reaction mixture was stirred at room temperature for 5 min, at which time TLC (5% MeOH in CH_2Cl_2) showed a single product to have formed (R_f = 0.75). The solvent was removed in vacuo, and the product **10** was obtained as a colorless oil without further purification. IR: 3415, 2990, 2930, 2706, 2117, 1638, 1132 cm^{-1}. ^1H NMR (400 MHz): δ 1.23−1.58 (m, 2H), 1.71 (t, 2H, J = 12.2 Hz), 1.99 (br d, 2H, J = 12.8 Hz), 2.33 (br d, 1H, J = 12.8), 2.62 (td, 1H, J = 3.3, 12.9 Hz), 2.80 (tt, 1H, J = 3.2, 12.4 Hz), 3.02 (br d, 2H, J = 6.7), 3.58 (br s, 2H), 3.66−3.73 (m, 4H), 3.90 (td, 1H, J = 4.0, 10.7 Hz), 6.72 (br s, 1H), 7.20−7.35 (m, 5H), 7.38 (d, 2H, J = 8.3 Hz), 7.84 (d, 2H, J = 8.3 Hz). LRMS (FAB^+): m/z 425 (MH^+).

Keyhole Limpet Hemocyanin (KLH) and Bovine Serum Albumin (BSA) Conjugates of 10. A solution of the protein (15 mg/mL for BSA, 10 mg/mL for KLH) was separated from any insoluble material by centrifugation at 14 000 rpm for 10 min, followed by filtration through a 0.2 μm filter. The solution was diluted to 5 mL total volume with water, and the pH was adjusted to 8.5−9.0 with 0.1 M NaOH. Dimethylformamide (0.5 mL) was added, followed by slow addition with gentle stirring of 1 mL of a 2 mg/mL solution of isocyanate **10**. Some cloudy precipitate formed during the addition, after which the pH was readjusted to 9.0 with 0.1 M NaOH. Considerably more precipitation occured with the KLH solution. After 3 h, the mixture was diluted slowly with 1 mL of phosphate-buffered saline solution (PBS, 138 mM NaCl, 10 mM NaH_2PO_4, 5 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) and filtered through a 0.45, μm filter. The protein conjugate was then dialyzed exhaustively against PBS. Protein concentration was determined by Bradford assay (based on a molecular weight of 59 000 for BSA, 77 000 for KLH), giving an epitope density of 15 for BSA and 10 for KLH (UV difference spectroscopy at 250 nm; ϵ_{250} (**10**) = 6500 M^{-1} cm^{-1}).

2-(4-Methyl benzoate)-1-propene (12a).[10] Freshly distilled DMSO (48 mL, 617 mmol) was added to NaH (0.713 g, 29 mmol), and the mixture was heated to 80 °C for 45 min, during which time H_2 evolved. The reaction was cooled to room temperature and $CH_3P(Ph)_3Br$ (10.6 g, 29.7 mmol) was added in 10 mL of DMSO; the resulting solution became dark green on stirring. Ketone **11** (2.14 g, 11.9 mmol) in 17 mL of DMSO was added, and the mixture was allowed to react for 20 min, at which time all of the ketone had been consumed. The reaction mixture was washed with 5 × 200 mL of hexanes. Water (30 mL) was added slowly to the hexanes with vigorous stirring, after which the hexanes were washed twice with 200 mL of brine, dried, and then concentrated. The resulting oil was chromatographed on silica gel (7 × 17 cm, 2% ethyl acetate in hexanes) to yield 2.06 g (98%)of **12a** as a white crystalline material (mp 48−51 °C). IR: 1716, 1276, 1123, 1105 cm^{-1}. ^1H NMR (400 MHz): δ 2.16 (s, 3H), 3.91 (s, 3H), 5.19 (s, 1H), 5.46 (s, 1H), 7.51 (d, 2H, J = 8.6 Hz), 7.99 (d, 2H, J = 8.6 Hz). LRMS (EI^+): m/z 176 (M^+). Anal. Calcd for $C_{11}H_{12}O_2$: C, 74.97; H, 6.87. Found: C, 74.84; H, 6.92.

2-(4-Methyl benzoate)-1-propene-*1,1-d*$_2$ (12b). To a well stirred slurry of methyltriphenylphosphonium iodide-d_3 (11.9 g, 29.4 mmol) in 184 mL of THF at 0 °C was slowly added 29 mL of 1 M *n*-butyllithium. The resulting translucent orange solution was stirred at 0 °C for 45 min. A solution of ketone **6** (4.4 g, 24.6 mmol) in 28 mL of THF was added slowly, resulting in a green solution which formed a white precipitate as it warmed to room temperature. After 0.5 h, the reaction was quenched by addition of 200 mL of water and extracted three times with 200 mL of ethyl acetate. The organic solvent was dried over Na_2SO_4 and concentrated into a red gum which was washed with approximately 1 L of hexanes. These washings were concentrated and chromatographed on silica gel (7 × 17 cm, 2% ethyl acetate in hexanes) to give 1.49 g (34%) of **12b** as a white solid (mp 53−56 °C). IR: 3420, 1637 cm^{-1}. ^1H NMR (400 MHz): δ 2.15 (s, 3H), 3.90 (s, 3H), 7.50 (d, 2H, J = 8.5 Hz), 7.98 (d, 2H, J = 8.5 Hz). ^{13}C NMR (101 MHz): δ 21.5, 52.0, 125.4, 128.9, 129.6, 142.2, 166.8,

(10) Alexander, J.; Rao, K. *Ind. J. Chem.* **1973**, *11*, 619.

170.0. LRMS (EI$^+$): *m*/*z* 178. HRMS: calcd for $C_{11}H_{10}D_2O_2$ (M^+) 178.0962, found 178.0966.

2-4-Methyl-*^{13}C* benzoate)-1-propene (12c). Using the procedure described for **12b**, isotopically labeled olefin **12c** was produced from the reaction of ketone **11** with $^{13}CH_3P(Ph)_3I$ in 50% yield as a white solid (mp 53−56 °C). IR: 3417, 1718, 1288, 908 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.15 (d, 3H, J = 7.10 Hz), 3.90 (s, 3H), 5.18 (d, 1H, J = 156.8 Hz), 5.31 (d, ^{1}H, J = 155.7 Hz), 7.50 (d, 2H, J = 8.5 Hz), 7.98 (d, 2H, J = 8.5 Hz). ^{13}C NMR (101 MHz): δ 21.2, 51.6, 114.6, 125.1, 129.3, 130.2, 142.4, 145.3, 166.5. LRMS (EI$^+$): *m*/*z* 177. HRMS: calcd for $C_{10}{}^{13}C_1H_{12}O_2$ (M^+): 177.0871, found 177.0874.

2-(4-Methyl benzoate)-1-propen-3-ol (13a). A solution of *t*-BuOOH (5.5 M in 2,2,4-trimethylpentane, 3.5 mL) was added slowly via syringe to a stirred slurry of SeO_2 (0.538 g, 4.9 mmol) in 8 mL of CH_2Cl_2 at room temperature. The mixture was allowed to stir for 5 min. A solution of **12a** (1.71 g, 9.7 mmol) in 7 mL of CH_2Cl_2 was added via syringe, and the mixture was stirred for 36 h at room temperature after which time TLC (3:1 hexanes/ethyl acetate) showed that the mixture contained primarily alcohol as product, along with aldehyde **14a** and unreacted starting material. Methylene chloride was added to bring the reaction volume up to 50 mL, and the solution was washed once with 50 mL each of H_2O, concentrated Na_2CO_3, 1 M Na_2SO_3, and finally brine. The organic solution was dried over Na_2SO_4 and concentrated in vacuo to a colorless oil. The crude mixture was chromatographed on silica gel (4.5 × 17 cm, 3:1 hexanes/ethyl acetate) to give 1.45 g of alcohol **13a** (77%) as a white solid (mp 72−75 °C) and 130 mg of aldehyde **14a** (7%) as a clear oil, with the remainder recovered as unreacted starting material. IR: 3436, 1659, 1642 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.91 (s, 3H), 4.56 (br s, 2H), 5.46 (s, 1H), 5.57 (s, 1H), 7.51 (d, 2H, J = 8.6 Hz), 8.01 (d, 2H, J = 8.6 Hz). ^{13}C NMR (101 MHz): δ 52.1, 64.9, 114.7, 126.0, 129.8, 143.1, 146.4, 166.6. LRMS (EI$^+$): *m*/*z* 192. HRMS: calcd for $C_{11}H_{12}O_3$ (M^+) 192.0912, found 192.0914.

2-(4-Methyl benzoate)-1-propen-3-ol-*1,1-d*$_2$ (13b). Using the procedure described for **13a,** 1.51 g of **12b** was oxidized with SeO_2 to give 914 mg of alcohol **13b** (56%) as a white solid (mp 73−76 °C) and 100 mg of aldehyde **14b** (6%) as a clear oil, with the remainder recovered as unreacted starting material. IR: 3400, 2091, 1641 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.91 (s, 3H), 4.56 (d, 2H, J = 6.1 Hz), 7.51 (d, 2H, J = 8.7 Hz), 8.01 (d, 2H, J = 8.7 Hz). ^{13}C NMR (101 MHz): δ 52.2, 64.8, 126.1, 129.5, 143.0, 146.4, 166.9. LRMS (EI$^+$): *m*/*z* 194. HRMS: calcd for $C_{11}H_{10}D_2O_3$ (M^+) 194.0912, found 194.0914.

2-(4-Methyl-*^{13}C* benzoate)-1-propen-3-ol (13c). Using the procedure described for **13a**, 954 mg of **12c** was oxidized with SeO_2 to give 511 mg of alcohol **13c** (50%). IR: 3418, 1718, 908 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.90 (s, 3H), 4.55 (s, 2H), 5.45 (d, 1H, J = 158.6 Hz), 5.56 (d, 1H, J = 157.5), 7.50 (d, 2H, J = 8.4 Hz), 8.00 (d, 2H, J = 8.4 Hz). ^{13}C NMR (101 MHz): δ 52.1, 64.8, 114.7, 117.3, 117.7, 126.0, 129.8, 166.6. LRMS (EI$^+$): *m*/*z* 193. HRMS: calcd for $C_{11}{}^{13}C_1H_{10}O_3$ (M^+) 193.0820, found 193.0819.

2-(4-Methyl benzoate)-1-propen-3-al (14a). Dess−Martin periodinane (2.39 g, 5.6 mmol)[11] was added as a solid to a solution of alcohol **13a** (0.903 g, 4.7 mmol) in 10 mL CH_2Cl_2 at 0 °C. The reaction was stirred for several hours, at which time the reaction was complete by TLC (3:1 hexanes/ethyl acetate). The reaction mixture was diluted with 20 mL of diethyl ether, and the reaction was quenched by addition of 15 mL of concentrated $NaHCO_3$. The emulsion was stirred vigorously while 9.8 mmol of $Na_2S_2O_3$ was added slowly. The organic layer was separated, dried over Na_2SO_4, and concentrated in vacuo. Silica gel chromatography (1.5 × 17 cm, 5:1 hexanes/ethyl acetate) provided 653 mg (73%) of aldehyde **14a** as a colorless oil. IR: 3438, 1720, 1288, 909 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.91 (s, 3H), 6.26 (s, 1H), 6.70 (s, 1H), 7.52 (d, 2H, J = 8.4 Hz), 8.04 (d, 2H, J = 8.4 Hz), 9.81 (s, 1H). ^{13}C NMR (101 MHz): δ 52.1, 53.4, 123.8, 125.6, 128.0, 129.5, 130.1, 136.8, 147.6, 166.6, 192.4. LRMS (EI$^+$): *m*/*z* 190. HRMS: calcd for $C_{11}H_{10}O_3$ (M^+) 190.0630, found 190.0626.

2-(4-Methyl benzoate)-1-propen-3-al-*1,1-d*$_2$ (14b). Using the procedure described for **14a**, a solution of **13b** (0.5 g, 2.5 mmol) was oxidized and chromatographed, providing 321 mg (68%) of aldehyde **14b** as a colorless oil. IR: 3368, 1721, 1288, 908 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.92 (s, 3H), 7.53 (d, 2H, J = 8.7 Hz), 8.05 (d, 2H, J = 8.6 Hz), 9.82 (s, 1H). ^{13}C NMR (101 MHz): δ 52.0, 128.0, 129.4, 130.1, 137.9, 147.3, 166.5, 192.3. LRMS (EI$^+$): *m*/*z* 192. HRMS: calcd for $C_{11}H_8D_2O_3$ (M^+) 192.0755, found 192.0750.

2-(4-Methyl benzoate)-1-propen-3-al-*1-*^{13}*C* (14c). Using the procedure described for **14a**, a solution of **13c** (0.5 g, 2.5 mmol) was oxidized and chromatographed, providing 420 mg (85%) of aldehyde **14c** as a colorless oil. IR: 2954, 1720, 1612, 1457, 909 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.91 (s, 3H), 6.28 (d, 1H, J = 173 Hz), 6.68 (d, 1H, J = 175 Hz), 7.52 (d, 2H, J = 8.4 Hz), 8.04 (d, 2H, J = 8.4 Hz), 9.81 (s, 1H). ^{13}C NMR (101 MHz): δ 52.0, 128.0, 129.4, 130.1, 137.9, 147.3, 166.5, 192.3. LRMS (EI$^+$): *m*/*z* 191. HRMS: calcd for $C_{11}{}^{13}C_1H_{10}O_3$ (M^+): 191.0663, found 191.0669.

3-Bromo-2-phenyl-1-propene (15). Using standard procedures,[12] **15** was prepared by reaction of α-methyl-styrene (47.2 g, 400 mmol) with *N*-bromosuccinimide (45 g, 240 mmol) using 5 mg of AIBN as a radical initiator. The compound was partially purified by fractional distillation (70−75 °C, 0.3 mmHg) to give material which was 70% pure by NMR. A portion of this material (5 g) was purified by silica gel chromatography (5 × 17 cm, 100% hexanes) to give 3 g of **15** as a colorless oil. ^{1}H NMR (400 MHz): δ 4.38 (s, 2H), 5.49 (d, 1H, J = 0.6 Hz), 5.55 (s, 1H), 7.27−7.40 (m, 3H), 7.49 (dd, 2H, J = 8.0, 1.6 Hz). LRMS (EI$^+$): *m*/*z* 196 (M^+).

2-(4-Methyl benzoate)-3-hydroxy-5-phenyl-1,5-hexadiene (16a). Aldehyde **14a** (0.720 g, 3.8 mmol) and bromide **15** (0.955 g, 4.8 mmol) were mixed in 12 mL of THF and cooled to 0 °C in an ice-water bath. An equal volume of freshly prepared, saturated aqueous NH_4Cl was added with stirring. Zinc dust (0.361 g, 5.6 mmol) was added in small portions over 1 min to the vigorously stirred solution. The mixture was stirred for 1.25 h at 0 °C, diluted with 10 mL of ethyl acetate, and warmed to room temperature. After filtering the solution through Celite and washing the filtrate once with 10 mL brine, the organic solution was dried over Na_2SO_4 and concentrated to a yellow oil. Silica gel chromatography (1.5 cm × 17 cm, 5:1 hexanes/ethyl acetate) gave 965 mg (82%) of **16a** as a colorless oil. IR: 3472, 3081, 2951, 1721, 1608, 1436, 1283, 1189, 1110 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.02 (br s, 1H), 2.51 (dd, 1H, J = 9.2, 14.4 Hz), 2.88 (dd, 1H, J = 2.3, 14.4 Hz), 3.92 (s, 3H), 4.66 (br d, 1H J = 8.7 Hz), 5.14 (s, 1H), 5.377 (s, 1H), 5.382 (s, 1H), 5.387 (s, 1H), 7.27−7.33 (m, 5H), 7.40 (d, 2H, J = 8.5 Hz), 7.99 (d, 2H, J = 8.5 Hz). ^{13}C NMR (101 MHz): δ 42.8, 52.1, 71.0, 114.5, 115.8, 126.2, 126.9, 127.8, 128.4, 129.2, 129.6, 140.0, 144.4, 145.0, 150.2, 166.8. LRMS (EI$^+$): *m*/*z* 308. HRMS: calcd for $C_{20}H_{20}O_3$ (M^+) 308.1410, found 308.1412.

2-(4-Methyl benzoate)-3-hydroxy-5-phenyl-1,5-hexadiene-*1,1-d*$_2$ (16b). Using the procedure described for **16a**, aldehyde **14b** (0.100 g, 0.52 mmol) and bromide **15** (0.130 g, 0.66 mmol) were reacted and chromatographed on silica gel (1.5 cm × 17 cm, 5:1 hexanes/ethyl acetate) to give 129 mg (80%) of product as a colorless oil. IR: 3456, 3081, 2951, 1722, 1608, 1436, 1282, 1184, 1113 cm $^{-1}$. ^{1}H NMR (400 MHz): δ 1.97 (d, ^{1}H, J = 2.5 Hz), 2.51 (dd, 1H, J = 9.1, 14.5 Hz), 2.88 (dd, 1H, J = 2.5, 14.5 Hz), 3.92 (s, 3H), 4.66 (m, 1H), 5.14 (s, 1H), 5.38 (s, 1H), 7.28−7.34 (m, 5H), 7.40 (d, 2H, J = 8.5 Hz), 7.99 (d, 2H, J = 8.5 Hz). ^{13}C NMR (101 MHz): δ 42.8, 52.0, 71.0, 115.8, 126.2, 126.9, 127.8, 128.4, 129.2, 129.6, 140.0, 144.3, 145.0, 150.0, 166.8. LRMS (EI$^+$): *m*/*z* 310. HRMS: calcd for $C_{20}H_{18}D_2O_3$ (M^+) 310.1538, found 310.1531.

2-(4-Methyl benzoate)-3-hydroxy-5-phenyl-1,5-hexadiene-*6-*^{13}*C* (16c). Using the procedure described for **16a**, aldehyde **14c** (0.42 g, 2.2 mmol) and bromide **15** (0.56 g, 2.8 mmol) were reacted in the presence of zinc dust (0.210 g, 3.2 mmol). Silica gel chromatography (1.5 cm × 17 cm, 5:1 hexanes/ethyl acetate) provided 116 mg (17%) **16c** as a colorless oil. IR: 3416, 1645, 1471, 1381, 1095, 908 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.03 (s, 1H), 2.52 (dd, 1H, J = 9.1, 14.5 Hz), 2.88 (dd, 1H, J = 2.5, 14.5 Hz), 3.93 (s, 3H), 4.66 (m, 1H), 5.14 (s, 1H), 5.37 (d, 1H, J = 157.6), 5.38 (s, 1H), 5.48 (d, 1H, J = 159.5), 7.28−7.33 (m, 5H), 7.39 (d, 2H, J = 8.3 Hz), 7.98 (d, 2H, J = 8.3 Hz). ^{13}C NMR (101 MHz): δ 29.9, 42.8, 52.0, 70.9, 114.4, 126.2, 126.9, 127.8, 128.3, 129.2, 129.6, 136.7, 140.0, 144.3, 145.0, 166.8.

(11) Dess, D. B.; Martin, J. C. *J. Org. Chem.* **1983**, *48*, 4156−4157.

(12) Reed, S. F. *J. Org. Chem.* **1965**, *30*, 3258−3258.

LRMS (EI$^+$): *m*/*z* 309. HRMS: calcd for $C_{19}{}^{13}CH_{20}O_3$ (M^+) 309.1446, found 309.1443.

2-(4-Benzoic acid)-3-hydroxy-5-phenyl-1,5-hexadiene (17a). Methyl ester **16a** (0.137 g, 0.5 mmol) was dissolved into dioxane (5 mL) and placed into an ice-water bath while it was vigorously stirred. After the addition of 3 mL of 1 N aqueous KOH over 1 min, the reaction was stirred for 3 h at room temperature. The mixture was diluted by the addition of 20 mL of H_2O and washed once with 20 mL of diethyl ether. The aqueous solution was acidified to pH 1 by the addition of 1 M $NaHSO_4$ and then extracted three times with 50 mL of ethyl acetate. The organic extracts were combined, dried over Na_2SO_4, and concentrated to yield 129 mg (97%) of the product as a light-yellow oil. IR: 3435, 2254, 1693, 1638, 1467 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.53 (dd, 1H, J = 9.3, 14.4 Hz), 2.89 (dd, 1H, J = 3.5, 14.4 Hz), 4.67 (dd, 1H, J = 3.5, 9.4 Hz), 5.15 (s, 1H), 5.40 (s, 1H), 5.41 (s, 1H), 5.51 (s, 1H), 7.28−7.32 (m, 5H), 7.44 (d, 2H, 8.5 Hz), 8.06 (d, 2H, 8.5 Hz). ^{1}H NMR (500 MHz, D_2O): δ 2.75 (dd, 1H, J = 8.2, 14.4 Hz), 2.97 (dd, 1H, J = 4.7, 14.4 Hz), 4.76 (dd, 1H, J = 4.9, 7.9 Hz), 5.20 (s, 1H), 5.39 (s, 1H), 5.43 (s, 1H), 5.44 (s, 1H), 7.37−7.43 (m, 7H), 7.86 (d, 2H, 7.9 Hz). ^{13}C NMR (101 MHz): 29.6, 42.7, 53.4, 71.1, 115.9, 126.2, 127.0, 127.8, 128.4, 128.5, 130.2, 140.0, 144.8, 145.2, 150.0, 171.6. LRMS (EI$^+$): *m*/*z* 294 (M^+). HRMS: calcd for $C_{19}H_{18}O_3$ (M^+) 294.1256, found 294.1258.

2-(4-Benzoic acid)-3(*S*)-hydroxy-5-phenyl-1,5-hexadiene (1). Enantiomerically pure substrate for kinetic assays was isolated using chiral reverse-phase HPLC and assigned absolute stereochemistry by derivatizing as the 3-hydroxy esters of phenyl (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)acetate, as previously described.[13]

2-(4-Benzoic acid)-3-hydroxy-5-phenyl-1,5-hexadiene-*1,1-d*$_2$ (17b). Using the procedure described for **17a**, methyl ester **16b** (0.250 g, 0.81 mmol) was hydrolyzed to yield 219 mg (91%) of **17b** as a light-yellow oil. IR: 3417, 2360, 2253, 1636, 913 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.15 (s, 1H), 2.53 (dd, 1H, J = 9.1, 14.4 Hz), 2.86 (dd, 1H, J = 3.5, 14.4 Hz), 4.64 (dd, 1H, J = 3.6, 9.0 Hz), 5.12 (s, 1H), 5.36 (s, 1H), 7.25−7.30 (m, 5H), 7.39 (d, 2H, 8.3 Hz), 8.00 (d, 2H, 8.3 Hz). ^{13}C NMR (101 MHz): δ 42.8, 67.0, 71.0, 115.8, 126.3, 127.0, 127.8, 128.2, 128.4, 130.1, 140.1, 145.0, 150.0. LRMS (FAB$^-$): *m*/*z* 295 (M − H). HRMS: calcd for $C_{19}H_{15}D_2O_3$ (M − H) 295.1303, found 295.1298.

2-(4-Benzoic acid)-3-hydroxy-5-phenyl-1,5-hexadiene-*1-*^{13}*C* (17c). Using the procedure described for **17a**, methyl ester **16c** (0.11 g, 0.4 mmol) was hydrolyzed to yield 90 mg (86%) of **17c** as a light-yellow oil. IR: 3430, 1638, 908 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.04 (s, 1H), 2.54 (dd, 1H, J = 9.1, 14.4 Hz), 2.89 (dd, 1H, J = 3.5, 14.4 Hz), 4.67 (m, 1H), 5.15 (s, 1H), 5.36 (s, 1H), 5.40 (d, 1H, J = 157.6), 5.51 (d, 1H, J = 159.6), 7.25−7.32 (m, 5H), 7.43 (d, 2H, 8.4 Hz), 8.06 (d, 2H, 8.4 Hz). ^{13}C NMR (101 MHz): δ 30.0, 42.9, 71.0, 114.9, 116.0, 126.3, 127.1, 127.9, 128.4, 128.5, 129.7, 130.2, 140.0, 145.0, 171.0. LRMS (EI$^+$): *m*/*z* 295. HRMS: calcd for $C_{18}{}^{13}C_1H_{18}O_3$ (M^+) 295.1289, found 295.1293.

2-(4-Benzoic acid methyl-*^{13}C* ester)-3-hydroxy-5-phenyl-1,5-hexadiene-*1,1-d*$_2$ (18). Isotopically labeled diazomethane, produced by reaction of *N*-methyl-*^{13}C*-*N*-nitroso-*p*-toluenesulfonamide with KOH, was distilled directly into a stirred solution of 20 mg (0.068 mmol) of acid **17b** in 3 mL of methanol at 0 °C. Distillation was continued until the solution retained a light-yellow color, at which point the reaction was quenched with acetic acid. Solvent was removed in vacuo to give 21 mg (99% yield) of **18** as a colorless oil. IR: 3446, 2947, 2360, 1721, 1608, 1432, 1281, 1107 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.46 (dd, 1H, J = 9.2, 14.4 Hz), 2.83 (dd, 1H, J = 3.4, 14.4 Hz), 3.87 (d, 3H, J = 147.1 Hz), 4.60 (dd, 1H, J = 3.4, 9.2 Hz), 5.09 (s, 1H), 5.34 (s, 1H), 7.08−7.27 (m, 5H), 7.35 (d, 2H, J = 8.5 Hz), 7.94 (d, 2H, J = 8.5 Hz). ^{13}C NMR (101 MHz): 42.9, 52.1, 53.3, 70.9, 89.2, 115.9, 126.3, 126.9, 127.8, 128.5, 129.6, 140.0, 145.1, 150.0, 160.0, 171.0. LRMS (EI$^+$): *m*/*z* 311. HRMS: calcd for $C_{19}{}^{13}C_1H_{18}D_2O_3$ (M^+) 311.1572, found 311.1578.

2-(4-Benzoic acid)-5-phenyl-5-pentenal (2). A solution of substrate **17a** (145 mg, 492, μmol) in 10 mL of distilled benzene in a sealed tube was degassed by three rounds of freeze-thawing under nitrogen. The tube was then heated to 180 °C in an oil bath for 4 h. After cooling, the solvent was removed in vacuo and the resulting oil was chromatographed on a silica gel column (2.5 × 17 cm, 2:1 hexanes/ethyl acetate) yielding 138 mg (95%) of aldehyde **2** as a colorless oil. IR: 2948, 1692, 1610, 1424, 1289 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.10 (m, 2H), 2.48 (m, 2H), 3.65 (t, 1H, J = 6.8 Hz), 5.29 (s, 1H), 5.30 (s, 1H), 7.30 (m, 7H), 8.10 (d, 2H, J = 8.1 Hz), 9.66 (s, 1H). ^{13}C NMR (101 MHz): δ 28.2, 32.5, 58.3, 113.4, 126.0, 127.6, 128.4, 129, 130.9, 140.4, 142.2, 147.1, 171.8, 199.7. LRMS (EI$^+$): *m*/*z* 294. HRMS: calcd for $C_{19}H_{18}O_3$ (M^+) 294.1256, found 294.1256.

2-(4-Benzoic acid)-5-phenyl-*3-*^{13}*C*-5-pentenal (19). Using the procedure for **2**, a solution of **17c** (90 mg, 305 μmol) in a sealed tube was degassed and then heated for 4 h at 180 °C. After cooling and removal of the solvent, the oil was chromatographed on silica gel (2 × 17 cm, 2:1 hexanes/ethyl acetate), yielding 70 mg (77%) of the aldehyde product **19** as a colorless oil. IR: 3422, 1705, 1638, 1288 cm^{-1}. ^{1}H NMR (400 MHz): δ 1.93 (m, 2H), 2.48 (m, 2H), 3.65 (m, 1H), 5.04 (s, 1H), 5.32 (s, 1H), 7.30 (m, 7H), 8.10 (d, 2H, J = 8.0 Hz), 9.66 (s, 1H). ^{13}C NMR (101 MHz): δ 28.1, 32.7, 58.4, 126.0, 127.5, 128.3, 128.6, 129.1, 130.7, 140.4, 169.9, 199.7. LRMS (EI$^+$): *m*/*z* 295. HRMS: calcd for $C_{18}{}^{13}CH_{18}O_3$ (M^+) 295.1289, found 295.1286.

Compounds **20a**, **20b**, and **20c** were obtained as a mixtures of inseparable diastereomers.

2-(4-Benzoic acid)-5-phenyl-5-pentenal Oxime (20a, 20a′). To a solution of the aldehyde **19a** (90 ma, 305 μmol) in 10 mL of ethanol were successively added 400 mg each of hydroxylamine hydrochloride (0.4 g, 5.9 mmol) and sodium acetate (0.4 g, 4.87 mmol). The resulting slurry was stirred vigorously and diluted with water after 2 h. The mixture was extracted with diethyl ether and then concentrated and dried over Na_2SO_4. The oil obtained was HPLC purlfied (C18 reverse-phase column; 43−60% gradient of CH_3CN (0.1% TFA) in H_2O (0.1% TFA)), providing 87 mg (91%) of a mixture of **20a and 20a′** as a colorless foam. IR: 3420, 1687, 1421, 911 cm^{-1}. ^{1}H NMR (400 MHz): (**20a**) δ 2.1 (m, 2H), 2.53 (m, 2H), 3.61 (m, 1H), 5.15 (d, 2H, J = 12.6), 6.84 (d, 1H, J = 6.51 Hz), 7.30 (m, 7H), 8.10 (d, 2H, J = 8 Hz); (**20a′**) δ 2.1 (m, 2H), 2.53 (m, 2H), 4.46 (m, 1H), 5.30 (d, 2H, J = 6.28), 7.30 (m, 7H), 7.53 (d, 1H, J = 6.67 Hz), 8.10 (d, 2H, J = 8 Hz). ^{13}C NMR (101 MHz): δ 33.6, 34.0, 113.3, 113.5, 127.5, 128.5, 129.1, 129.4, 131.2, 142.2, 148.2, 149.4, 153.6, 170.0. LRMS (FAB$^+$): *m*/*z* 310 (MH^+). HRMS: calcd for $C_{19}H_{20}NO_3$ 310.1443, found 310.1443.

2-(4-Methyl-*^{13}C* benzoate)-5-phenyl-5-pentenal Oxime (20b, 20b′). To a heated solution of KOH pellets (500 mg, 8.9 mmol) dissolved in 0.8 mL of water and 1 mL of ethanol was gradually added 500 mg of *N*-methyl-*^{13}C*-*N*-nitroso-*p*-toluenesulfonamide dissolved in 4.5 mL of diethyl ether. The resulting ^{13}C isotopically labeled diazomethane was added to a stirred solution of the hydroxylamine oxime **20a** (12.5 mg, 40 μmol) in CH_2Cl_2 until the solution retained a light-yellow color. The mixture was then quenched with a solution of acetic acid in diethyl ether and concentrated in vacuo to yield 15.3 mg (>99%) of methyl esters **20b** and **20b′**. IR: 3405, 2946, 2360, 1721, 1610, 1432, 1282, 1108 cm^{-1}. ^{1}H NMR (400 MHz): (**20b**) δ 2.0 (m, 2H), 2.48 (m, 2H), 3.60 (m, 1H), 3.86 (d, 3H, J = 38 Hz), 5.04 (d, 2H, J = 12.68 Hz), 6.81 (d, 1H, J = 6.51), 7.30 (m, 7H), 7.99 (d, 2H, J = 8 Hz); (**20b′**) δ 2.0 (m, 2H), 2.48 (m, 2H), 3.86 (d, 3H, J = 38 Hz), 4.39 (m, 1H), 5.29 (d, 2H, J = 6.28 Hz), 7.30 (m, 7H), 7.48 (d, 1H, J = 6.67 Hz), 7.99 (d, 2H, J = 8 Hz). ^{13}C NMR (101 MHz): δ 31.9, 32.2, 32.8, 32.9, 45.7, 52.1, 113.1, 127.5, 127.8, 128.0, 128.3, 128.7, 130.0, 130.1, 153.2, 160.0, 172.0. LRMS (EI$^+$): *m*/*z* 324. HRMS: calcd for $C_{19}{}^{13}C_1H_{21}NO_3$ (M^+): 324.1555, found 324.1559.

2-(4-Benzoic acid)-5-phenyl-*3-*^{13}*C*-5-pentenal oxime (20c, 20c′). To a solution of aldehyde **19b** (30 ma, 101 μmol) in 9 mL of ethanol were successively added 300 mg each of hydroxylamine hydrochloride (4.65 mmol) and sodium acetate (3.65 mmol). The resulting slurry was diluted with 25 mL of water after 2 h and extracted with ethyl acetate. The phases were separated, and the aqueous portion was acidified with 1 M $NaHSO_4$ and then extracted three times with 50 mL portions of ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated in vacuo, to give 29 mg (99%) of the ^{13}C-labeled oxime **20c** as a mixture of diastereomers. ^{1}H NMR

(13) Ulrich, H. D.; Driggers, E. M. G.; Schultz, P. G. *Acta Chem. Scand.* **1996**, *50*, 328−332.

(400 MHz): (**20c**) δ 2.1 (m, 2H), 2.48 (m, 2H), 3.60 (m, 1H), 5.28 (d, 2H, J = 3.3 Hz), 7.29 (m, 7H), 7.50 (d, 1H, J = 6.7 Hz), 8.02 (d, 2H, J = 3.08 Hz); (**20c′**) δ 2.1 (m, 2H), 2.48 (m, 2H), 3.60 (m, 1H), 5.04 (d, 2H, J = 8.2 Hz), 6.83 (d, 1H, J = 7.51 Hz), 7.29 (m, 7H), 8.02 (d, 2H, J = 3.08 Hz). ^{13}C NMR (101 MHz): δ 31.0, 32.0, 32.6, 45.8, 113.2, 127.5, 127.9, 128.0, 128.3, 128.5, 130.6, 130.7, 140.6, 146.2, 147.3, 152.9, 170.9. LRMS (FAB$^+$): *m*/*z* 311 (MH$^+$). HRMS: calcd for $C_{18}{}^{13}C_1H_{20}NO_3$ (MH$^+$) 311.1477, found 311.1476.

4-Phenyl-3-hydroxy-1-(4-methyl benzoate)-4-penten-1-one-*5,5-d*$_2$. To 1.5 mL of a 1 M solution of LiN(Si(CH$_3$)$_3$)$_2$ in THF cooled to −78 °C was slowly added a solution of 364 μL (3.12 mmol) of acetophenone in 1.5 mL of THF. After 45 min, **14b** dissolved in 1 mL of THF was added dropwise to the mixture. The reaction was quenched after 20 min by the addition of 3 mL of methanol. The solution was washed with brine, and the aqueous phase was extracted three times with ethyl acetate. The combined organic phases were dried over Na_2SO_4 and concentrated. The resulting oil was chromatographed on silica gel (2 × 17 cm, 4:1 hexanes/ethyl acetate), yielding 0.190 g (58%) of the hydroxy ketone as a white solid (mp 102−106 °C). IR: 3063, 1719, 1681, 1448, 1436, 1184 cm^{-1}. ^{1}H NMR (400 MHz): δ 3.13 (m, 2H), 3.63 (d, 1H, J = 3.55 Hz), 3.90 (s, 3H), 5.30 (m, 1H), 7.49 (m, 5H), 7.84 (d, 2H, J =9Hz), 7.99 (d, 2H, J =8.6 Hz). ^{13}C NMR (101 MHz): δ 44.1, 52.1, 68.9, 126.8, 128.0, 128.4, 128.6, 129.8, 133.6, 136.0, 145.0, 149.0, 166.5, 200.0. LRMS (EI$^+$): *m*/*z* 312. HRMS: calcd for $C_{19}D_2H_{16}O_4$ 312.1331, found 312.1339.

4-Phenyl-3-((triisopropylsilyl)oxy)-1-(4-methyl benzoate)-4-penten-1-one-*5,5-d*$_2$ (21). Triisopropyl triflic acid (0.574 mL, 2.13 mmol) and 2,6-lutidine (0.478 mL, 4.11 mmol) were added to a stirred solution of 4-phenyl-3-hydroxy-1-(4-methyl benzoate)-4-penten-1-one-*5,5-d*$_2$ in 2 mL of CH_2Cl_2. The reaction mixture was washed with 10 mL of brine and dried over Na_2SO_4. Silica gel chromatography (3.5 × 17 cm, 2% ethyl acetate in hexanes) provided 0.197 g (76%) of **21** as a colorless oil. IR: 2945, 2867, 1716, 908 cm^{-1}. ^{1}H NMR (400 MHz): δ 1.09 (m, 21H), 3.02 (dd, 1H, J = 4.9, 11.2 Hz), 3.29 (dd, 1H, J = 6.7, 9.4 Hz), 5.51 (m, 1H), 7.36 (d, 2H, J = 7.5 Hz), 7.51 (d, 2H, J = 7.27 Hz), 7.55 (d, 2H, J = 8.3 Hz), 7.80 (d, 2H, J = 7.27 Hz), 7.96 (d, 2H, J = 8.3 Hz). ^{13}C NMR (101 MHz): δ 11.9, 12.2, 12.4, 12.5, 17.6, 18.0, 46.8, 52.0, 126.9, 128.1, 128.3, 129.1, 129.6, 133.0, 137.1, 143.4, 149.7, 166.9, 198.2. LRMS (EI$^+$): *m*/*z* 468. HRMS: calcd for $C_{28}D_2H_{36}O_4Si$ 468.2665, found 468.2666.

2-(4-Methyl benzoate)-3-((triisopropylsilyl)oxy)-5-phenyl-1,5-hexadiene-*1,1,6,6-d*$_4$ (22). To a well stirred slurry of methyl-d_3-triphenylphosphonium iodide (0.208 g, 0.51 mmol) in 4 mL of THF at 0 °C was slowly added 2.05 mL of *n*-butyllithium (2.5 M). The solution was stirred at 0 °C for 0.5 h, at which time a solution of ketone **21** (0.20 g, 0.427 mmol) in 3 mL of THF was slowly added and the mixture allowed to stir for 0.5 h as it slowly warmed to room temperature. Water (6 mL) was added to quench the reaction. The aqueous mixture was extracted twice with 15 mL of ethyl acetate, washed with 15 mL of brine, dried over Na_2SO_4, and concentrated. The resulting oily residue was chromatographed on silica gel (2 × 17 cm, 1.5% ethyl acetate in hexanes) to give 78 mg (39%) of **22** as a light oil. IR: 2948, 1381, 1283, 909, 735 cm^{-1}. ^{1}HNMR(400 MHz): δ 1.02 (m, 21H), 2.80 (dd, 2H, J =7.7, 6.2 Hz), 3.91 (s, 1H), 4.72 (dd, 1H, J =5.5, 2.1 Hz), 7.20 (m, 5H), 7.47 (d, 2H, J = 8.5 Hz), 7.92 (d, 2H, J = 6.7 Hz). ^{13}C NMR (101 MHz): δ 12.3, 18.0, 43.6, 51.9, 126.3, 127.1, 127.3, 127.9, 128.7, 129.1, 140.7, 144.1, 144.2, 149.4, 166.9. LRMS (EI$^+$): *m*/*z* 468. HRMS: calcd for $C_{29}H_{36}D_4O_3Si$ (M$^+$) 468.2998, found 468.2988.

2-(4-Benzoic acid)-3-hydroxy-5-phenyl-1,5-hexadiene-*1,1,6,6-d*$_4$ (23). To a stirred solution of **22** (0.08 g, 1.3 mmol) in 10 mL of THF at 0 °C was added 148 μL of a tetra-*N*-butylammonium fluoride solution (1 M in THF). Upon completion, 2 mL of a saturated NH_4C1 solution was added and the aqueous mixture was extracted with CH_2Cl_2 (4 × 10 mL). The organic extractions were combined, washed twice with 20 mL of brine, dried over Na_2SO_4, and concentrated to an oily residue which was chromatographed on silica gel (2 × 17 cm, 3:1 hexanes/ethyl acetate) to give 29 mg (49%) of the deprotected alcohol as a colorless oil. This material was combined with the product of a second identical reaction to give 59 mg of compound, which was hydrolyzed in a basic aqueous solution as described for compounds **17a**−**c** to give carboxylic acid **23** (83% yield). IR: 3649, 1792, 1472, 1381, 911 cm^{-1}. ^{1}H NMR (400 MHz): δ 2.55 (dd, 1H, J = 9.1, 14.4 Hz), 2.87 (dd, 1H, J = 2.2, 14.4 Hz), 4.67 (dd, 1H, J = 2.2, 9.1 Hz), 7.32 (m, 5H), 7.43 (d, 2H, J = 8.3 Hz), 8.06 (d, 2H, J = 8.3 Hz). ^{13}C NMR (101 MHz): δ 14.1, 21.0, 42.7, 60.4, 71.0, 126.4, 127.0, 127.8, 128.4, 130.2, 140.0, 144.8, 149.2, 170.1. LRMS (EI$^+$): *m*/*z* 298. HRMS: calcd for $C_{19}H_{14}D_4O_3$ (M$^+$) 298.1507, found 298.1508.

Antibody Production and Purification. Monoclonal antibodies were generated against hapten **3** using standard hydridoma technology.[14,15] Swiss Webster mice were injected intraperitoneally with KLH-**3** (100 mg) emulsified in complete Freund's adjuvant. After 2 weeks, the mice were boosted with KLH-**3** emulsified in incomplete Freund's adjuvant, followed 2 weeks later by intravenous injection of KLH-**3** in phosphate-buffered saline solution (PBS, 138 mM NaCl, 10 mM NaH_2PO_4, 5 mM KCl, 1.8 mM KH_2PO_4, pH 7.4). Three days after this final immunization, 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 under standard HAT selective conditions. Screening by enzyme-linked immunosorbent assay (ELISA) for binding to BSA-**3** identified 21 cell lines specific for the hapten, which were then cloned by limiting dilution. Antibody containing ascites fluid was produced for each of these monoclonal cell lines in irradiated, pristane primed Swiss Webster mice using standard protocols.[16]

Antibodies were purified by protein-G affinity chromatography using a procedure adapted from that of Fredriksson et al.[17] A given volume of ascites fluid was diluted 2:1 with binding buffer (aqueous 1.5 M glycine, 3.0 M NaCl, pH 8.9), filtered through a glass wool plug to eliminate particulate matter, and applied at 0.5 mL min^{-1} to an 8 mL bed of agarose-linked protein-G already equilibrated in binding buffer. Nonspecific binders were eluted from the column with binding buffer until the OD_{280} of the eluent was <0.05 absorbance units. Antibody was eluted from the column with 100 mM sodium citrate, pH 3.0. Column fractions were collected, neutralized with 1.0 M Tris, pH 9.0, and the protein concentration determined by absorbance at 280 nm (1 mg mL^{-1} = 1.37 absorbance units, with a molecular weight of 150 000 for immunoglobulin-G). Approximately 4 mg of AZ-28 were recovered per milliliter of ascites fluid. The eluted antibody was dialyzed exhaustively against PBS and stored at 4 °C following sterile (0.2 μm) filtration. Antibodies were judged to be >95% pure by polyacrylamide gel electrophoresis.

NMR Sample Preparation. For production of large quantities of antibody AZ-28, ascites was produced in 30 Swiss Webster mice using standard protocols.[16] Antibody was purified from ascites as described above and then exchanged into deuterated PBS buffer (dPBS, produced by three successive lyophilizations from D_2O of a known volume of PBS). It was found that lyophilization inactivated antibody AZ-28, so exchange into deuterated buffer was accomplished by four sequential concentration and dilution (1:10) steps using a centriprep-10 concentration apparatus (Amicon). The final antibody concentration of the resulting antibody stock solution was determined by UV absorbance at 280 nm and typically ranged from 250 to 700 μM in binding sites. In the NMR experiments, Girard's Reagent T was used as an aldehyde trapping agent to minimize product affinity for the antibody combining site. Stock solutions of substrate **1** and trapping agent Girard's Reagent T were prepared separately by repeated lyophilization from D_2O solution at 10× the desired final concentration. Immediately prior to NMR experiments, these stock solutions were combined with the antibody stock solution and diluted with dPBS to give samples containing 50 μM whole antibody, 2 mM substrate, and 5 mM trapping agent (pD 7.0) in a volume of 500 μL. Kinetic competence of these samples was independently confirmed using the described HPLC assay.

(14) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor: Cold Spring Harbor: Plainview, NY, 1988.

(15) Goding, J. W. *MonoclonalAntibodies: Principles andPractice*, 3rd ed.; Academic Press, Ltd.: San Diego, CA, 1996; pp 141−180.

(16) Shokat, K. M. Ph.D. Thesis, University of California, Berkeley, CA, 1991.

(17) Fredriksson, U. B.; Fagerstam, L. G.; Cole, A. W. G. *Protein A-Sepharose C1-4B Affinity Purification of Ig G Monoclonal Antibodies from Mouse Ascites*; Pharmacia AB: Uppsala, Sweden, 1986.

Transferred NOE Spectroscopy. NMR studies were performed on a Bruker AMX-600 spectrometer at a frequency of 600.14 MHz and a GE Omega500 spectrometer at a frequency of 500.13 MHz, using 5 mm ^{1}H probes maintained at 18 °C by variable temperature units. Spectra were processed using the Felix 95.0 (Molecular Simulations Inc.) program on Silicon Graphics Inc. workstations. Chemical shift assignments are referenced to the residual water present in the samples (4.88 ppm at 18 °C).

Spectra for resonance assignments were acquired on a 3 mM sample of **1** on the GE Omega500 spectrometer. Assignments were made using a combination of ^{1}H lD presaturation and ^{1}H NOESY experiments. ^{1}H lD spectra were acquired with 4096 complex points, 5000 Hz spectral width, 1s recycle delay, and 3 s water presaturation for 64 scans. ^{1}H NOESY spectra were acquired with 4096 × 256 complex points, 6024.1 Hz spectral width, 1 s recycle delay, and 2 s water presaturation for 32 scans per t_1 increment.

For transferred-NOE experiments, a steady-state nuclear Overhauser effect (NOE) difference pulse sequence was used, with a 1-1 jump-return to achieve water suppression. A 1 s preirradiation pulse was applied to selected proton frequencies. Reference spectra (no preirradiation) were interwoven with preirradiated spectra to average out effects due to substrate turnover during experiment acquisition. Spectra were acquired with 4096 complex points, 5555.56 Hz spectral width, and 1 s recycle delay for a total of 3520 transients.

For CAMELSPIN experiments, a lD ROE pulse sequence was used with a 1-1 jump-return to achieve water suppression. Spectra were acquired with 4096 complex points, 6024.10 Hz spectral width, and 4.0 s recycle delay for a total of 2560 transients per resonance. A 50 ms DANTE pulse was utilized for the selective inversion followed by a 50 ms spin lock at 6.19 kHz.

Kinetic Assays. Catalyzed and uncatalyzed reaction rates for the rearrangement of **1** to **2** were measured using a reverse-phase HPLC assay. Reactions were initiated by addition of a concentrated stock solution of substrate **1** or the racemate **17a** in DMSO or acetonitrile to an aqueous solution, such that the final solution contained 20 mM MES, 100 mM NaCl, 5 mM NH_2OH, 5% CH_3CN or DMSO, pH 6.0, ±500 nM AZ-28. Reaction volumes were typically 200 μL. Reaction progress was monitored by periodic injection of a 30 μL sample from the reaction mixture onto a reverse-phase C-18 HPLC column (Rainin Microsorb) with UV detection at 240 nm, using an elution gradient of 43−60% CH_3CN (0.1% TFA) in H_2O (0.1% TFA) over 17 min at a flow rate of 1 mL min^{-1} to resolve the product. Hydroxylamine was introduced into the reaction buffer as a fast trapping agent for aldehyde **2**; therefore, product was quantified as the hydroxylamine oxime of **2** (**20a**). All rates are initial rates, determined at less than 4% product conversion based on product peak area, and calibrated to either 3-nitrobenzoic acid or *o*-nitroanisole as internal standards.

Determination of Activation Parameters. The kinetic assay described above was carried out at a variety of temperatures (13−47 °C uncatalyzed, 5−25 °C catalyzed) in order to determine energies of activation for the conversion of **1** to **2**. For consistency, the catalyzed and uncatalyzed temperature ranges were overlapped as much as possible. Solutions were pre-equilibrated to the desired temperature using a recirculating water bath, and maintained at that temperature during the course of the assay. Temperature was monitored using a dual channel thermometer (0.3% accuracy, Fisher Scientific) referenced to 0 °C with a well-stirred ice-water bath. Antibody-catalyzed temperature dependence assays were run with enantiomerically pure (*S*)-**1** at a concentration of 2 mM to insure V_{max} conditions (K_m = 75 μM). Uncatalyzed temperature dependence assays were carried out using 2 mM **17a**. All reactions were carried out in quadruplicate, and the data converted to enthalpies and entropies of activation by fitting it to the standard Eyring relationships.[18]

Kinetic Isotope Effect (KIE). a. Competition Experiments. The secondary deuterium kinetic isotope effects on the antibody-catalyzed reaction ($^{\alpha-D}(V/K)$) was measured by the method of internal competition. A solution containing approximately a 1:1 ratio of substrates **18**/**16a** was prepared by mixing equal volumes (20 mM each in CH_3CN) of the two substrates. This stock solution was added to a stock concentration of antibody AZ-28 such that the final solution consisted of 10 μM Ig, 500 μM each of **18** and **16a**, 20 mM MES, 100 mM NaCl, 5 mM NH_2OH, 15% CH_3CN, pH 6.0. The additional acetonitrile was added to solubilize the methyl ester substrates. Total reaction volumes were 2 mL. The antibody-catalyzed reaction proceeded for 16 h, at which time the solutions were frozen at −78 °C. Preparative reverse-phase HPLC was used to isolate reaction products using a mobile phase gradient of 35−100% CH_3CN in unbuffered H_2O, which allowed recovery of both substrate and aldehyde product, trapped as the oxime. Preparative samples were concentrated by lyophilization, resuspended in 1:1 CH_3CN/H_2O, 1% AcOH, and analyzed by electron impact mass spectrometry (EIMS) to determine the ratio of labeled to unlabeled substrate and product. Six such reactions were analyzed for the catalyzed and uncatalyzed reactions, and the catalyzed isotope effect calculated as $^{\alpha-D}(V/K) = (P_H/P_D)/(S_H/S_D)$. Isotope effect reported is the average ± standard deviation to 95% confidence. **b. V_{max} Effect.** For determination of the secondary deuterium kinetic isotope effect on V_{max} ($^{\alpha-D}V$), a 2 mM solution of **23** was converted to hydroxylamine-trapped product in the presence of 500 nM AZ-28 and the rate determined using the HPLC assay described above. This V_{max} rate was compared to the V_{max} rate for conversion of **17a** with the same antibody preparation. Rates were determined in quadruplicate and the isotope effect calculated as $^{\alpha-D}V = k_{cat}H/k_{cat}D$, reported as the average ± standard deviation to 95% confidence.

Results and Discussion

Synthesis. Hapten **3** was designed to mimic the chairlike transition state for the oxy-Cope rearrangement of diene **1** to aldehyde **2**.[1] An antibody generated against this hapten was expected to catalyze this reaction by overcoming the negative entropy of activation typically associated with a [3,3]-sigmatropic rearrangement.[18,19] Incorporation of the 2,5-diaryl substituents was expected to provide a large hydrophobic binding surface for the antibody. In addition, phenyl substituents at the 2 and 5 positions of the corresponding substrate have been shown to substantially lower the activation energy of this reaction, facilitating kinetic analysis of the reaction at temperatures compatible with a protein catalyst.[21] The 3-hydroxyl group in both hapten **3** and substrate **1** might be expected to participate in hydrogen-bonding interactions in the antibody combining site that result in increased electron density on the oxygen and, as a consequence, an increased the rate of rearrangement.[22] A linker was incorporated at the para position of the 2-phenyl substituent for attachment of the hapten to the carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). Linkage at this position to the protein, via a thiourea group, was expected to maximize binding interactions with the 2,5-diarylcyclohexyl core of hapten **3**. Product inhibition was expected to be minimized as a result of the rapid conversion of the enol group in the product to the aldehyde. Trapping of the aldehyde with hydroxylamine further minimizes product inhibition and prevents addition of the aldehyde product to lysine ϵ-amino groups present on the antibody.

The synthesis of hapten **3** is illustrated in Scheme 1. The disubstituted cyclohexanol core of **3** was generated by a Stille coupling[23] between aryl tin derivative **5** and vinyl triflate **4**. Subsequent hydroboration of **6** establishes a trans relationship

(18) Foster, G. E.; Cope, A. C.; Daniels, F. J. *J. Am. Chem. Soc.* **1947**, *69*, 1893−1896.

(19) Doering, W. V. E.; Roth, W. R. *Tetrahedron* **1962**, *18*, 67−74.

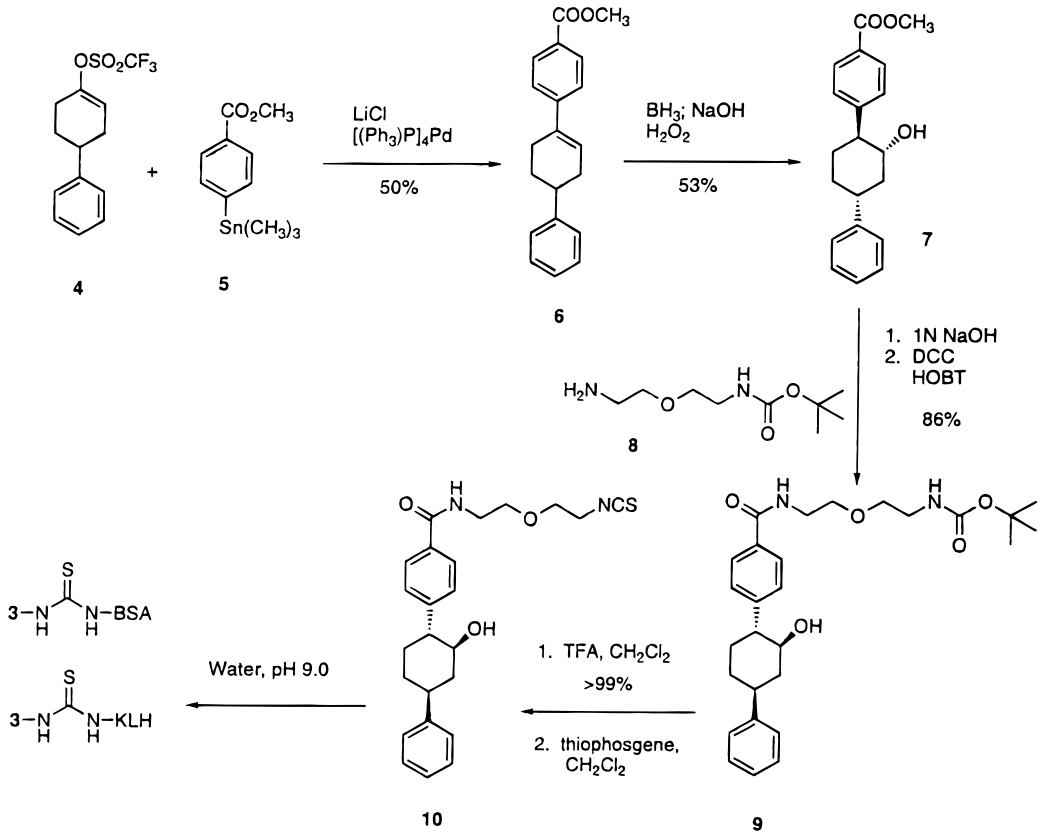
(20) Although the entropies determined by Evans and Golob are on the order of −1 cal mol^{-1} K^{-1}, these values are for a constrained norbornene system. A more appropriate comparison would be to Foster et al.[17]

(21) Dewar, M. J. S.; Wade, L. E. J. *J. Am. Chem. Soc.* **1977**, *99*, 4417−4424.

(22) Steigerwald, M. L.; Goddard, W. A. I.; Evans, D. A. *J. Am. Chem. Soc.* **1979**, *101*, 1994−1997.

(23) Scott, W. J.; Crisp, G. T.; Stille, J. K. *J. Am. Chem. Soc.* **1984**, *106*, 4630−4632.

Scheme 1



between the hydroxyl group at C1 and the aryl substituent at C2 due to the syn, anti-Markovnikov addition of borane to olefins.[24] While the C5 stereochemistry was not set, separation of the diastereomers was found to be possible by repeated elution on a preparative TLC plate. Stereochemical assignments were made through a combination of ^{1}H NMR and synthetic methods following separation of the diastereomers. First, NMR assignments were made on material which had been equilibrated in DBU to the equatorially substituted ring system. Stereochemical assignments of the separated diastereomers were then made by NMR proton decoupling and comparison of coupling constants with the equilibrated material.[25] Hydrolysis of the methyl ester **7** and condensation of the resulting free acid with monoprotected diamine **8** using 1,3-dicyclohexylcarbodiimide afforded hapten **9** in protected form. Acid-catalyzed deprotection of **9**, followed by condensation with thiophosgene, and coupling to carrier proteins in aqueous solution (pH 9) produced the final protein conjugates with epitope densities of 15 (BSA) and 10 (KLH).

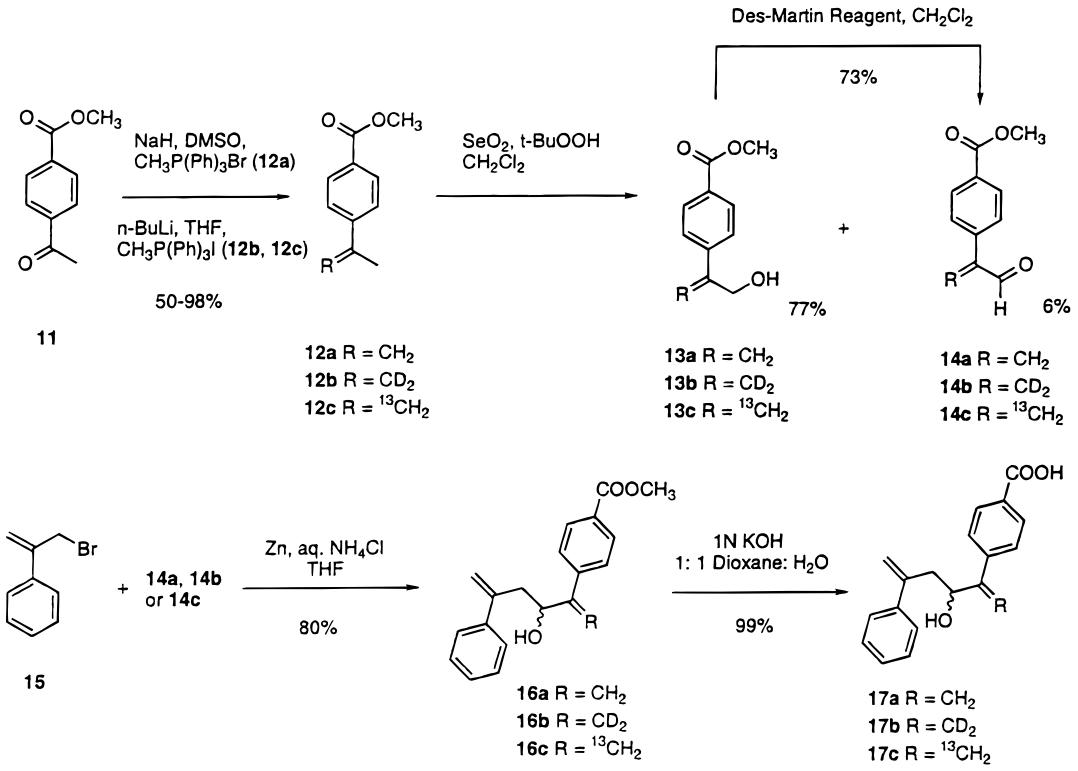
The syntheses of substrate **1** and isotopically substituted derivatives are illustrated in Schemes 2 and 3. 4-Acetylbenzoic acid was converted to olefin **12a** by means of a Wittig reaction with the ylide of methyltriphenylphosphonium bromide in DMSO. Oxidation to the allylic alcohol with SeO_2[26] gave a mixture of alcohol **13a** and aldehyde **14a**. Purification of the alcohol was followed by Dess−Martin oxidation[11,27] to give aldehyde **14a**; recovery and reoxidation of starting material resulted in 70% overall conversion of compound **12a** to **14a**. Coupling of **14a** to bromide **15** using zinc dust[28] followed by base hydrolysis provided racemic substrates **17a**−**c**; **17a** was resolved by chiral reverse-phase HPLC to give substrate **1** as reported.[13] Racemichexadiene **17a** was heated in a sealed tube to give aldehyde **2** by oxy-Cope rearrangement, and the aldehyde was trapped as the hydroxylamine oxime by reaction in sodium acetate buffered ethanol to afford compounds **20a** and **20a′** (inseparable diastereomers), the final assayed product of the antibody catalysis reaction mixture. Preparative reverse-phase HPLC purification of **20a** provided material of sufficient purity for kinetic assays.

In preparing deuterated substrate **18** for the measurement of kinetic isotope effects, we found it necessary to introduce the deuterium label in the initial step of the synthesis. While the procedure for the Wittig reaction of **11** to **12a** had provided good yields using the unlabeled $CH_3P(Ph)_3Br$ salt, it was found that this method produced scrambling of the deuterium atoms in reaction with $CD_3P(Ph)_3I$. Therefore, the phosphorus ylide was generated using *n*-BuLi in THF, giving >99% specific incorporation of deuterium. The same procedure gave specific incorporation of ^{13}C in **12c** as well. Subsequent transformations were as described for the unlabeled material and proceeded without measurable scrambling to give completely specific deuterium and ^{13}C incorporation in the final substrates and products as determined by integration of the relevant NMR resonances.

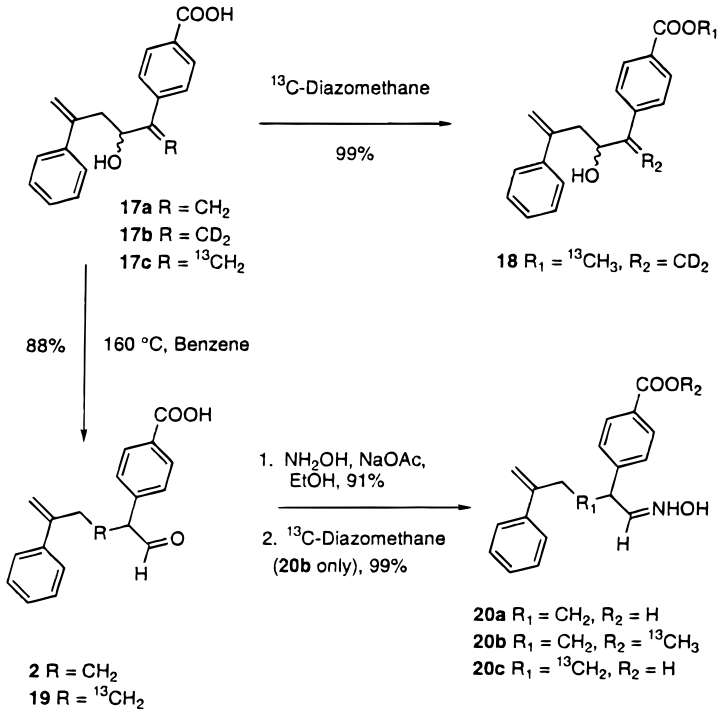
In order to generate material which would give a sufficient secondary deuterium kinetic isotope effect for direct detection in kinetic assays, tetra-deuterated substrate **23** was synthesized as shown in Scheme 4. Aldehyde **14b** was coupled to acetophenone using an aldol condensation. Following protection of the alcohol with triisopropyl triflate, the ketone was easily

(24) Brown, H. C.; Zweifel, G. *J. Am. Chem. Soc.* **1961**, *83*, 2544−2551.
(25) Braisted, A. B. Ph.D. Thesis, University of California, Berkeley, CA, 1994.
(26) Warpehoski, M. A.; Chabaud, B.; Sharpless, K. B. *J. Org. Chem.* **1982**, *47*, 2897−2900.
(27) Ireland, R. E.; Liu, L. *J. Org. Chem.* **1993**, *58*, 2899−2900.
(28) Wilson, S. R.; Guazzaroni, M. E. *J. Org. Chem.* **1989**, *54*, 3087−3091.

Scheme 2



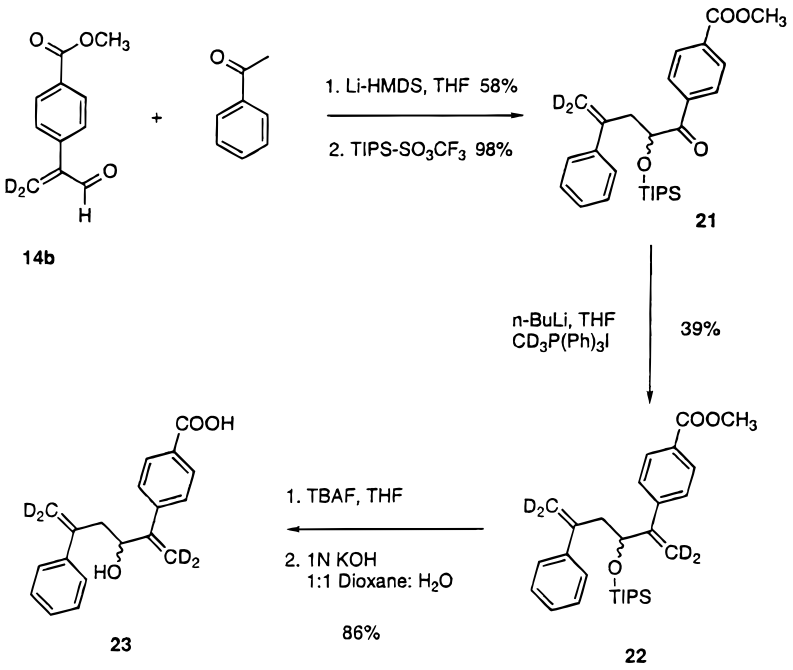
Scheme 3



converted into the desired deutero-olefin **22** by Wittig reaction with $CD_3P(Ph)_3I$ using conditions identical to those for the synthesis of **12b**. The Wittig reaction resulted in approximately 90% specific deuterium incorporation at the terminal olefin. Deprotection with tetra-*n*-butylammonium fluoride (TBAF) in THF, followed by base hydrolysis, afforded tetra-deutero substrate **23**, which was used as the racemate after HPLC purification.

Catalytic Properties. Following immunization, a total of 21 monoclonal antibodies were isolated which bound hapten **3** in ELISA assays using BSA−**3** conjugate. Kinetic screening revealed nine of the antibodies to have measurable activity in the assays of oxy-Cope rearrangement of **1** to **2**; as the most efficient catalyst, antibody AZ-28 was chosen for further characterization. As previously reported,[1] AZ-28 was found to catalyze the rearrangement of substrate **1** to aldehyde **2** with a k_{cat} of 0.02 min^{-1} and a K_m (**1**) of 74 μM, corresponding to a rate enhancement (k_{cat}/k_{uncat}) of 5300 over the uncatalyzed reaction ($k_{uncat} = 4.9 \times 10^{-6}$ min^{-1}). The antibody catalyzed reaction was competitively inhibited by hapten **3** with a K_i of

Scheme 4



17 nM. In addition, the antibody shows a kinetic preference for the *S* configuration at C3 of the substrate ($k_{cat}S/k_{cat}R = 15.1$:1). In the development of a robust HPLC kinetic assay, it was found to be necessary to trap the aldehyde product of the reaction **2** with hydroxylamine to achieve reliable integration of the product. It was subsequently determined in NMR studies that catalysis in the absence of the trapping agent resulted in a time-dependent inactivation of the antibody, presumably due to imine formation of the product aldehyde with surface lysines. The exchange rate of oxime-trapped product was determined using ^{13}C NMR with isotopically labeled product **20c** and shown to be at least 45 s^{-1}, consistent with the lack of product inhibition.[1] Among the less-efficient oxy-Cope catalysts which were generated by immunization with hapten **3** and characterized kinetically, the values of k_{cat} ranged from 4.4×10^{-4} to 9.4×10^{-4} min^{-1}, with K_m values varying from 15 to 66 μM, making AZ-28 by far the most efficient of the isolated antibodies.

Substrate Conformational Analysis. It was expected that immunization with the equatorially substituted cyclohexanol derivative **3** would result in an antibody that restricts the acyclic substrate in a conformation resembling the pericyclic transition state (Figure 1). However, geometric differences exist between the transition state and hapten **3** that could preclude binding of the substrate in the desired conformation. For example, C2 and C5 in the transition state (for either a concerted or stepwise reaction) are sp^2 hybridized, while the C2 and C5 positions in the hapten are sp^3 hybridized. In addition, studies on the solution structure of equatorially substituted phenylcyclohexanes have shown that in the most stable conformation, the phenyl ring lies in the symmetry plane bisecting the cyclohexyl ring,[29] again geometrically different from the transition state. Consequently, the antibody bound conformation of the substrate was examined by NMR in order to demonstrate that the antibody does indeed bind the substrate in the cyclic conformation dictated by the hapten.

Structural information on rapidly exchanging bound complexes is readily available through transferred NOE spectroscopy (tr-NOE),[30] and can be compared with NMR data for the unbound substrate or ligand. For example, in a system similar to AZ-28 an antibody that catalyzes a Claisen rearrangement was shown to bind its substrate in a "high energy" conformation.[31] The use of tr-NOE spectroscopy to probe the conformation of the antibody−substrate complex requires that the ligand be in fast exchange relative to the NOE relaxation rate ($\tau_{exchange}/T_{1bound} < 0.2$).[30] To determine if the fast exchange requirement was satisfied by the AZ-28 system, line-broadening experiments were performed at low substrate/antibody stoichiometries (1:1 to 4.4:1). A titration series shows the sharpening and shifting of the substrate resonances as the substrate:antibody ratio is increased (Figure 2), confirming rapid exchange on the NMR time scale. Addition of hapten **3** (1.1 equiv relative to antibody) to displace the substrate from the antibody binding site gave the final titration point. The difference in shift between the free and bound resonances is 12 Hz, providing a lower limit of 12 s^{-1} for the substrate exchange rate.

Initial resonance assignments and studies on the feasibility of applying tr-NOE to the AZ-28 system employed 2D-NOESY spectroscopy. Spectra ($\tau_m = 600$ ms) of the free substrate **1** in the absence of antibody AZ-28 exhibit observable crosspeaks between olefinic protons and their nearest aromatic neighbors, allowing complete assignment of each olefin proton resonance. Additionally, the NOE crosspeaks are of opposite sign from the diagonal peaks, characteristic of the short correlation times observed in small molecule NMR spectroscopy. No crosspeaks were observed between the terminal olefins pairs ($H_{a,b}$ and $H_{c,d}$; see Figure 1), indicating the solution structure of **1** is approximated by an extended conformation. NOESY spectra ($\tau_m = 200$ ms) of the substrate in the presence of antibody exhibit crosspeaks of the same sign as the diagonal peaks, verifying that AZ-28 binds the substrate and greatly increases its correlation time. However, attempts to observe an NOE crosspeak between the terminal olefin pairs of the substrate in the bound

(29) Hodgson, D. J.; Rychlewska, U.; Eliel, E. L.; Manoharan, M.; Knox, D. E.; Olefirowicz, E. M. *J. Org. Chem.* **1985**, *50*, 4838−4843.

(30) Sykes, B. D. *Curr. Opin. Biotech.* **1993**, *4*, 392−396.

(31) Campbell, A. P.; Tarasow, T. M.; Massefski, W.; Wright, P. E.; Hilvert, D. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8663−8667.

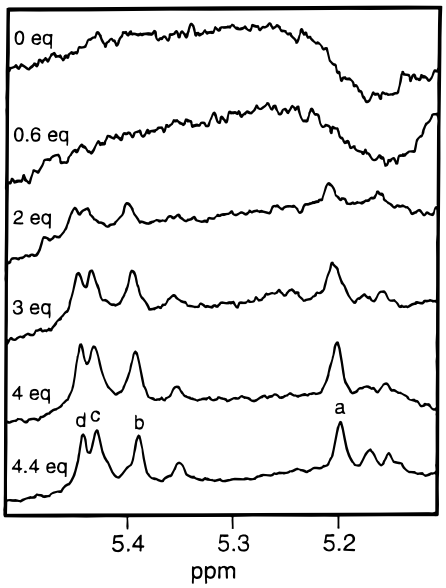


Figure 2. Spectra, from top to bottom, following the progress of a titration series of substrate **1** into antibody AZ-28. Indicated on the spectra are the equivalents of substrate present with respect to antibody concentration. The gradual appearance and steady shift of substrate resonances over the duration of the titration series confirms the fast-exchange nature of this system. The final titration point also included the addition of 1.1 equiv (with respect to antibody) of hapten **3**, to fully compete substrate out of the antibody binding site. Resonances growing in at 5.36, 5.17, and 5.15 ppm are those of product trapped by Girard's Reagent T.

state via NOESY were complicated by antibody catalysis of the rearrangement, limiting useful sample lifetime to under 5 h.

To overcome the extended data acquisition times required for 2D-NOESY analysis, 1D saturation tr-NOE experiments were performed. One-dimensional spectra of substrate **1** in the presence of antibody (10:1 ratio) clearly show an NOE between the $H_{a,b}$ and $H_{c,d}$ olefin pairs (Figure 3d,e,f). This result indicates that the terminal olefins of the substrate are in close proximity to each other (within 5 Å) in the antibody−substrate complex. In order to achieve such close proximity, the substrate must adopt a cyclic conformation, in accord with the structure of hapten **3**. The NOE intensities indicate that H_b is closer in proximity to $H_{c,d}$ than is H_a, with the possibility that transfer from H_a is mediated by spin diffusion through H_b. Saturation tr-NOE experiments were performed between the terminal olefins in both directions ($H_{a,b}$ to $H_{c,d}$ and $H_{c,d}$ to $H_{a,b}$) to rule out the possiblity that the observed NOEs are due to magnetization transfer from amino acid side chain protons within the saturation frequency envelope. One-dimensional tr-NOE experiments performed on the substrate in the presence of antibody, 20 equiv of substrate, and 1.1 equiv of hapten **3** revealed no tr-NOEs between the $H_{a,b}$ and $H_{c,d}$ olefin pairs (Figure 3c), confirming that the terminal olefins of the hexadiene are spatially distant in the unbound state and that nonspecific binding does not contribute to any NOEs observed for the antibody−substrate complex. Additionally, it was noted that the preirradiation was selective enough to avoid spillover excitation of the H_b proton when selectively saturating H_d.

To assess the contribution of spin diffusion to the observed NOEs, 1D ROE experiments were conducted under the same solution conditions used for the NOE experiments. Quantitation of the spin diffusion in this experiment was hindered by limited selectivity in excitation and $T_{1\rho}$ relaxation during the spin lock.

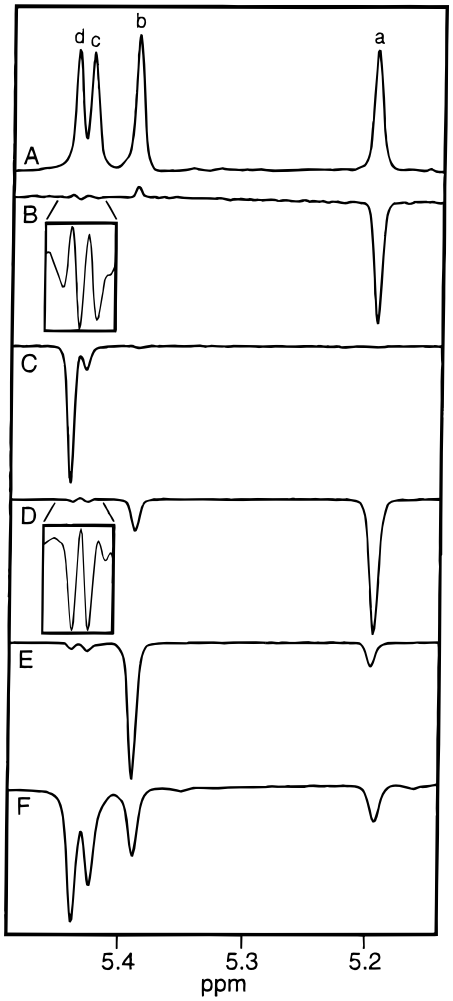


Figure 3. ^{1}H NMR spectra at 600 MHz of the olefin region of substrate **1**. (A): reference spectrum. (B,C): difference NOE spectra of substrate competitively displaced from the active-site by hapten **3** with H_a and H_d irradiated, respectively. No signal transfer is observed between olefin pairs. (D−F): difference NOE spectra of bound substrate with H_a, H_b, and H_d irradiated, respectively. Observed transfer between olefin pairs indicate the allylic termini of the substrate molecule are in close proximity while bound by the antibody. Relative NOE intensities indicate that H_b is closer in proximity to $H_{c,d}$ than is H_a. The positive amplitude of the H_b peak in B is indicative of an NOE attributable to a small molecule, confirming that substrate is effectively displaced from the binding site by hapten. In contrast, the negative amplitude of the transferred NOE's in (D), (E), and (F) result from antibody binding the substrate molecule and lowering its overall correlation time. The inset in B displays a dispersive subtraction artifact, while the inset in D highlights a tr-NOE of interest. Spectra B−F represent the difference between spectra which were acquired with 1 s of preirradiation of the indicated protons and spectrum A. The preirradiation pulse is not selective enough to avoid perturbing H_c while irradiating H_d (as evidenced by the inverted H_c peak in C). However, the pulse does resolve H_b as no spillover signal is observed in C.

However, the 1D ROE results support the conclusion that the H_b to $H_{c,d}$ transfer is direct, while that from H_a is partially via spin diffusion through H_b. In conclusion, saturation tr-NOE and tr-ROE results clearly show that the substrate is preorganized into a cyclic conformation in the antibody−substrate complex, as intended by the hapten design.

Activation Parameters. To determine the extent to which conformational restriction of the substrate in the antibody−substrate complex contributes to the energetics of catalysis, the temperature dependence of both the uncatalyzed and the

antibody-catalyzed reactions was investigated. Rates of the uncatalyzed rearrangement in 20 mM MES, 100 mM NaCl, pH 6.0, were measured at elevated temperatures between 37 and 50 °C, whereas rates of the antibody-catalyzed oxy-Cope rearrangement were determined in the same buffer at temperatures between 5 and 25 °C. The temperature dependence of the uncatalyzed rearrangement was fit to the standard Eyring relationship, affording values for the enthalpy and entropy of activation ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) of 27.4 ± 1.3 kcal/mol and −3 ± 8 cal/mol K, respectively. This value of $\Delta H^{\ddagger}$ is in reasonable agreement with reported values (32−38 kcal/mol) for similar oxy-Cope rearrangements.[32] The value for $\Delta S^{\ddagger}$, however, is higher than those found in previous studies using less polar solvents[20] and may reflect a decrease in ordered solvent around the substrate (including water hydrogen bonded to the hydroxyl group of **1**) as the electron density in **1** becomes more delocalized in the transition state.

The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the antibody-catalyzed reaction were determined to be 15.4 ± 2.4 kcal/mol and −23 ± 8 cal/mol K, respectively. These values were determined under conditions of saturating substrate (500 μM) and therefore correspond to the kinetic constant k_{cat}. This result indicates that the antibody functions primarily by lowering $\Delta H^{\ddagger}$ rather than $-T\Delta S^{\ddagger}$, a surprising result in light of the NMR structural data which indicate that the substrate is preorganized into a cyclic conformation upon binding to the antibody. These data argue that the mechanism of catalysis is more complex than the simple conformational restriction of substrate revealed by the NMR experiments.

Kinetic Isotope Effects. To gain greater insight into the mechanism of the antibody-catalyzed reaction, secondary deuterium kinetic isotope effects (KIE's) on both k_{cat} (^{D}V) and k_{cat}/K_m ($^{D}V/K$) were measured. Due to the rapid tautomerism of enol to keto form in oxy-Cope rearrangement products, the reaction is generally treated as irreversible, and to date only a single thermal retro-oxy-Cope rearrangement has been observed.[33] This irreversibility allows an $^{\alpha-D}(V/K)$ kinetic isotope effect (KIE) determined under V_{max} conditions to be interpreted as a specific measure of the isotope effect on the pericylic rearrangement, the last step prior to the tautomerization. Competition experiments to determine $^{\alpha-D}(V/K)$ were carried out between the deuterated and nondeuterated substrates **18** and **16a**, respectively, under conditions of saturating substrate in the presence of 10 μM antibody. Electron impact mass spectral analysis afforded a value of $^{\alpha-D}(V/K) = 0.72 \pm 0.03$. The magnitude of the isotope effect indicates a significant change in hybridization at the allylic termini in the transition state and is comparable to the value 0.64 ± 0.04 determined previously for the Cope rearrangement of 1,1,6,6-tetradeuterio-2,5-diphenyl-1,5-hexadiene[34] (this value is 0.80 ± 0.03 if normalized to the dideuterio compound). In the latter case, the size of these isotope effects have been interpreted as supporting a mechanism in which there is a significant degree of bonding between the allylic termini at the transition state, with the possibility of a marginally stable biradicaloid intermediate. In contrast, secondary deuterium isotope effects measured for the rearrangement of the potassium alkoxide of 3-methyl-1,5-hexadien-3-ol in organic solvents have been interpreted in terms of a mechanism involving substantial bond breaking of the C3−C4 bond and little bond making at the C1 and C6 termini.[35] The differences between our results and the previously reported isotope effects likely reflect the ability of the diphenyl substituents in **1** to stabilize a biradicaloid transition state, versus anionic weakening of the C3−C4 bond alone. Thus, it appears in the case of substrate **1** that the antibody-catalyzed reaction involves significant bond making in the transition state, consistent with an activated complex having considerable biradical character.

Kinetic isotope effects should also allow us to determine whether the chemical rearrangement step is partially or wholly rate limiting in the antibody-catalyzed reaction, as no significant kinetic isotope effect would be expected for either isomerization of the enol product to the aldehyde or the release of product from the binding site. In order to directly measure the effects of a secondary isotope effect on the rate of the antibody-catalyzed reaction, substrate **23** with deuterium substitution on both allylic termini was synthesized. The $^{\alpha-D}V$ KIE was determined to be 0.61 ± 0.1 under conditions of saturating substrate (1 mM) at room temperature, again similar to values determined by Gajewski for the analogous Cope rearrangement[34] (this value normalizes to 0.78 ± 0.1 per D_2 for comparison with the $^{\alpha-D}(V/K)$ KIE determined in the competition experiments). The similarity of the $^{\alpha-D}V$ and $^{\alpha-D}(V/K)$ KIEs indicates that the transition state is the same for both k_{cat} and k_{cat}/K_m, further confirming that the chemical rearrangement step contributes significantly to the observed rate of the antibody-catalyzed reaction. Thus, the measured activation parameters correspond to the energetics of the pericyclic rearrangement step rather than to a product dissociation step, a result consistent with the observed exchange kinetics (given the substrate and oxime-trapped product exchange rates, >12 s^{-1} and >45 s^{-1}, respectively, it would be surprising if product release was rate determining in this reaction).

Mechanistic Considerations. The three-dimensional crystal structure of the recombinant AZ-28 Fab-hapten **3** complex determined to 2.6 Å resolution has recently been reported, contributing to a complete picture of catalysis in antibody AZ-28.[2] The active site is shown in Figure 4 and described briefly below as a framework for the mechanistic discussion that follows. The structure reveals that the hapten is bound in a deep cylindrical cavity in the antibody combining site and retains the chair-like geometry expected in the ground state of an equatorially substituted cyclohexanol. The phenyl substituents of hapten **3** are rotated with respect to each other by a dihedral angle of 17 ° and make extensive contacts with the active site residues. The 5-phenyl group of hapten **3** is tightly packed at the bottom of the cavity and surrounded by a large number of aromatic and hydrophobic side chains. The 2-phenyl substituent is located near the opening of the binding pocket and its orientation is fixed by a π-stacking interaction with the imidazole ring of HisH96 (heavy-chain histidine 96) and van der Waals interactions with the side chain of TyrL91 (light-chain tyrosine 91). The cyclohexyl ring of hapten **3** is rotated out of the planes of the 5- and 2-phenyl rings by 81° and 85°, respectively, an expected conformation based on known structures of phenylcyclohexanes.[29] Its position is fixed by hydrogen-bonding interactions between the hydroxyl substituent of hapten **3** and both the imidazole ring and backbone amide NH group of HisH96. There are also hydrogen-bond contacts with the carboxylate groups of GluH35 and GluH50, with the latter interaction bridged by a water molecule. The cyclohexyl ring

(32) Evans, D. A.; Golob, A. M. *J. Am. Chem. Soc.* **1975**, *97*, 4765.
(33) Elmore, S. W.; Paquette, L. A. *Tetrahedron Lett.* **1991**, *32*, 319−322.
(34) Gajewski, J. J.; Conrad, N. D. *J. Am. Chem. Soc.* **1979**, *101*, 6693−6704.
(35) Gajewski, J. J.; Gee, K. R. *J. Am. Chem. Soc.* **1991**, *113*, 967−971.

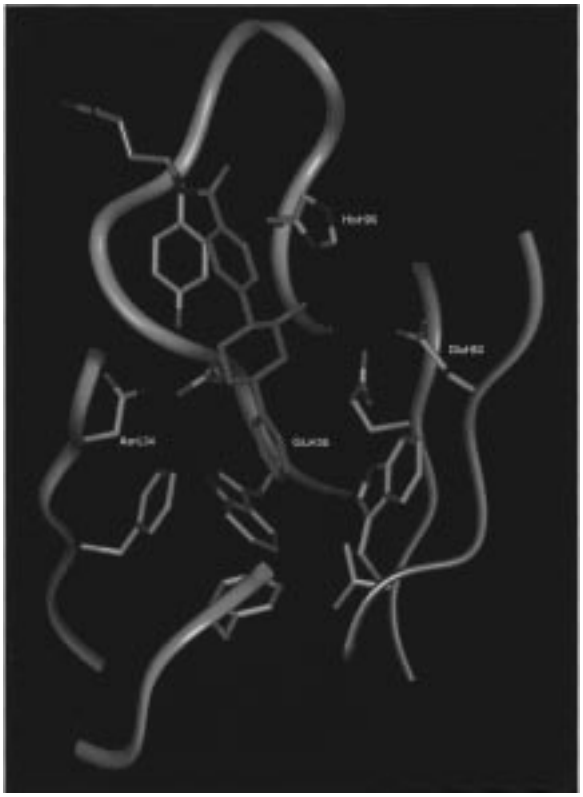


Figure 4. Active site of AZ-28 Fab-hapten **3** crystal structure showing the orientation of the 2- and 5-phenyl substituents relative to the cyclohexadienol core, as well as the position of key residues which contact the hapten. Side chains and peptide backbone are brown, and the hapten is lavender. Heteroatoms are color-coded blue for nitrogen and red for oxygen.

of hapten **3** is also in van der Waals contact with the hydrophobic side chains of the light chain and the hydrophilic side chains of AsnL34 and AspH101.

In the context of this structural information, the NMR and kinetic experiments described above allow one to formulate the following mechanistic proposal for the antibody-catalyzed reaction. The substrate, which exists predominantly in an extended conformation in solution, is bound in a rapid, reversible step in the active site and its conformation is fixed in a cyclic geometry. This conformational restriction likely involves extended interactions with the aryl substituents, which orient the C2 and C5 carbons, as well as hydrogen-bonding interactions with the hydroxyl group of **1**. The latter interactions are consistent with the kinetic preference of the antibody for the (*S*)-substrate stereochemistry at C3. Binding interactions with the active site thus lead to a cyclic alignment of the $4\pi + 2\sigma$ orbital system of the hexadiene core, contributing to the overall rate enhancement of the antibody-catalyzed reaction.

The V_{max} kinetic isotope effect experiments indicate that the [3,3]-sigmatropic rearrangement is rate limiting in the antibody-catalyzed reaction, while the magnitude of the $^{\alpha-D}V/K$ secondary isotope effect points to a significant degree of bond formation between carbons C1 and C6 in the transition state. As discussed above, this effect can arise from either a biradicaloid transition state leading to a resonance stabilized 1,4-biradical intermediate or from a concerted reaction in which bond breaking and formation occur simultaneously in a single transition state. In the case of the Cope rearrangement, it is known that phenyl substituents at the 2 and 5 positions of 1,5-hexadiene reduce the activation energy by approximately 10 kcal/mol relative to the unsubstituted diene,[21] attributable to conjugation of electron density at C2 or C5 with the aromatic rings in a transition state with considerable biradicaloid character. In the crystal structure of the antibody−hapten **3** complex, the two phenyl substituents of **3** bind in an almost parallel orientation with respect to each other, allowing both to conjugate with the $4\pi + 2\sigma$, orbital system simultaneously. However, if substrate **1** binds to AZ-28 in the same conformation as the hapten in the crystal structure, then the 1,5-hexadiene group must rotate with respect to the phenyl substituents to achieve conjugative stabilization in the transition state. This rotation would require reorganization of the extended network of van der Waals and hydrogen-bonding interactions between substrate and the active site residues and is consistent with the large negative entropy of activation observed for the k_{cat} step.

This interpretation is consistent with immunological studies on the affinity maturation of AZ-28 from its germline precursor. The germline-encoded antibody binds hapten **3** with 40-fold lower affinity and has a rate enhancement (k_{cat}/k_{uncat}) of 163 000.[2] This difference in rate largely results from the somatic mutation of active site serine 34L in the germline antibody to an asparagine in AZ-28. Asparagine 34L directly contacts the cyclohexyl ring and also interacts with a number of residues that help fix the conformation of bound hapten by fixing the configuration of complementarily determining region (CDR) 3 of the heavy chain. Thus, this residue might be expected to play a significant role in restricting substrate rotation around the C2 and C5 aryl bonds. Consequently, mutation of Asn 34L in AZ-28 to serine may facilitate rotation about these bonds, increasing the bound concentration of the favorable rotamer and enchancing k_{cat} in the germline antibody. Consistent with this model, changes in the structure of CDR 3 of the heavy chain, which plays an important role in fixing the orientation of the 2-phenyl substituent, also lead to large variations in catalytic activity in a series of related antibodies.[36] While it is unclear whether the enhanced rate of the germline-encoded antibody results from improved overlap between the hexadiene system and one or both aryl substituents, reorganization in the case of AZ-28 likely involves rotation of the hexadiene core and one or both of the aryl rings relative to each other.

In addition to this π-overlap of the aryl substituents, electronic effects arising from the 3-hydroxy substituent may lower the enthalpy of the [3,3]-sigmatropic rearrangement reaction. The anionic substituent effect accelerates the oxy-Cope rearrangement through hyperconjugation of electron density on oxygen.[22] Consequently, the side chains HisH96, GluH35, and GluH50, which hydrogen bond to the 3-hydroxyl group of the substrate, might influence the rate of the rearrangement by increasing or decreasing the electron density on the oxygen substituent of the substrate. In the case of anionic catalysis of the oxy-Cope rearrangement of 3-methyl-1,5-hexadien-3-ol in solution, a small secondary deuterium kinetic isotope effect is observed for isotopic substitution at the allylic termini (approximately 0.96 $\pm$ 0.05).[35] However, the magnitude of the C1−C6 isotope effect in a C2,C5-aryl-substituted hexadienol undergoing anionic catalysis has never been studied and may be closer to the value observed here as a result of the aryl substitution.

The final steps in the antibody-catalyzed reaction involve tautomerism of the resulting enol group to an aldehyde and release of the product, both of which are rapid in comparison to the rate of the rearrangement reaction. It is necessary to trap this aldehyde with an added nucleophile to prevent time-dependent inactivation (see above), presumably due to imine formation between antibody and product. It has not been

(36) Ulrich, H. D.; Schultz, P. G. *J. Mol. Biol.* In press.

determined whether the antibody contains a specific site for hydroxylamine binding or whether the trapping reaction occurs in the active site or after diffusion of substrate from the active site. Ultimately, this trapping reaction could provide the basis for a screen of AZ-28 mutants with enhanced catalytic activity.

The mechanistic proposal presented here suggests a number of experiments both to test this model and to enhance the efficiency of the AZ-28-catalyzed reaction. Specifically, modifications to the hapten structure which restrict the aryl substituents to be coplanar with the cyclohexyl ring may afford antibodies with increased rates. In addition, site-directed mutagenesis experiments which convert HisH96, GluH35, and GluH50 to residues with modified hydrogen-bonding capacities should provide insight into the role of electron density on the oxygen of the 3-hydroxyl substituent in catalysis. Determination of activation parameters for the germline antibody would help to validate the mechanistic model, as would the crystal structure of the germline antibody. Several of these investigations are currently underway.

Finally, the mechanism of antibody AZ-28 can be compared in general to those of other proteins that catalyze [3,3]-sigmatropic rearrangements. Structural and mechanistic studies have been carried out on one antibody (1F7)[37] and three enzymes (*Bacillus subtilis*, *Escherichia coli*, and yeast chorismate mutases)[38,39] that catalyze the [3,3]-sigmatropic rearrangement of chorismate to prephenate. Similar studies have been carried out on an antibody which catalyzes a Diels−Alder reaction (39A11).[40] In each of these cases, the protein appears to preorganize the substrate into a reactive conformation by a network of hydrogen-bonding, electrostatic, and van der Waals interactions. In the case of AZ-28, however, additional unanticipated conformational adjustments appear to be required to reach the transition state. In the active sites of the *B. subtilis* chorismate mutase and antibody 1F7, hydrogen bonds from an active site arginine to the enol ether oxygen of chorismate have been postulated to stabilize the developing charge at this center in the transition state. Similarly, the crystal structure of the 39A11 Fab−hapten complex shows a specific hydrogen-bonding interaction which likely serves to reduce the electron density on the substrate dienophile and accelerate the antibody-catalyzed reaction. In the case of AZ-28, the role of active site hydrogen bonding is less clear, and further studies will be required to determine if hydrogen-bonding interactions with the 3-hydroxy substituent of the substrate serve to accelerate or retard the reaction. It appears, however, that, for all of these pericyclic reactions, Diels−Alder, Claisen, and oxy-Cope, catalysis involves both the restriction of rotational and translational entropy in the substrate, as well as hydrogen-bonding interactions which modulate electron densities on key substituents in the transition state.

The studies reported here demonstrate the role antibodies can play in understanding biological catalysis, particularly when information from small-molecule NMR is combined with more traditional kinetic and structural probes of enzyme mechanism. They also provide a basis for further improvement in the rates of antibody-catalyzed reactions.

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(37) Haynes, M. R.; Stura, E. A.; Hilvert, D.; Wilson, I. A. *Science* **1994**, *263*, 646−652.

(38) Lee, A. Y.; Stewart, J. D.; Clardy, J.; Ganem, B. *Chem. Biol.* **1995**, *2*, 195−203.

(39) Guilford, W. J.; Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5013−5019.

(40) Romesberg, F. R.; Spiller, B.; Schultz, P. G.; Stevens, R. C. Submitted for publication.