Catalytic Antibodies with Lipase Activity and *R* or *S* Substrate Selectivity

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The specific hydrolysis of unactivated esters bearing an *R* or *S* enantiomeric alcohol has been achieved by two separate classes of catalytic antibodies induced to bind either the *R* or *S* substrates. The antibodies exhibit rate accelerations (10^3 to 10^5) above background hydrolysis that, coupled with their antipodal specificity, provide a novel set of reagents for use in synthesis.

THE NUMBER OF CHEMICAL TRANSFORMATIONS CATALYZED by antibodies (abzymes) is rapidly increasing (*1–9*). Antibodies have been shown to catalyze a variety of acyl transfer reactions including lactonization, aryl amide and ester hydrolysis, and amide bond formation, as well as carbon-carbon bond formation and redox reactions. Recently an antibody that catalyzes sequence-specific peptide cleavage has been generated (*10*). Many of these reactions proceed with high rates (*3, 9*) and high enantioselectivity (*2, 5, 6*) and, for some reactions, mechanistic details are beginning to emerge (*3, 9*).

One of the challenges at present is to develop catalytic antibodies of practical significance. For example, lipases are of considerable interest because of their potential importance in organic synthesis. Up until now, lipases have been successfully used as transesterification catalysts for stereoselective acylation and kinetic resolution of alcohols (*11*). An important difference in terms of reaction energetics and specificity between the catalytic antibodies generated thus far and lipases is the ability of lipases to hydrolyze the unactivated esters of complex alcohols. Moreover, lipases show a preference for substrates at the interface between water and an immiscible organic phase. In order to induce antibodies with lipase activities, we synthesized an antigen **4** that is a transition-state analog for hydrolysis of the ester of α-methylbenzyl alcohol **1** (Fig. 1). The phosphonate moiety imparts the oxyanionic and tetrahedral features of the transition state for ester hydrolysis to the antigen, whereas substitution of the methyl group on the benzylic carbon confers asymmetry on the alcoholic fragment of the molecule. The binding specificities induced by this antigen should be similar to authentic lipases, such as Amano P from *Pseudomonas fluorescens*, which can catalyze the hydrolysis of (*R*,*S*)-α-phenethyl acetate to selectively form (*R*)-α-phenethyl alcohol (*12*). A critical feature of our experiment is the use of a racemic antigen, which should simultaneously induce antibodies that may exclusively bind to either the *R* or *S* substrates and thereby extend the potential for kinetic resolution to both enantiomers. We have used this strategy to generate catalytic antibodies that are lipases, in that they hydrolyze unactivated esters and show exquisite specificity for substrates with either the *R* or *S* configuration at the alkoxide leaving group.

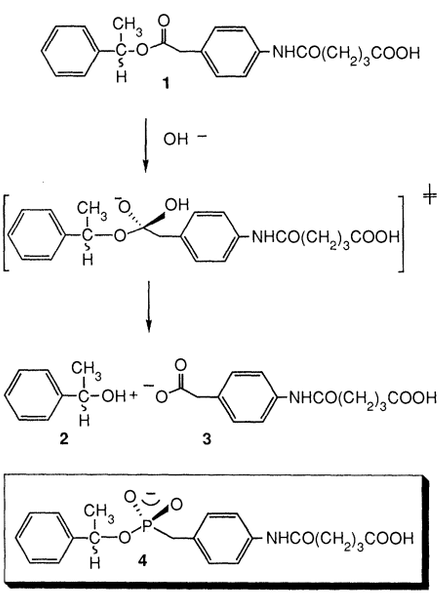

Fig. 1. Possible mechanism for ester hydrolysis reaction.

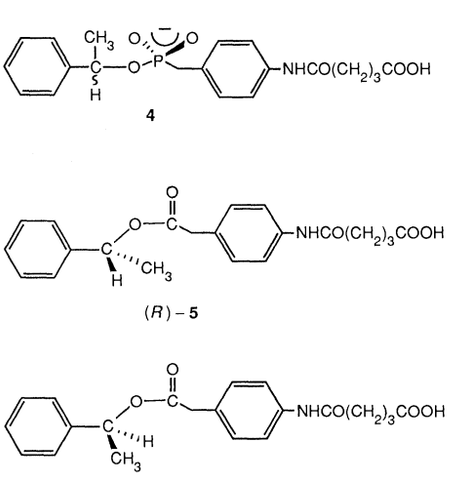

Fig. 2. Compound **4** represents the phosphonate used to induce monoclonal antibodies and inhibit the reaction. The α-methylbenzyl ester (*R*)-**5** and (*S*)-**5** act as substrates for antibodies obtained to antigen **4**.

Immunogen design. A probable pathway for hydrolysis of unactivated esters is illustrated in Fig. 1. At slightly alkaline *p*H,

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several zwitteronic tetrahedral species are developed (*13*). Just as for amide hydrolysis (*9*), an effective catalytic antibody should stabilize the oxyanion, but it is not necessary to protonate the leaving alkoxide. Methyl substitution on the benzylic carbon imparts *R* or *S* chirality to the alcoholic fragment of **4** or **5** (Fig. 2). The hapten **4** includes a five-carbon spacer, a derivative of glutaric anhydride, which is attached to carrier protein through the ε-amino moiety of lysine by the water-soluble coupling agent, EDC [1-(3-dimethylaminopropyl)-3-ethyl carbodiimide]. Racemic **4** was synthesized in eight steps from 4-aminobenzyl phosphonate and α-methylbenzyl alcohol (Fig. 3). The pure (*R*)-(+)-**5** or (*S*)-(−)-**5** enantiomers of the ester substrate were synthesized in four steps from 4-aminophenylacetic acid and either (*R*)-(+)- or (*S*)-(−)-α-methylbenzyl alcohol (Fig. 4).

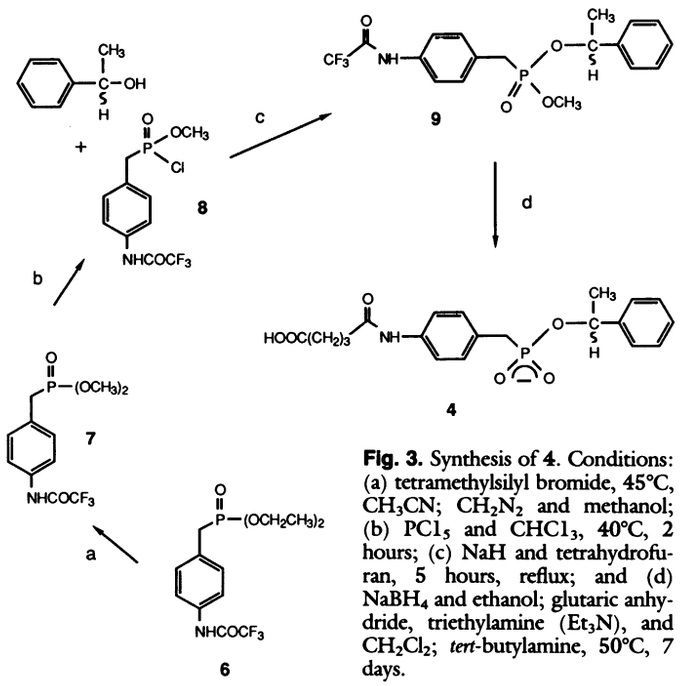

Fig. 3. Synthesis of **4**. Conditions: (a) tetramethylsilyl bromide, 45°C, CH_3CN; CH_2N_2 and methanol; (b) PCl_5 and $CHCl_3$, 40°C, 2 hours; (c) NaH and tetrahydrofuran, 5 hours, reflux; and (d) $NaBH_4$ and ethanol; glutaric anhydride, triethylamine (Et_3N), and CH_2Cl_2; *tert*-butylamine, 50°C, 7 days.

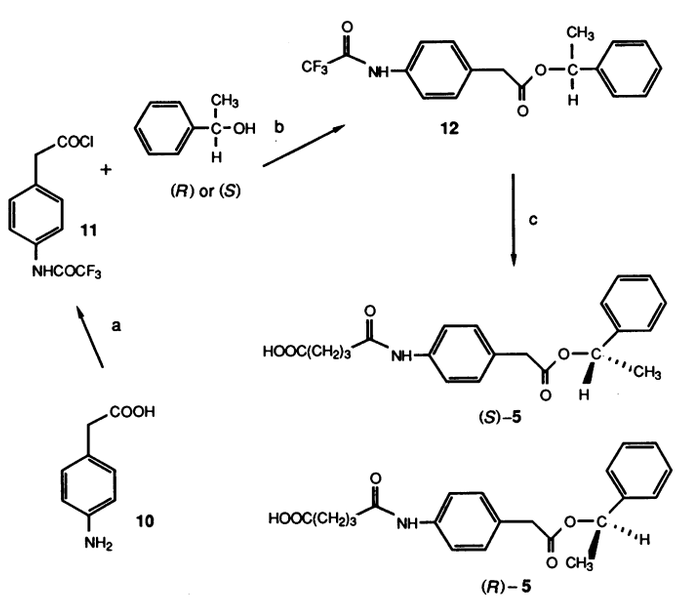

Fig. 4. Synthesis of (*R*)-**5** or (*S*)-**5**. Conditions: (a) Trifluoroacetic anhydride (TFA) in a mixture of CH_3CN and H_2O (97 : 3), Na_2CO_3, $SOCl_2$, 45°C; (b) Et_3N and CH_2Cl_2; and (c) $NaBH_4$, ethanol, glutaric anhydride, Et_3N, and CH_2Cl_2. Rotation of (*S*)-**5** $[\alpha]_D$: −30.3 (*C* = 0.03892, methanol), and of (*R*)-**5** $[\alpha]_D$: +30.6 (*C* = 0.04266, methanol).

Induction of antibodies. Racemic **4** was coupled to keyhole limpet hemocyanin (KLH) and the conjugate was used to immunize AJ mice for production of monoclonal antibodies (*14*). Eighteen monoclonal antibodies were shown by an enzyme-linked immunosorbent assay (ELISA) (*15*) to bind to **4** conjugated to bovine serum albumin (*16*). All 18 cell lines were cloned and injected separately into mice for production of ascites fluid. Antibody from each sample of ascites fluid was purified by salt precipitation, anion exchange, and affinity chromatography (Fig. 5).

***R* or *S* abzyme specificity and kinetic analysis**. All 18 antibodies at a concentration of 20 μ*M* were initially screened against racemic alkyl ester **1** (*17*) for the production of the diacid hydrolysis product **3**. In our study, 11 of the 18 antibodies were identified as catalysts for the hydrolysis of **1**. These 11 antibodies were tested for their potential stereospecificities in separate experiments with either the enantiomerically pure (*R*)-**5** or (*S*)-**5** ester. Nine of the 11 antibodies were enantiospecific for the hydrolysis of ester (*R*)-**5**, whereas the other two were stereospecific for hydrolysis of the antipodal ester (*S*)-**5**. Hydrolysis of less than 2 percent of the opposite stereoisomer would have been detected. Kinetic studies then were performed on 2 of these 11 antibodies chosen on the basis of their rate of reaction and enantiomeric selectivity.

The monoclonal antibody 2H6 was completely enantiospecific for ester (*R*)-**5**. Its initial rate of hydrolysis measured as a function of substrate (*R*)-**5** concentrations followed Michaelis-Menten kinetics (Eq. 1), a finding consistent with pre-equilibrium substrate binding, subsequent rate-limiting hydrolytic ester cleavage, and product dissociation (see Fig. 6 and Table 1).

$$\begin{array}{l} 2H6 + (R)\text{-}5 \xrightarrow{k_{uncat}} \text{products} \\ \quad\uparrow\downarrow\ K_m \\ 2H6 \cdot (R)\text{-}5 \xrightarrow{k_{cat}} 2H6 + \text{products} \end{array} \quad (1)$$

Comparison of k_{cat}/k_{uncat} (the ratio of the rates of the catalyzed and uncatalyzed reactions) (*p*H 9.0, ATE) gives a rate acceleration for hydrolysis of (*R*)-**5** of ~80,000. Although the observed Michaelis constant K_m (4 m*M*) suggests weak substrate binding, increased product binding ("product inhibition") was noted with diacid **3**, which competitively inhibited the hydrolysis for (*R*)-**5** with a K_i of

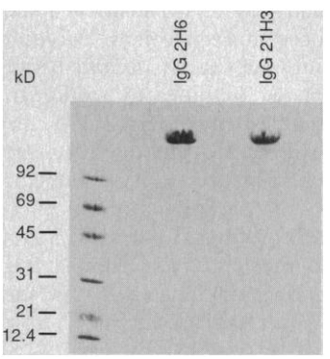

Fig. 5. SDS–polyacrylamide gel electrophoresis of purified IgG 2H6 and 21H3. The globular fractions from ascitic fluid were precipitated by dropwise addition of saturated ammonium sulfate at 4°C, *p*H 7.2, to achieve a final concentration of 45 percent. The ammonium sulfate was removed by dialysis against 10 m*M* tris, *p*H 8. The concentrated antibody was next purified by anion exchange chromatography on DEAE-Sephacel and eluted with a stepwise salt gradient (50 to 500 m*M* NaCl). The antibody eluted in the 100 m*M* NaCl fraction was then concentrated by ultrafiltration for affinity purification on a protein G–Sepharose column. After loading onto the column, nonadherent material was removed by extensive washing (20 to 30 column volumes) with 0.1*M* phosphate buffer, *p*H 7.0. The adherent antibody was eluted with 0.05*M* citric acid, *p*H 3.0, and immediately neutralized by collecting the active fractions into 1*M* tris, *p*H 9.0. The antibody was then dialyzed into 75 m*M* tris-HCl, *p*H 8.0, and eluted from a Mono Q column with a linear salt gradient. The purified antibody was then dialyzed into ATE buffer, *p*H 9, and assayed by HPLC (see Table 1).

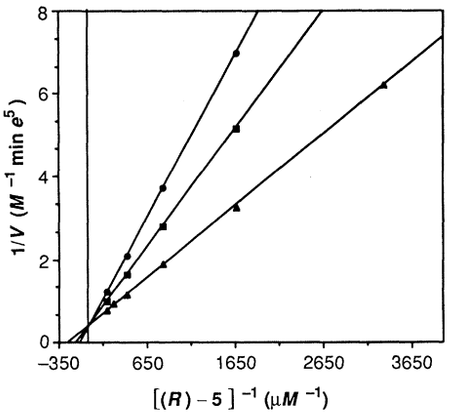

Fig. 6. Lineweaver-Burke plot for hydrolysis of substrate (*R*)-**5** by the monoclonal antibody 2H6. Velocities were determined as described in Table 1: (▲) no inhibitor present; (■) inhibited by $2.5 \times 10^{-6}M$ phosphonate **4**; and (●) inhibited by $5 \times 10^{-6}M$ phosphonate **4**.

Table 1. Kinetic parameters for the hydrolysis of (*R*)-**5** by 2H6 and (*S*)-**5** by 21H3. Velocities were determined by following the formation of diacid **3** by an HPLC assay (reverse-phase C-18 column, with a mixture (15:85) of acetonitrile and water, 0.5 percent TFA). The antibody at 5 μ*M*, [average value determined from a bicinchoninic acid (BCA) assay and absorbance at 280 nm and by assuming a molecular weight of 150,000 for IgG] was preincubated at 21°C in ATE [see (*17*)], *p*H 9.0. The reactions were initiated by addition of varying aliquots of a 50 or 100 m*M* solution of ester (*R*)-**5** or (*S*)-**5** in DMF to give a substrate concentration of 150 to 5000 m*M* (total organic phase 5 percent DMF). Aliquots (0.3 ml) of the reaction mixture containing antibody were removed and quenched with a 10 percent perchloric acid solution, final *p*H of solution ~4.5. The first-order rate constant for the hydrolysis in the absence of antibody was also measured by HPLC and determined to be 5.56×10^{-5} min^{-1}. Initial rates determined by HPLC are uncorrected, as the background rate of hydrolysis did not contribute to the antibody-catalyzed rate under typical assay conditions. Standard deviations are reported in parentheses.

Antibody	K_m ($10^{-6}M$)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}	K_i ($\times 10^{-6}$)
21H3	394 (8.0)	0.09 (0.008)	1,619	0.19 (0.01)
2H6	3,994 (40.0)	4.6 (0.4)	82,733	2.0 (0.04)

0.05 m*M*. Competitive inhibition by the phosphonate **1** was characterized by a K_i of 1.98 μ*M* (Fig. 6) (*18*).

In contrast to the *R* stereospecificity of monoclonal antibody 2H6, 21H3 was completely specific for the hydrolysis of the antipodal (*S*)-α-methylbenzyl ester **5**. It likewise displayed saturation kinetics based on the initial rates of hydrolysis of the alkyl ester (*S*)-**5**, although the rate accelerations ($k_{cat}/k_{uncat} = 1600$) were only modest (Table 1). In this case, product inhibition by either diacid **3** or **2** [(−)-α-methylbenzyl alcohol] or both was not detected, so that $K_i > 0.2$ m*M*. Studies of the inhibition of hydrolysis by phosphonate **4** revealed competitive inhibition with $K_i = 0.19$ μ*M* (*18*).

Implications for organic synthesis. On the basis of our knowledge of antibody-catalyzed reactions, it seems reasonable to assume that abzymes make use of specific interactions with high-energy species that lie along the reaction coordinate to achieve their enzyme-like catalysis. Recent evidence implies that abzyme site residues may sometimes act as general acid-base catalysts (*3, 9*). The extent to which factors over and above the interactions expected from antigen binding data may contribute to antibody catalysis have been estimated from a thermodynamic cycle derived from transition-state theory (*6, 19*). In essence, the ratio of k_{cat}/k_{uncat} may be predicted from K_m/K_i for a typical abzyme reaction, provided that the hapten is an ideal transition-state mimic. Kinetic values obtained for 21H3 appear to be in close agreement with this hypothesis ($K_m/K_i = 2 \times 10^3$ and $k_{cat}/k_{uncat} = 1.6 \times 10^3$). In contrast, 2H6 appears to deviate from the predicted behavior ($K_m/K_i = 2 \times 10^3$ and $k_{cat}/k_{uncat} = 8.2 \times 10^4$). In this latter case, some interactions not accounted for in the complex of 2H6 and **4** but expressed in the transition states for 2H6 and the substrate **5** may be associated with general acid-base catalysis. In abzyme-catalyzed hydrolysis of an amide bond, similar deviations from predicted values were also encountered (*9, 20*). In either case, the magnitude of the rate accelerations (10^3 to 10^4) are sufficient to make the use of abzymes feasible for organic synthesis.

The novel properties of the abzymes generated in our study are their abilities to hydrolyze alkyl esters with exquisite selectivity for one or the other antipodal substrate. Except for the preference for water-organic interfaces, these are the same chemical properties which make lipases that act only on one stereoisomer such a valuable class of enzymes for organic chemistry. Moreover, there is no a priori reason to rule out the use of abzymes in immiscible organic solvents, thereby encompassing all of the properties of lipases. Indeed, abzymes have recently been shown to function in reverse micelles (*8*).

In the field of organic synthesis, one can imagine the generation of pairs of antibodies, each capable of selectively removing an asymmetric blocking group that is required during chemical transformations. By performing immunizations with a racemic mixture of antigens, we can already generate two distinct catalysts that may be used for selective removal of antipodal blocking groups. Synthesis of new antigens in which the benzylic carbon is differentially alkylated should allow induction of additional pairs of specific lipases so that stereospecific unmasking of the desired functionality can be incorporated into the synthetic strategy.

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by slowly adding 100 μl of 1*M* EDC in H_2O at *p*H 4.8 to 4 mg of **4** in 200 μl of dimethylformamide (DMF) and 100 μl of H_2O, *p*H 4.0. This solution was stirred for 1 hour at 0°C and added slowly to 4 mg of KLH in 1 ml of H_2O, *p*H 4.0. This solution was gently stirred overnight at 4°C. Four 8-week-old mice each received an intraperitoneal (IP) injection of 100 μg of **4** conjugated to KLH with RIBI adjuvant (MPL and TDM emulsion). A 50-μg IP injection of **4**-KLH conjugate in alum was given 2 weeks later. One month after the second injection, the mouse with the highest titer (12,800 to 25,600) was injected intravenously with 50 μg of the **4**-KLH conjugate; 3 days later the spleen was taken for the preparation of hybridomas. Spleen cells (1.0×10^8) were fused with 1.4×10^2 SP2/0 and 2.3×10^7 HL myeloma cells. Cells were plated into 30 96-well plates; each well contained 150 μl of hypoxanthine, aminopterin, thymidine–Dulbecco's minimal essential medium (HAT-DMEM) containing 1 percent nutridoma and 2 percent bovine serum albumin.

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16. After 2 weeks, the antibodies produced by wells containing macroscopic colonies were assayed by ELISA for binding to **4**. Colonies that initially produced antibodies that bound **4** were subcloned twice, after which 18 remained active. The subtype distribution of the 18 monoclonal antibodies was 14 IgG1, 3 IgG2a, and 1 IgG2b. All 18 monoclonal antibodies were injected into pristane-primed BALB/c × AJ mice to generate ascitic fluid. For initial studies, the IgG monoclonal antibodies were purified from ascitic fluid by salt precipitation and affining chromatography. Each purified monoclonal antibody was dialyzed into ATE buffer, *p*H 9.0 (*17*).
17. The antibodies were initially screened against **1** (1 m*M*), *p*H 9.0, in 0.1*M* aces {2-[(carbamoylmethyl)amino]ethanesulfonic acid}, 0.052*M* tris, and 0.052*M* ethanolamine (ATE buffer) by high-performance liquid chromatography [HPLC; reverse-phase C-18 column with a mixture (15:85) of acetonitrile and water, 0.5 percent trifluoroacetic acid].
18. Since phosphonate **4** was racemic, the K_i value determined from the Lineweaver-Burke plot was divided by 2.
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