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Antibody Catalysis of Multistep Reactions: An Aldol Addition Followed by a Disfavored Elimination

Thomas Koch, Jean-Louis Reymond,* and Richard A. Lerner*

Contribution from the Departments of Molecular Biology and Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

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Abstract: The intramolecular aldol condensation of keto-aldehyde **1** yields a substituted 2-benzyl-3-hydroxy-cyclohexanone **2** and subsequently 2-benzyl-2-cyclohexenone (**3**). The sequence involves four individual reaction steps. Three of these steps can be accelerated using general acid–base catalysis to effect proton transfer at or near the α-carbon of the ketone involved in the condensation, which is at the homobenzylic position relative to the aromatic group of the substrate (Ar). An antibody to the corresponding *N*-benzyl-*N*-methylpiperidinium hapten **5** was found to catalyze the entire reaction sequence. This antibody seems to act purely as a general base and does not catalyze the carbon–carbon bond forming step. Catalysis of the aldol elimination is selective for the disfavored *trans*-elimination with a single enantiomer of stereoisomer **2a**. Catalysis is suppressed by incubating the antibody with a carboxyl-specific reagent, suggesting that a carboxyl group acts as a general base to catalyze the sequence. The antibody is approximately 2.0×10^5 times more reactive than acetate for catalysis of the sequence. These experiments demonstrate that catalysis of reactions with several consecutive transition states is possible using catalytic antibodies.

Introduction

One of the attractive properties of antibody catalysis is the fact that specific features of the catalyst are induced by the experimenter, thus allowing strategic positioning of critical functionalities in the binding pocket. This has lead to the generation of many diverse catalysts, many of which are highly efficient and/or may catalyze different reactions depending on the choice of the substrate.[1] However, most catalytic antibodies catalyze single-step transformations in which the first, and often only, bond-making or bond-breaking event is rate determining. Given that many important chemical transformations represent sequences of steps with several consecutive high-energy transition states, each of which must be stabilized in order to obtain catalysis, we wished to expand the catalytic antibody methodology to encompass such multistep reactions. We report here the first antibody-catalyzed intramolecular aldol condensation which proceeds via sequential stabilization of three distinct transition states.[2,3]

Results and Discussion

The intramolecular aldol condensation of keto-aldehyde **1** in water (pH > 7) yields aldols **2a,b** and subsequently enone **3** as the only detectable products (Scheme 1). Since formation of **3** is negligible (<1%) below 10% conversion of **1** to **2**, aldolization (**1** to **2**) and elimination (**2** to **3**) can be followed separately. The aldolization is catalyzed by buffers with saturation kinetics (Figure 1).[4] In dilute buffer, enolization (step A) is rate limiting[5] and the reaction is first order in buffer, which acts as a general base to catalyze that step.[6] In concentrated buffer, enolization

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(1) (a) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659–667. (b) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391.

(2) Earlier attempts to catalyze multistep processes with antibodies were not successful, see: Cochran, A. G.; Pham, T.; Sugasawara, R.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 6670 and references cited therein.

(3) While this work was in progress, catalysis of a similar aldol condensation by cyclodextrins was reported: Desper, J. M.; Breslow, R. *J. Am. Chem. Soc.* **1994**, *116*, 12081.

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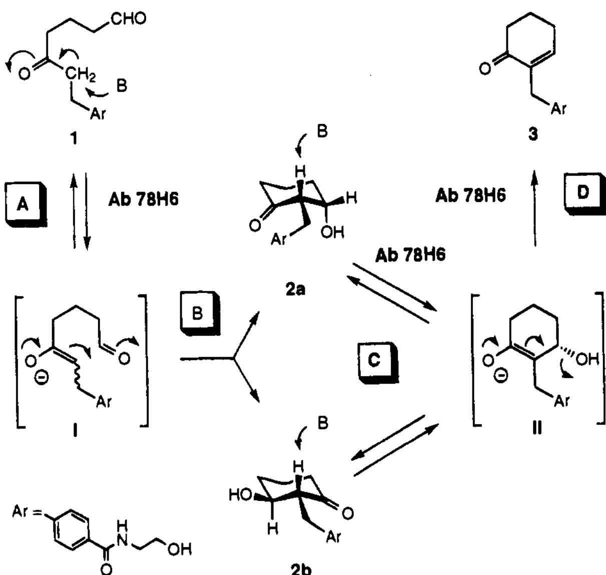
Scheme 1. Antibody 78H6 (anti-**4**) Catalyzes Three of the Four Individual Steps Involved in the Aldol Condensation of Keto-Aldehyde **1** to Aldols **2a,b** and Enone **3**



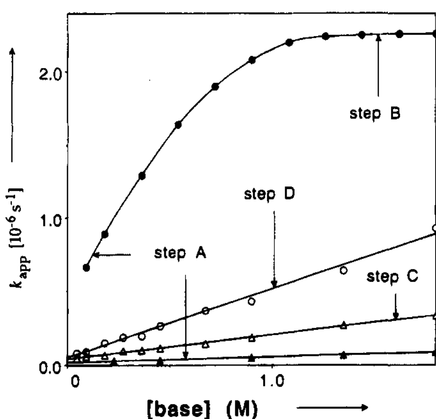


Figure 1. Apparent first-order rate constants for the initial rate of aldolization and elimination as a function of total buffer concentration at 20 °C: (▲) aldolization (**1** → **2**) with sodium acetate at pH = 7.50; (●) aldolization (**1** → **2**) with triethanolamine at pH 7.90; (△) interconversion (**2a** → **2b**); and (○) elimination (**2a** → **3**) with sodium acetate at pH = 7.50. Measured at 20 °C with [**1**] or [**2a**] = 250 μM. The ionic strength was held constant at 1.8 M using NaCl. See also footnote 4.

is fast and the carbon−carbon bond formation (step B) becomes rate limiting. This step is not general acid−base catalyzed, and the reaction shows zero order in buffer.[7] Under the influence of buffers, aldols **2a** and **2b** interconvert into each other (stepC) and eliminate to enone **3** (step D) but do not revert to ketone **1**. The ratio of interconversion (C) to elimination (D) depends on the nature and concentration of the buffer.[8] Quite unusually, the *trans*-elimination of **2a** is strongly disfavored relative to the *syn*-elimination of **2b**.[9] These observations imply that elimination of **2** to **3** proceeds by an ElcB mechanism with two independent steps (C and D) separated by a stable intermediate **II**.[10]

In the reaction described above, three out of the four steps in the sequence require general acid−base catalysis at or near the homobenzylic position relative to the aromatic nucleus (Ar). Among the growing repertoire of antibody catalysts there are several that were induced against haptens **4**−**6** and are known to have a functionality that promotes efficient acid−base catalysis in the homobenzylic region of a variety of substrates.[11] We reasoned that some of these antibodies might catalyze the reaction sequence by virtue of an induced general acid−base residue (presumably a carboxyl group) promoting these steps.[12] Furthermore, the piperidine ring in these haptens ensured that the antibodies would accommodate the aldol product **2a** or **2b**.

A set of monoclonal antibodies (22 anti-**4**, 24 *anti*-**5**, and 13 *anti*-**6**) was first assayed for catalysis of the formation of aldol **2** from **1**. Two *anti*-**4** antibodies catalyzed the reaction.

(4) Saturation was observed for catalysis of aldolization of **1** to **2** by phosphate (pH 6.90), *N*-methyl morpholine (pH 7.50), and triethanolamine (pH 7.90, Figure 1). The reaction rates also increased with higher ionic strength. Therefore, the rate obtained by extrapolation to zero buffer concentration at 1.8 M ionic strength (Figure 1) is higher than that observed in the presence of 25 mM Bistris and 100 mM NaCl in the antibody assay (Figure 2).

(5) Whether or not initially formed by the deprotonation step, the enolate of either **1** or **2** is in diffusion-controlled equilibrium with its O-protonated enol form, an equilibrium which is much faster than any of the steps discussed here and governed by the pH. The neutral enol form is predominant at pH 7.5 (e.g., pK_a (enol) > 10, see: Chiang, Y.; Kresge, A. J.; Tang, Y. S.; Wirz, J. *J. Am. Chem. Soc.* **1984**, *106*, 460). Nevertheless, the enolate should be more reactive for either C−C bond formation (step B) or β-elimination (step D).

(6) Reversible enolization presumably also takes place on the other side of the ketone and at the α-position of the aldehyde, but without further reaction.

(7) Aldol condensations are not catalyzed by general acid−bases when the C−C bond forming step is rate limiting, that is under conditions of fast preequilibrium enolization. Notable exceptions are primary and secondary amines which catalyze aldol condensations via enamine intermediates. (a) Fischer, F. G.; Marschall, A. *Ber. Dtsch. Chem. Ges.* **1931**, *64*, 2825. (b) Langenbeck, W.; Borth, G. *Ber. Dtsch. Chem. Ges.* **1942**, *75*, 951. (c) Spencer, T. A.; Neel, H. S.; Flechtner, T. W.; Zayle, R. A. *Tetrahedron Lett.* **1965**, 3889. (d) Gutsche, C. D.; Redmore, D.; Buriks, R. S.; Nowotny, K.; Grassner, H.; Armbruster, C. W. *J. Am. Chem. Soc.* **1967**, *89*, 1235.

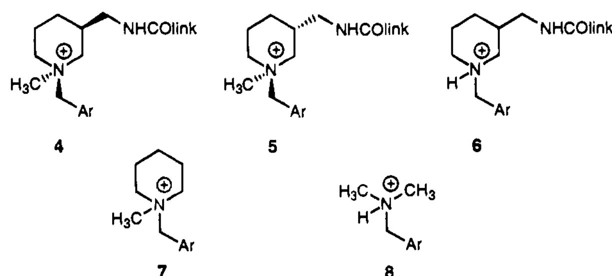
(8) For isomer **2a**, the ratios of elimination (**2a** → **3**) to interconversion (**2a** → **2b**) are 4.5:1 (100 mM triethanolamine, pH 7.8), 1.6:1 (100 mM *N*-methyldiethanolamine, pH 9.0), 1:1.5 (100 mM phosphate, pH 6.9), and 5:1 (100 mM acetate, pH 7.5). The rate constants for triethanolamine catalysis at pH 9.0 (calculated for total buffer concentration) are k(**2b** → **2a**) = 1.5×10^{-5} M^{-1} s^{-1}, k(**2b** → **3**) = 3.2×10^{-5} M^{-1} s^{-1}. k(**2a** → **2b**) = 2.9×10^{-6} M^{-1} s^{-1}, and k(**2a** → **3**) = 7.8×10^{-6} M^{-1} s^{-1}.

(9) Both **2a,b** are in a chair conformation in solution with the benzylic substituent in α-position to the ketone in equatorial position. The stereochemistry of **2a,b** is assigned unambiguously from the ^3J coupling constants of H-C(3) at the secondary alcohol center. (**2a**: δ 4.07 ppm, br s. **2b**: δ 3.47 ppm, td, ^3J = 10.1, 4.0 Hz.)

(10) For such a mechanism, the reactivity of each aldol isomer depends on its thermodynamic stability relative to this intermediate. Ground state stabilization by *trans*-diaxial electronic interactions between the axial α C−H bond and the axial β C−O bond in **2a** might explain the unusual unreactivity of the *trans*-isomer **2a** in this elimination. For detailed kinetic studies on general acid−base catalyzed aldol eliminations, see: (a) Hupe, D. J.; Kendall, M. C. R.; Sinner, G. T.; Spencer, T. A. *J. Am. Chem. Soc.* **1973**, *95*, 2260. (b) Fedor, L. R.; Glave, W. R. *J. Am. Chem. Soc.* **1971**, *93*, 985.

(11) (a) Reymond, J.-L.; Janda, K. D.; Lerner, R. A. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1711. (b) Reymond, J.-L.; Janda, K. D.; Lerner, R. A. *J. Am. Chem. Soc.* **1992**, *114*, 2257. (c) Reymond, J.-L.; Jahangiri, G. K.; Stoudt, C.; Lerner, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 3909. (d) Sinha, S. C.; Keinan, E.; Reymond, J.-L. *J. Am. Chem. Soc.* **1993**, *115*, 4893. (e) Sinha, S. C.; Keinan, E.; Reymond, J.-L. *Proc. Natl. Acac. Sci. U.S.A.* **1993**, *90*, 11910. (f) Koch, A.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1994**, *116*, 803. (g) Reymond, J.-L.; Reber, J.-L.; Lerner, R. A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 475. (h) Chen, Y.; Reymond, J.-L.; Lerner, R. A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1607. (i) Jahangiri, G. K.; Reymond, J.-L. *J. Am. Chem. Soc.* **1994**, *116*, 11264. (k) Shabat, D.; Itzaky, H.; Reymond, J.-L.; Keinan, E. *Nature* **1995**, *374*, 143. (l) Sinha, S. C.; Keinan, E. *J. Am. Chem. Soc.* **1995**, *117*, 3653. (m) Reymond, J.-L.; Chen, Y. *Tetrahedron Lett.* **1995**, *36*, 2575.

(12) For other haptens inducing general acid−base functionality in catalytic antibodies, see: (a) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Nature* **1989**, *338*, 269. (b) Janda, K. D.; Weinhouse, M. I.; Schloeder, D. M.; Lerner, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 1274. (c) Jackson, D. Y.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2319. (d) Uno, T.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 6573. (e) Shokat, K.; Uno, T.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2261. (f) Cravatt, B.; Ashley, J.; Janda, K. D.; Boger, D. L.; Lerner, R. A. *J. Am. Chem. Soc.* **1994**, *116*, 6013. (g) Thorn, S. N.; Daniels, R. G.; Auditor, M.-T. M.; Hilvert, D. *Nature* **1995**, *373*, 228.



Catalysis was quantitatively inhibited by hapten **7**, demonstrating that the reaction was taking place specifically in the antibody combining site. One of them, antibody 78H6, was characterized in detail. Catalysis followed Michaelis–Menten kinetics (Figure 2). Analysis of product **2** on chiral HPLC columns revealed that the antibody catalyzed the formation of all four aldol stereoisomers in equal amounts, suggesting that the antibody did not catalyze the chirality-inducing aldol step B and acted solely as general base in step A. Antibody 78H6 also catalyzed the elimination reaction to enone **3**, now with complete stereoselectivity for the disfavored *trans*-elimination on a single enantiomer of isomer **2a**. The catalyzed elimination was not only *stereo-* and *enantioselective* but also *chemo*-selective in that interconversion of **2a** and **2b** was not catalyzed by the antibody. This implied that antibody 78H6 catalyzed both the enolization (step C) and the ensuing β-elimination of intermediate **II** (step D), possibly in concerted fashion.[13]

The dual catalysis observed with antibody 78H6 suggested that both catalytic activities might result from a single general acid–base residue induced in response to the positive charge in the hapten. Covalent modification experiments were carried out to substantiate this hypothesis. While 70% of the catalytic activity of antibody 78H6 was abolished by incubation with the carboxyl-specific reagent EDC/1 M glycine methyl ester,[14] only 30% of the activity disappeared under the same conditions in the presence of 1 mM **8**, a weak active-site-specific inhibitor of this antibody. On that basis it is reasonable to propose that catalysis is triggered by a carboxyl residue within the antibody active site.

Further evidence for a simple general base catalysis in the antibody reaction was obtained by excluding covalent catalysis by an imine mechanism. Aldolase enzymes catalyze aldol condensations by using the ϵ-amino group of a lysine residue as catalytic residue, which engages in covalent catalysis via imine and enamine intermediates.[15] Imine catalysis of aldol eliminations by simple amines is also well documented.[16] These catalysts are very rapidly and irreversibly inhibited by covalent modification of the imine intermediate by reduction with $NaBH_4$ or $NaBH_3CN$ to form the corresponding alkylated amine or with cyanide to form an amino nitrile.[17] Following these published procedures, antibody 78H6 was incubated with $NaBH_3CN$ at pH 7.4 in the presence of either aldehyde **1** (400 μM) or aldol **2a** (500 μM) and then purified by gel filtration and assayed for catalysis of the aldolization of **1** to **2**. In both cases, no loss of catalytic activity could be detected for antibody 78H6, despite the fact that the antibody active site was approximately two-thirds saturated with each substrate under the reduction conditions. Furthermore, the aldol elimination step of **2a** to **3** was assayed at pH 7.6 in the presence of cyanide (15 mM). No loss of activity could be detected over a period of several days. These experiments clearly showed that antibody 78H6 catalysis could not be explained on the basis of an enamine mechanism involving covalent intermediates.

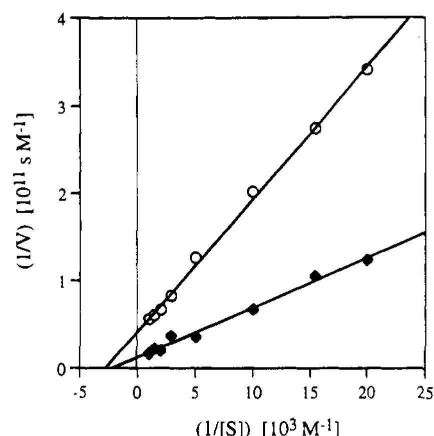


Figure 2. Lineweaver–Burk plot of reaction rates: (○) aldolization (**1** → **2**), $k_{cat} = 2.4 \times 10^{-6}$ s^{-1}, $K_M = 360$ μM; (◆) elimination (**2a** → **3**), $k_{cat} = 8.2 \times 10^{-6}$ s^{-1}, $K_M = 470$ μM. Measured in 25 mM Bistris buffer, 100 mM NaCl at pH 7.50, with 10 μM antibody 78H6. Under these conditions, catalysis by the buffer is negligible; the background reaction is due to hydroxide catalysis and amounts to $k_{app}(OH^-) = 1.2 \times 10^{-8}$ s^{-1} for aldolization of **1** to **2** and $k_{app}(OH^-) = 2.1 \times 10^{-8}$ s^{-1} for the elimination of **2a**. See also footnote 4.

Antibody 78H6 is an efficient general base catalyst. Its reactivities toward the substrates, given by the specificity constants $k_{cat}/K_M = 0.0065$ M^{-1} s^{-1} for reaction with **1** and $k_{cat}/K_M = 0.017$ M^{-1} s^{-1} for reaction with **2a**, are 2.0×10^5 and 3.6×10^4, respectively, times that of acetate in solution, $k_{AcO^-} = 3.3 \times 10^{-8}$ M^{-1} s^{-1} for the aldolization of **1** and $k_{AcO^-} = 4.7 \times 10^{-7}$ M^{-1} s^{-1} for the elimination of **2a**. Since the aldol step B is not catalyzed, it might be rate limiting for the antibody. Antibody 78H6 probably achieves maximum efficiency as a general acid–base catalyst for aldolization of **1** to **2**, as suggested by the similarity between the reaction rate under antibody catalysis ($k_{cat} = 2.4 \times 10^{-6}$ s^{-1}) and at saturating concentration of general bases ($k_{inf} = 2.2 \times 10^{-6}$ s^{-1}, Figure 1). The relatively lower catalytic efficiency for elimination ($(k_{cat}/k_M)/k_{AcO^-} = 3.6 \times 10^4$) compared to aldolization ($(k_{cat}/K_M)/k_{AcO^-} = 2.0 \times 10^5$) might be due to unfavorable interactions of the bulkier aldol substrate within the antibody active site, which could prevent its optimal alignment with the general base for enolization.

Conclusion

In summary, we have shown that antibody 78H6 catalyzes three out of four steps in the aldol condensation sequence leading from **1** to **3**, each of which represent significant kinetic barriers along the reaction pathway. Most significantly, this multistep catalysis was induced with a single transition state analog motif, which is the positive charge in hapten **4**. These experiments demonstrate that a single haptenic design can induce catalytic antibodies stabilizing several consecutive transition states along a reaction pathway and that multistep transformations are indeed within reach of antibody catalysis.

(13) This stands in contrast to enzymes, which usually catalyze aldol eliminations with *syn*-selectivity. Gertl, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928 and references cited therein.

(14) Hoare, D. G.; Koshland, D. E. *J. Am. Chem. Soc.* **1966**, *88*, 2058.

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(16) Studies on imine-mediated aldol β-eliminations: (a) Spencer, T. A.; Kendall, M. C. R.; Hupe, D. J. *J. Am. Chem. Soc.* **1972**, *94*, 1254; **1973**, *95*, 2271. (b) Spencer, T. A.; Gula, M. J.; Ferran, H. E.; Roberts, R. D. *J. Am. Chem. Soc.* **1980**, *102*, 7054. See also: (c) Gassman, P. G.; Gerlt, J. A. *J. Am. Chem. Soc.* **1993**, *115*, 11552.

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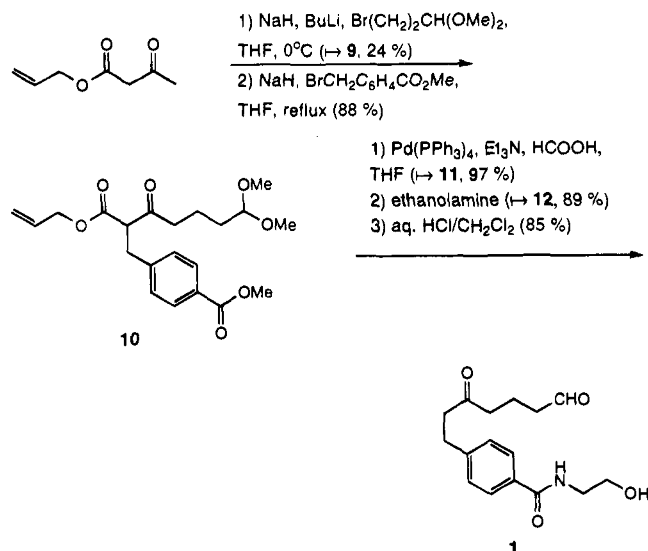
Scheme 2. Synthesis of Substrate **1** Proceeds in Five Steps from Allyl Acetoacetate



Experimental Section

A. Synthesis. General Remarks. Reagents were purchased from Aldrich or Fluka. Solvents were A.C.S. grade from Fisher. All chromatographies (flash) were performed with Merck Silicagel 60 (0.040−0.063 mm). Preparative HPLC was done with Fisher Optima grade acetonitrile and ordinary deionized water using a Waters prepak cartridge 500g installed on a Waters Prep LC 4000 system from Millipore, flow rate 100 mL/min, gradient +0.5% min^{-1} CH_3CN, detection by UV at 254 nm. TLC was performed with fluorescent Merck F254 glass plates. NMR spectra were recorded on a Brucker AM-300 MHz or AM-500 MHz instrument. Chemical shifts δ are given in ppm and coupling constant ^3J or ^2J in hertz. MS and HRMS (high-resolution mass spectra) were provided by the Scripps Research Institute facility (Gary Siuzdak). Gel filtration was performed with Pharmacia Sephadex PD-10 (G-25M) columns.

Ketoaldehyde **1** was prepared from allyl acetoacetate in a five-step sequence outlined in Scheme 2.

Allyl 7,7-Dimethoxy-3-oxoheptanoate (9). NaH (156 mg, 6.5 mmol) was combined with allyl acetoacetate (853 mg, 6 mmol) in 25 mL of THF. The mixture was stirred for 5 min at room temperature and cooled to 0 °C, and BuLi (2.5 M, 6.3 mmol) was added. After further stirring for 10 min at 0 °C, 3-bromopropionaldehyde dimethyl acetal (1.10 g, 6 mmol) was added and the reaction mixture was allowed to warm to room temperature. The reaction was terminated after 20 h by quenching with 2 mL of aqueous NH_4Cl solution. The mixture was then poured on water and extracted with CH_2Cl_2. The organic layer was dried and the solvent removed under reduced pressure. The residue obtained was purified by flash chromatography (hexane/ethyl acetate, 2:1) to give **9** as a colorless oil (348 mg, 1.43 mmol, 24%). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.94-5.83 (m, 1H), 5.36-5.22 (m, 2H), 4.64−4.60 (m, 2H), 4.33 (t, J = 5.5 Hz, 1H), 3.45 (s, 2H), 3.29 (s, 6H), 2.57 (t, J = 6.7 Hz, 2H), 1.70−1.56 (m, 4H).

Allyl 7,7-Dimethoxy-2-((4′-(methoxycarbonyl)phenyl)methyl)-3-oxoheptanoate (10). NaH (34 mg, 1.41 mmol) was added to a solution of **9** (312 mg, 1.28 mmol) in 8.5 mL of THF. After 5 min, methyl 4-(bromomethyl)benzoate (295 mg, 1.28 mmol) was added and the reaction mixture was heated to reflux for 3.5 h. The mixture was cooled, treated with aqueous NH_4Cl solution, poured on water, and extracted with CH_2Cl_2. The organic layer was dried and the solvent removed under reduced pressure. Purification of the crude material by flash chromatography (hexane/ethyl acetate, 3:1) gave **10** as a colorless oil (440 mg, 1.12 mmol, 88%). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.93−7.88 (m, 2H), 7.24−7.11 (m, 2H), 5.86−5.73 (m, 1H), 5.27−5.14 (m, 2H), 4.62−4.54 (m, 2H), 4.28 (t, J = 5,5 Hz, 1H), 3.87 (s, 3H), 3.79 (t, J = 7.6 Hz, 1H), 3.31−3.12 (m, 2H), 3.26 (s, 6H), 2.59−2.53 (m, 1H), 2.39−2.23 (m, 1H), 1.64−1.44 (m, 4H).

Methyl 4-(7′,7′-Dimethoxy-3′-oxohept-1′-yl)benzoate (11). To a solution of **10** (130 mg, 0.33 mmol) in 1.5 mL of THF were added Et_3N (115 μL, 0.83 mmol), formic acid (25 μL, 0.66 mmol), and a trace of $Pd(PPh_3)_4$. After stirring at 30 °C for 40 min, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (hexane/ethyl acetate, 3:1) to give **11** as a colorless oil (98 mg, 0.32 mmol, 97%). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.92 (d, J = 8.1, 2H), 7.26 (d, J = 8.1, 2H), 4.30 (t, J = 5.2, 1H), 3.87 (s, 3H), 3.27 (s, 6H), 2.91 (t, J = 7.4 Hz, 2H), 2.71 (t, J = 7.4, 2H), 2.39 (t, J = 6.8 Hz, 2H), 1.61−1.49 (m, 4H).

4-(7′7′-Dimethoxy-3′-oxohept-1′-yl)-*N*-(2′-hydroxyethyl)benzamide (12). A solution of **11** (82 mg, 0.27 mmol) in ethanolamine (2 mL) was stirred vigorously for 2 days. The reaction mixture was separated directly by flash chromatography (5% MeOH in CH_2Cl_2) to give **12** as colorless oil (81 mg, 0.24 mmol, 89%). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.71 (d, J = 8.3, 2H), 7.25 (d, J = 8.3, 2H), 6.78 (t, br., 1H), 4.33 (t, J = 5.5 Hz, 1H), 3.83 (t, J = 4.7, 2H), 3.62 (q, J = 4.7, 2H), 3.31 (s, 6H), 2.95 (t, J = 7.4, 2H), 2.75 (t, J = 7.4 Hz, 2H), 2.43 (t, J = 6.8 Hz), 1.64−1.54 (m, 5H).

4-(2′,7′-Dioxohept-1′-yl)-*N*-(2′-hydroxyethyl)benzamide (1). A biphasic mixture of **12** (18 mg, 0.053 mmol) in 2 mL of CH_2Cl_2 and 5 mL of aqueous 1 N HCl was stirred vigorously. After 30 min, the organic layer was separated and dried and the solvent removed under reduced pressure to give aldehyde **1** (13 mg, 0.045 mmol, 85%). The crude product was purified by preparative RP-HPLC. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.68 (s, 1H), 7.66 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 8.2, 2H), 6.78 (s, br., 1H), 3.77 (t, J = 4.7 Hz, 2H), 3.56 (q, J = 4.8 Hz, 2H), 3.52 (s, 2H), 2.88 (t, J = 7.4, 2H), 2.69 (t, J = 7.4, 2H), 2.47−2.38 (m, 5H), 1.82 (quint., J = 7.0 Hz, 2H). ^{13}C-NMR ($CDCl_3$, 500 MHz): δ 210.5 (s), 202.4 (s), 168.5 (s), 144.7 (s), 131.7 (s), 128.2 (d, 2C), 127.1 (d, 2C), 65.6 (t), 60.8 (t), 42.5 (t), 39.7 (t), 35.7 (t), 18.5 (t), 15.6 (t). HRMS (FAB): m/z calcd for ($[C_{16}H_{21}NO_4 + H]^+$) 292.1549, found 292.1540.

(2′*R,3′*S**)- and (2′*R**,3′*R**)-*N*-(Hydroxyethyl)-4-[(3′-hydroxy-1′-oxocyclohex-2′-yl)methyl]benzamide (2a,b).** A solution of **1** (35 mg, 0.12 mmol) in 2 mL of MeOH and 1 mL of THF was poured into 200 mL of aqueous 100 mM *N*-methyldiethanolamine buffered at pH 9.1. After 15 h the mixture contained 11% **2a**, 23% **2b**, 4% **3**, and 56% unreacted **1**. The solution was acidified with TFA to pH 3.5, and the mixture was separated by preparative RP-HPLC to give the aldol products **2a** (3.5 mg, 10%) and **2b** (8 mg, 23%). Aldol **2a**: ^{1}H-NMR ($CDCl_3/CD_3OD$, 7:1; 500 MHz): δ 7.67 (d, J = 8.2 Hz 2H), 7.23 (d, J = 8.2 Hz 2H), 4.07 (s, br., 1H), 3.71 (t, J = 5.2 Hz, 2H), 3.51 (t, J = 5.2 Hz, 2H), 3.12 (dt, J = 5.4, 13.9 Hz, 1H), 2.75−2.71 (m, 1H), 2.63−2.61 (m, 1H), 2.39−2.29 (m, 2H), 2.14−2.09 (m, 1H), 1.90−1.74 (m, 3H). 2D-COSY-NMR: ($CDCl_3/CD_3OD$, 7:1, 500 MHz): J (H-C(2)−H-C(3)) = 3.0 Hz. ^{13}C-NMR ($CDCl_3/CD_3OD$, 7:1; 500 MHz): δ 211.4 (s), 168.5 (s), 144.3 (s), 131.7 (s), 129.3 (d), 129.2 (d), 127.1 (d), 127.0 (d), 70.9 (t), 61.3 (d), 56.9 (d), 42.3 (t), 41.7 (t), 32.4 (t), 31.0 (t), 21.4 (t). HRMS (FAB): m/z calcd for ($[C_{16}H_{21}NO_4 + H]^+$) 292.1549, found 292.1541. Aldol **2b**: ^{1}H-NMR ($CDCl_3/CD_3OD$, 7:1; 500 MHz): δ 7.60 (d, J = 8.2 Hz 2H), 7.21 (d, J = 8.2 Hz 2H), 3.63 (t, J = 5.3 Hz, 2H), 3.51−3.45 (dt, J = 4.0, 10.1 Hz, 1H), 3.42 (t, J = 5.3 Hz, 2H), 2.95 (t, J = 5.9 Hz, 2H), 2.59−2.54 (m, 1H), 2.28−2.04 (m, 3H), 1.94−1.86 (m, 1H), 1.67−1.63 (m, 1H), 1.43−1.39 (m, 1H). ^{13}C-NMR ($CDCl_3/CD_3OD$, 7:1; 500 MHz): δ 210.9 (s), 167.8 (s), 143.8 (s), 132.0 (s), 129.5 (d, 2C), 126.9 (d, 2C), 73.2 (t), 61.5 (d), 61.0 (d), 42.5 (t), 40.7 (t), 33.4 (t), 31.3 (t), 20.8 (t). HRMS (FAB) m/z calcd for ($[C_{16}H_{21}NO_4 + H]^+$) 292.1549, found 292.1539.

***N*-(Hydroxyethyl)-4-[(1′-oxocyclohex-2′-en-2′-yl)methyl]benzamide (3).** Treatment of **1** with aqueous 1 N KOH for 1 h and purification by preparative RP-HPLC gave enone **3**. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.66 (d, J = 8.2 Hz, 2H), 7.21 (d, J = 8.2 Hz, 2H), 6.65 (s, br., 1H), 6.59 (t, J = 4,2, 1H), 3.79 (t, J = 4.7 Hz, 2H), 3.58 (q, J = 4.7 Hz, 2H), 2.71 (s, br., 1H), 2.43 (t, J = 7.1 Hz, 2H), 2.32 (dt, J = 4.2, 7.1 Hz, 2H), 1.96 (quint., J = 7.1, 2H). ^{13}C-NMR ($CDCl_3$, 500 MHz): δ 199.2 (s), 174.0 (s), 147.3 (d), 143.7 (s), 138.7 (s), 131.9 (s), 129.2 (d, 2C), 127.1 (d, 2C), 61.7 (t), 42.6 (t), 38.3 (t), 35.4 (t), 26.0 (t), 22.9 (t). HRMS (FAB): m/z calcd for ($[C_{16}H_{19}NO_3 + H]^+$) 274.1443, found 274.1455.

B. Antibody Assays. Antibodies. Monoclonal antibodies against haptens **4**, **5**, and **6**, which were raised in conjunction with a previous project,[11a] were obtained from ascites fluid grown from the individual hybridoma cell lines and purified to homogeneity by ammonium sulfate

precipitation followed by anion exchange and protein G chromatography.[18] The concentration of antibody was measured by UV at 280 nm as c (mg/mL) = abs/1.4.

Kinetic Assays. All substrates were purified by preparative RP-HPLC before use and then used as stock solutions of 10 mM in 9:1 water–acetonitrile. Assay conditions: 20 °C, 25 mM (bis(2-hydroxyethyl)amino)tris(hydroxymethyl)methane (Bistris) buffer (pH 7.5), 100 mM NaCl, 50–1000 μM **1** or **2a**, followed by RP-HPLC (Vydac 218TP54 C-18, 0.45 × 22 cm, 1.5 mL/min of 88% H_2O, 12% CH_3CN, 0.1% trifluoroacetic acid, detection by UV 240 nm, t_R(**2a**) = 4.5 min; t_R(**2b**) = 5.0 min; t_R(**1**) = 7.5 min; t_R(**3**) = 20.5 min). The concentration of active sites for catalytic antibody 78H6 was measured in the catalytic assay by quantitative titration with hapten **7** as described before.[11c] Quantitative titration at [Ab] = 10 μM was possible as the apparent K_i for **7** with 78H6 was below 0.5 μM. For [Ab78H6] = 1.5 mg/mL (by UV 280 nm), a linear decrease of catalytic activity down to zero was observed when the concentration of **7** was gradually increased from 0 to 10 μM. This corresponds to only one catalytic site per antibody molecule. SDS-PAGE electrophoresis shows >90% purity for Ab 78H6. In another study, it was shown that the myeloma cell line used to prepare the hybridoma expresses another antibody light chain, and the decreased concentration of active sites can be attributed to the fact that both a hapten-specific, catalytic antibody and a myeloma, hapten nonspecific antibody are present in the preparation.[19] Assay setup and Lineweaver–Burk analyses were carried out as described before.[11c,i]

Isolation of Aldols 2a,b and Characterization by Chiral HPLC. The product of a semipreparative experiment (0.2 mL of 300 μM **1**, 33 μM Ab 78H6 in 25 mM Bistris (pH 7.5), 100 mM NaCl, 20 °C) was isolated by RP-HPLC at 30% conversion and analyzed (hexane/ethanol, 6:1; 1.0 mL/min, UV 240 nm) on a chiralpak AS column (0.45 × 22 cm, from Daicel): t_R (*rac*-**2b**) = 11.6 min and t_R (**2a**) = 19.1 and 37.1 min. The peak at 11.6 min was collected and analyzed on a chiracell OD column (Daicel): t_R (**2b**) = 15.6 and 18.9 min. The absolute configurations were not determined.

For the determination of the selectivity of antibody 78H6 with respect to aldol **2**, each stereoisomer was isolated in pure form by chiral column. Thus the fractions for each isomer were collected from four 200 μg injections on the analytical column. The solvent (approximately 10 mL each) was removed by evaporation, and the residues were redissolved in 50% aqueous acetonitrile to give stock solutions of approximately 10 mM, as measured by UV. Each stereoisomer was then assayed separately for catalysis. The **2a** enantiomer accepted as the substrate elutes at t_R = 37.1 min on chiralpak AS.

Antibody Modification. Glycine/EDC. A solution of 1 mg/mL Ab 78H6 (2 mL) in 50 mM morpholinoethanesulfonic acid, 1 M glycine methyl ester, and 100 mM EDC (*N*-ethyl-*N*-((diethylamino)propyl)-carbodiimide hydrochloride) was incubated in the presence (sample A) or absence (sample B) of 1 mM inhibitor **8** for 18 h at 20 °C, then treated with 1 M sodium acetate (0.2 mL) at 20 °C for 3.5 h. A control sample was similarly treated with 1 M glycine methyl ester without EDC, followed by 1 M sodium acetate (sample C). The three samples were then extensively dialyzed against 25 mM Bistris and 100 mM NaCl (pH 7.5) and reconcentrated by diyalysis–centrifugation in Centricon tubes. The antibody concentration was determined by UV at 280 nm, and each sample was then diluted to a standard concentration of 1.5 mg/mL and assayed for catalysis against an untreated sample of antibody 78H6. The apparent activity for the aldolization of **1** to **2** relative to that of the untreated antibody sample was 70% for sample A (derivatized, protected by inhibitor **8**), 30% for sample B (derivatized, unprotected), and 95% for sample C (nonderivatized, submitted to glycine methyl ester and NaOAc).

Attempted Modification with $NaBH_3CN$. Three 2.5 mL samples of a solution of 2.5 mg/mL Ab 78H6 in 25 mM Bistris and 100 mM NaCl (pH 7.5) were treated with 1 mM $NaBH_3CN$ in the presence of 400 μM **1** (sample D) or 500 μM **2** (sample E) or without added substrate (sample F). After 10 min at 4 °C, each sample was purified by gel filtration on a PD-10 column (1.5 × 10 cm) equilibrated with the same buffer. The antibody fractions were collected and their concentrations measured by UV. As checked by HPLC analysis, samples D and E contained no traces of remaining **1** or **2**, attesting the efficiency of the purification by gel filtration. Each sample was diluted to 1.5 mg/mL and assayed against an untreated sample of antibody 78H6 for catalysis of the aldolization of **1** to **2**. There was no significant decrease in catalytic activity in the treated samples vs the untreated sample. Aldolases are completely deactivated by a similar treatment.[17]

Antibody 78H6 Catalysis in the presence of CN^-. Antibody 78H6 (1.5 mg/mL) in 25 mM Bistris and 100 mM NaCl (pH 7.5) was assayed for catalysis of the elimination of **2a** to **3** in the presence or absence of 15 mM sodium cyanide (mainly HCN at that pH). Aldolization could not be assayed in this case because quantitative conversion of the aldehyde group in **1** to a cyanohydrin is observed. There was no significant difference in the catalytic activity of antibody 78H6 in the presence or absence of cyanide over a period of 3 days. By comparison, aldolases reportedly experience 50–75% deactivation after only 30 min in the presence of 10–25 mM cyanide, this between pH 6.0 and 9.0.[17]

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Supporting Information Available: Figures showing RP and chiral HPLC traces for the separation of **1**, **2**, and **3** (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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