Relaxing Substrate Specificity in Antibody-Catalyzed Reactions: Enantioselective Hydrolysis of *N*-Cbz-Amino Acid Esters

Fujie Tanaka, Keiko Kinoshita, Ryuji Tanimura, and Ikuo Fujii*

Contribution from the Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan

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Abstract: For a catalytic antibody to be generally useful for organic synthetic chemistry, it must be able to accept a broad range of substrates, yet retain high selectivity. In this work, we propose a hapten design to endow antibody catalysts with two opposing qualities, such as high enantioselectivity and broad substrate specificity. Racemic hapten **2** induced two separate classes of catalytic antibodies to hydrolyze either the L- or D-isomers of *N*-Cbz-amino acid esters **1**. In the kinetic resolution of racemic ester **9**, antibodies 7G12 and 3G2 gave 96% ee of L-**10** and 94% ee of D-**10**, respectively. In addition, antibody 7G12 displayed broad substrate specificity, hydrolyzing the L-esters of Ala (**1a**), Leu (**1b**), Norleu (**1c**), Met (**1d**), Phe (**1e**), Val (**1f**), and phenylglycine (**1g**) with high enantioselectivity. Antibody 3G2 also hydrolyzed the D-isomers of these esters without sacrificing the enantioselectivity. This observation suggests that the use of haptens that fit snugly into the antigen-combining site, and leave the linker moiety outside, is an effective approach for the generation of catalytic antibodies with high selectivity and broad substrate applicability.

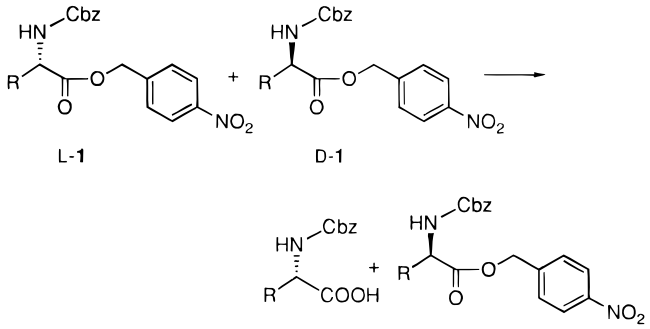
Introduction

Since antibody-catalyzed reactions display high regio- and stereoselectivity, according to the reaction pathway programmed in the hapten,[1] catalytic antibodies have the potential to be powerful tools in the field of organic synthetic chemistry. However, the applicability to a broad range of substrates is still restricted within narrow limits, due to the inherent binding specificity of antibodies. For a catalytic antibody to be generally useful, it must be able to accept a broad range of substrates, yet retain high selectivity. Therefore, we have focused on the issue of whether catalytic antibodies can be developed to combine the aforementioned two opposing qualities. Recently, we described catalytic antibodies that discriminate between chemically identical functional groups in the same molecule to catalyze regioselective deprotection of a variety of acylated carbohydrates,[2] and others have shown antibody-catalyzed chemoselective reactions with various substrates.[3] Herein, we demonstrate the generation of two separate classes of catalytic antibodies that enantioselectively hydrolyze either the L- or D-isomers of amino acid ester derivatives possessing various α-substituents (Scheme 1). Despite the many efforts that have been mounted to prepare chiral α-amino acids,[4,5] simple access to a wide range of them remains limited.[4a] These antibody-catalyzed kinetic resolutions with broad substrate specificity will provide a convenient route to a wide range of natural and unnatural, enantiomerically pure α-amino acids.

Results and Discussion

Design and Synthesis of Hapten. To design a hapten that would induce broad substrate specificity in an enantioselective antibody-catalyzed reaction, it seemed necessary to consider the molecular basis of antibody−antigen interactions. X-ray structural analyses of antibody−antigen complexes have shown that relatively small haptens, such as fluorescein,[6a] progesterone,[6b] and progesterone 11α-hemisuccinate,[6b] are buried deep in the antigen-combining sites, with surface areas of 270−353 Å^2. Therefore, we thought that the size range of these haptens would be sufficient to display highly specific antibody−antigen

Scheme 1



* To whom correspondence should be addressed.

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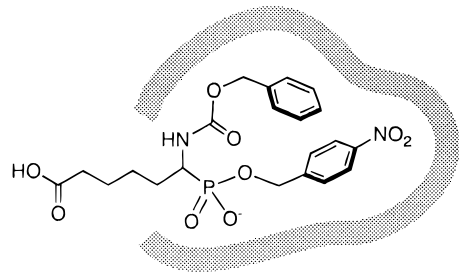
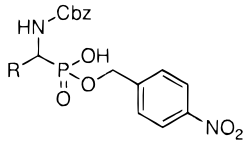


Figure 1.

interactions and would be enough to occupy only the antigen-combining site, while leaving the linker moiety free from antibody−antigen interactions. To induce high enantioselectivity in antibody-catalyzed reactions, an immunizing haptenic epitope including a chiral center should be within the 270−353 Å^2 range so that the haptenic epitope occupies the antigen-combining site. In addition, since the linker to the hapten is outside of the antigen-combining site, the position of attachment of the linker to the hapten may be chosen to allow broad substrate specificity.

According to these ideas, we designed haptenic phosphonate transition state analog **2** to generate catalytic antibodies that enantioselectively hydrolyze amino acid ester derivatives possessing various α-substituents. In hapten **2**, relatively bulky protecting groups, carbobenzoxy (Cbz) and 4-nitrobenzyl groups, were used to adjust the size of the surface area of the immunizing haptenic epitope, and the linker moiety was attached at the Cα position (Figure 1). In practice, a ^{1}H NMR analysis of hapten **2** has shown a NOE (0.6%) of the phenyl proton in the Cbz group, with irradiation of the *ortho* proton of the nitro group,[7] suggesting a packed conformation of these two aromatic groups of **2** in aqueous media. The surface area (solvent accessible area) of the packed conformation of **3**, which lacks the linker moiety, was calculated to be 330 Å^2.[8]

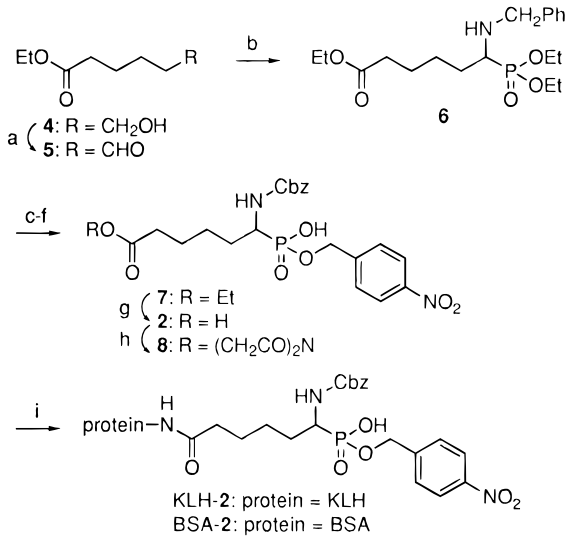


2: R = HOC(O)(CH$_2$)$_4$-
3: R = CH$_3$-

To generate two separate classes of catalytic antibodies that catalyze the hydrolysis of either the L- or D-isomers of amino acid esters, hapten **2** was synthesized as a racemic mixture.[9] An imine derived from aldehyde **5** and benzylamine was treated with triethyl phosphite to give (aminoalkyl)phosphonate **6**, an intermediate with a linker moiety to a carrier protein at the Cα position. Deprotection and protection of the amino group of **6**, dealkylation of the diethyl phosphonate, condensation with 4-nitrobenzyl alcohol, and hydrolysis of the ester group afforded **2**. Finally, hapten **2** was conjugated to carrier proteins, keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA), via an activated ester method (Scheme 2).

Antibody Production. Balb/c mice were immunized with KLH-**2**, and monoclonal antibodies were generated by standard

Scheme 2[a]



[a] Reagents: (a) PDC/CH$_2$Cl$_2$, 40%; (b) PhCH$_2$NH$_2$, P(OEt)$_3$/EtOH, 48%; (c) H$_2$, Pd−C/HCOOH−MeOH; (d) CbzCl, Et$_3$N/THF; (e) TMSBr/CH$_3$CN; (f) 4-nitrobenzyl alcohol, DCC, 1*H*-tetrazole/CH$_2$Cl$_2$−pyridine, 6% from **6**; (g) NaOH/H$_2$O−MeOH, 99%; (h) *N*-hydroxysuccinimide, WSC, DMAP/CH$_3$CN, 79%; (i) KLH or BSA/phosphate buffer (pH 7.4)−DMF.

Table 1. Kinetic Parameters of Anti-**2** Antibody-Catalyzed Hydrolysis of Substrate **9**[a]

antibody	substrate	K_m, μM	k_{cat},[b] min^{-1}	k_{cat}/k_{uncat}[c]	K_i, nM (inhibitor)
7G12	L-**9**	13	7.0×10^{-2}	3700	19 ((*R*)-**3**)
3G2	D-**9**	5.4	3.3×10^{-2}	1700	47 ((*S*)-**3**)

[a] Reaction conditions: 10% DMSO/50 mM Tris (pH 8.0), 25 °C. [b] See ref 13. [c] The first-order kinetic constant of the background reaction (k_{uncat}) was 1.9×10^{-5} min^{-1}.

protocols.[10] Hybridoma supernatants were screened for antibodies with binding affinity to BSA-**2** by ELISA. Monoclonal antibodies were purified from the hybridoma supernatants via cation-exchange chromatography (Mono S) followed by affinity chromatography (Protein G), as described previously.[11]

Catalytic Assay and Kinetics. Thirty-nine monoclonal antibodies that bound to BSA-**2** were screened for the ability to catalyze the hydrolysis of the racemic fluorogenic substrate **9**. The reaction was performed using 1.5 μM of antibody and 15 μM of racemic **9** in 10% DMSO/50 mM Tris (pH 8.0) at 25 °C, and the increase in fluorescence (λ_{ex} 340 nm, λ_{em} 415 nm) was monitored.[12] As a result, fourteen antibodies were found to be catalytic. These antibodies were examined for their enantioselectivities in separate experiments with 15 μM of either chiral L-**9** or D-**9**, under the same conditions described above. Ten of them were enantioselective for the hydrolysis of L-**9**, whereas the other four were enantioselective for the hydrolysis of D-**9**. Hydrolysis of less than 3% of the opposite isomer was detected. Antibodies 7G12 and 3G2 were highly active for the hydrolysis of L-**9** and D-**9**, respectively, and were characterized in more detail.

Antibodies 7G12 and 3G2 displayed saturation kinetics described by the Michaelis−Menten equation in the hydrolysis of L-**9** and D-**9**, respectively. The kinetic parameters are shown in Table 1. The rate accelerations (k_{cat}/k_{uncat}) with 7G12 and 3G2 above background hydrolysis were 3700- and 1700-fold,

(7) A ^{1}H NMR NOE experiment of phosphonate **2** in D$_2$O showed 0.6% NOE of the Cbz phenyl proton and 9.6% NOE of the *meta* proton of the nitro group when the *ortho* proton of the nitro group was irradiated.

(8) The packed conformation of phosphonate **3** was optimized by Discover CVFF, and the value was calculated using Insight II (Biosym Technologies) with a 1.7 Å probe sphere and van der Waals radii.

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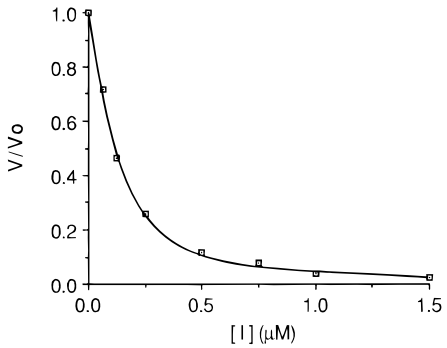


Figure 2. Representative plot of tight-binding inhibition of antibody 7G12 by phosphonate (*R*)-**3**. Initial velocities were measured at increasing concentrations of (*R*)-**3** in the presence of 0.22 μM (active site) antibody 7G12 and 10 μM L-**9**. [I] is the (*R*)-**3** concentration. *V* and V_o are, respectively, the velocities in the presence and absence of (*R*)-**3**.

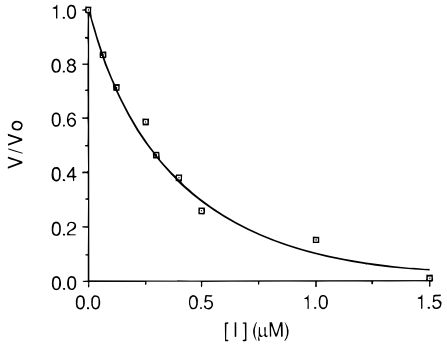


Figure 3. Representative plot of tight-binding inhibition of antibody 3G2 by phosphonate (*S*)-**3**. Initial velocities were measured at increasing concentrations of (*S*)-**3** in the presence of 0.27 μM (active site) antibody 3G2 and 10 μM D-**9**. [I] is the (*S*)-**3** concentration. *V* and V_o are, respectively, the velocities in the presence and absence of (*S*)-**3**.

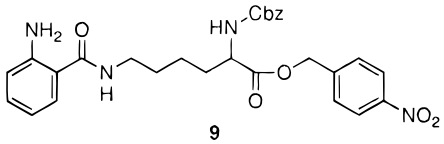
Table 2. Binding Affinity of Anti-**2** Antibodies with Chiral Phosphonate **3**[a]

antibody	K_d of (*R*)-**3**, μM	K_d of (*S*)-**3**, μM	ratio of affinitites[b]	$\Delta\Delta G$,[c] kcal/mol
7G12	2.5	10	81:19	0.8
3G2	11	0.17	2:98	2.5

[a] K_ds were determined by competition ELISA.[15] See ref 16. [b] Ratio of affinities = [1/K_d of (*R*)-**3**]:[1/K_d of (*S*)-**3**]. [c] $\Delta\Delta G = |RT \ln\{[1/K_d \text{ of } (R)\text{-}\mathbf{3}]/[1/K_d \text{ of } (S)\text{-}\mathbf{3}]\}|$.

respectively.[13] These antibodies catalyzed the hydrolyses with multiple turnovers, and no product inhibition was detected in the reactions. The antibody-catalyzed hydrolysis of L-**9** with 7G12 was competitively inhibited with the addition of chiral phosphonate (*R*)-**3**, which possesses the same Cα configuration as L-**9** (Figure 2). Similarly, the hydrolysis of D-**9** with 3G2 was inhibited with (*S*)-**3** (Figure 3). Henderson plots[14] of the tight-binding inhibition afforded the K_i values for these antibody-catalyzed reactions (Table 1).

Kinetic Resolution. To demonstrate that antibodies 7G12 and 3G2 have the potential to be useful for kinetic resolution of racemic substrates, the enantiomeric excess (ee) of the reaction product, Cbz-amino acid with **10**, was determined in the antibody-catalyzed hydrolysis of racemic substrate **9** by HPLC assay with a chiral column. When the reaction was performed using antibody 7G12 (20 μM of active site) and racemic **9** (150 μM) in 5% DMSO/50 mM Tris (pH 8.0) at 25 °C, 96% ee of L-**10** (59 μM conversion after 137 min) was obtained. Similarly, 94% ee of D-**10** (34 μM conversion after 177 min) was obtained in the reaction of antibody 3G2 (40 μM of active site) and racemic **9** (150 μM) under the same conditions.



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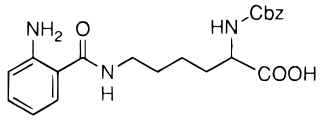

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Correlation of Hapten and Substrate Chirality. In this study, racemic hapten **2** was used for immunization, so that each enantiomer of the hapten could elicit catalytic antibodies specific to the corresponding enantiomer of the substrate. To correlate the hapten and substrate chirality, the binding affinities of antibodies 7G12 and 3G2 to chiral phosphonates (*R*)-**3** and (*S*)-**3**, in which their chiral centers are the same configuration (Cα) as L-**9** and D-**9**, respectively, were determined by competitive ELISA.[15,16] The values of the dissociation constants (K_d) are shown in Table 2. As expected from the observed enantioselectivity to L-**9** in hydrolysis, antibody 7G12 displayed an affinity to (*R*)-**3** that was approximately 4-fold higher than that to the opposite isomer (*S*)-**3**. Similarly, antibody 3G2 preferentially bound to (*S*)-**3** with 60-fold higher affinity than to (*R*)-**3**. This result suggests that the enantioselectivity observed in the antibody-catalyzed reactions is associated with binding in the antibody-combining site and that antibodies 7G12 and 3G2 should be elicited against the corresponding enantiomers of the transition state analog. However, the molecular mechanism of the enantioselectivity may not so simple because these catalytic antibodies bind the corresponding enantiomers of the transition state analog, with cross-reactivity to the opposite enantiomers. In the case of antibody 7G12, the difference ($\Delta\Delta G = 0.8$ kcal/mol) in the binding energy between (*R*)-**3** and (*S*)-**3** is not consistent with the difference ($\Delta\Delta G = 2.3$ kcal/mol) calculated from the enantiomeric excess of product L-**10** in the antibody-catalyzed kinetic resolution. Probably, antibody 7G12 interacts with (*S*)-**3** by a binding mode different from that of (*R*)-**3**.[19]

Substrate Specificity and Enantioselectivity. In addition to having the high enantioselectivity described above, the antibodies 7G12 and 3G2 were able to accept a broad range of substrates, as determined using *N*-Cbz-amino acid esters **1** possessing various α-substituents. The initial velocity of the antibody-catalyzed hydrolysis of each enantiomer was determined, and its ratio was used to estimate the enantioselectivity

(13) The k_{cat}s were obtained by dividing by the measured active site concentrations. The active site concentrations were determined by extrapolating from Henderson plots[14] of the activity titrations, with phosphonate (*R*)-**3** for antibody 7G2 or (*S*)-**3** for antibody 3G2.

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(15) Friguet, B.; Chaffotte, A. F.; Djavadi-Ohaniance, L.; Goldberg, M. E. *J. Immunol. Methods* **1985**, *77*, 305−319.

(16) Competition ELISA of an antibody often gives lower affinity values (higher values of K_d) than true binding because of the avidity of the bivalent IgG.[17] However, this method is useful in determining the ratio of the K_ds.[18]

(17) For an example: Tarasow, T. M.; Lewis, C.; Hilvert, D. *J. Am. Chem. Soc.* **1994**, *116*, 7959−7963.

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(19) In the X-ray structural analysis of anti-progesterone antibody, some of the cross-reactivity of the antibody with structurally different steroids was observed through different binding modes of the steroids.[6b]

Table 3. Enantioselective Hydrolysis of Esters **1** with Antibody 7G12[a]

substrate		velocities,[b] μM/min		ratio of velocities
1	R-	L-**1**	D-**1**	L-**1**:D-**1**
1a	CH_3-	2.52×10^{-1}	3.44×10^{-2}	88:12
1b	$(CH_3)_2CHCH_2$-	2.87×10^{-1}	7.30×10^{-3}	98:2
1c	$CH_3(CH_2)_3$-	1.83×10^{-1}	3.85×10^{-3}	98:2
1d	$CH_3SCH_2CH_2$-	3.58×10^{-1}	1.80×10^{-2}	95:5
1e	$PhCH_2$-	1.78×10^{-1}	3.44×10^{-3}	98:2
1f	$(CH_3)_2CH$-	5.54×10^{-3}	5.42×10^{-4}	91:9
1g	Ph-	7.83×10^{-1}	ND[c]	>99:<1
1h	4-HOPh-	3.33×10^{-1} [d]	6.86×10^{-3}	
1i	$H_2N(CH_2)_4$-	4.36×10^{-1}	1.49×10^{-2}	97:3

[a] Reaction conditions: [L- or D-substrate] 100 μM for **1a**,**c**,**f**,**g**,**i** and 200 μM for **1b**,**d**,**e**,**h**, [antibody 7G12] 10 μM (active site), 10% DMSO/50 mM Tris (pH 8.0), 25. °C. The first-order kinetic constants of the background reactions: **1a**, 1.06×10^{-4} min^{-1}; **1b**, 1.26×10^{-5} min^{-1}; **1c**, 1.71×10^{-5} min^{-1}; **1d**, 3.83×10^{-5} min^{-1}; **1e**, 2.24×10^{-5} min^{-1}; **1f**, 6.47×10^{-6} min^{-1}; **1g**, 5.62×10^{-3} min^{-1}; **1h**, 1.84×10^{-4} min^{-1}; **1i**, 1.40×10^{-4} min^{-1}. [b] Initial velocities of the hydrolysis were determined by HPLC assay. [c] Not detected. [d] This velocity was determined by the use of DL-**1h** instead of L-**1h**.

Table 4. Enantioselective Hydrolysis of Esters **1** with Antibody 3G2[a]

substrate		velocities,[b] μM/min		ratio of velocities
1	R-	L-**1**	D-**1**	L-**1**:D-**1**
1a	CH_3-	9.68×10^{-3}	1.86×10^{-1}	5:95
1b	$(CH_3)_2CHCH_2$-	6.67×10^{-3}	1.16×10^{-1}	5:95
1c	$CH_3(CH_2)_3$-	3.98×10^{-3}	9.12×10^{-2}	4:96
1d	$CH_3SCH_2CH_2$-	2.44×10^{-2}	2.15×10^{-1}	6:94
1e	$PhCH_2$-	2.46×10^{-3}	3.08×10^{-2}	7:93
1f	$(CH_3)_2CH$-	5.70×10^{-4}	1.96×10^{-3}	23:77
1g	Ph-	ND[c]	5.83×10^{-2}	<1:>99
1h	4-HOPh	1.84×10^{-1} [d]	4.08×10^{-1}	
1i	$H_2N(CH_2)_4$-	2.04×10^{-1}	2.20×10^{-1}	48:52
1j	$H_2NCH_2CONH(CH_2)_4$-	3.85×10^{-3}	1.63×10^{-1}	2:98

[a] Reaction condditions: [L- or D-substrate] 100 μM, except for **1e** (50 μM), [antibody 3G2] 10 μM (active site), 10% DMSO/50 mM Tris (pH 8.0), 25 °C. The first-order kinetic constants of the background reactions: **1a**−**i**, see Table 3; **1j**, 1.21×10^{-4} min^{-1}. [b] See Table 3. [c] Not detected. [d] This velocity was determined by the use of DL-**1h** instead of L-**1h**.

(Tables 3 and 4).[20] The reaction in 10% DMSO/50 mM Tris (pH 8.0) at 25 °C was followed by monitoring the production of 4-nitrobenzyl alcohol by HPLC. Antibody 7G12 displayed broad substrate specificity, hydrolyzing the L-ester derivatives of Ala (**1a**), Leu (**1b**), Norleu (**1c**), Met (**1d**), Phe (**1e**), and Lys (**1i**) with high enantioselectivity (Table 3). In all cases, product inhibition was not detected. Interestingly, amino acid esters with bulky substituents on Cβ, such as Val (**1f**), phenylglycine (**1g**), and 4-hydroxyphenylglycine (**1h**), were also enantioselectively hydrolyzed by 7G12.[21] Antibody 3G2 also accepted a broad range of amino acid esters and enantioselectively hydrolyzed the D-isomers. When the hydrolysis of the lysine derivative (**1i**) with antibody 3G2 was examined, we were surprised to find that no enantioselectivity was detected. Since the *N*ϵ-glycyl-D-lysine ester (**1j**) was enantioselectively hydrolyzed with 3G2, the ϵ-amino group of lysine may influence the substrate recognition with antibody 3G2, but the details remain unclear. Neither antibody 7G12 nor 3G2 catalyzed the hydrolysis of proline ester derivatives.

Table 5. Kinetic Parameters of Antibody 7G12-Catalyzed Hydrolysis of Ester **1**[a]

substrate	R-	K_m, μM	k_{cat},[b] min^{-1}
L-**1a**	CH_3-	13	2.8×10^{-2}
L-**1b**	$(CH_3)_2CHCH_2$-	23	3.7×10^{-2}
L-**1c**	$CH_3(CH_2)_3$-	36	2.8×10^{-2}
L-**1e**	$PhCH_2$-	4.9	2.4×10^{-2}
L-**1h**	4-HOPh-	64[c]	1.2×10^{-1} [c]

[a] Reaction conditions: 10% DMSO/50 mM Tris (pH 8.0), 25 °C. [b] See ref 13. [c] The K_m and k_{cat} values were determined by the use of DL-**1h** and by disregarding the presence of D-**1h**, for example, [DL-**1h**] = 50 μM was regarded as [L-**1h**] = 25 μM.

Table 6. Kinetic Parameters of Antibody 3G2-Catalyzed Hydrolysis of Ester **1**[a]

substrate	R$^-$	K_m, μM	k_{cat},[b] min^{-1}
D-**1a**	CH_3-	16	2.1×10^{-2}
D-**1b**	$(CH_3)_2CHCH_2$-	21	1.0×10^{-2}
D-**1c**	$CH_3(CH_2)_3$-	41	8.6×10^{-3}
D-**1e**	$PhCH_2$-	5.5	3.0×10^{-3}
D-**1h**	4-HOPh-	27	6.2×10^{-2}

[a] Reaction conditions: 10% DMSO/50 mM Tris (pH 8.0), 25 °C. [b] See ref 13.

To examine the effects of the α-substituents of the substrates in more detail, the K_m values of the amino acid esters Ala (**1a**), Leu (**1b**), Norleu (**1c**), Phe (**1e**), and 4-hydroxyphenylglycine (**1h**), which were chosen on the basis of the size (bulkiness) of the α-substituents, were determined. As shown in Tables 5 and 6, the K_m values for these substrates were found to be in the narrow ranges of 4.9−64 μM for 7G12 and 5.5−41 μM for 3G2. Thus, alteration of the α-substituents of the substrates has little effect on the binding affinity to the antibodies. This observation suggests that the use of haptens that fit snugly into the antigen-combining site, leaving the linker moiety (involving Cβ) outside, is an effective approach for the generation of catalytic antibodies with broad substrate applicability. Although Hirschmann *et al*. also attempted to generate catalytic antibodies (for peptide-bond formation) that accomodate the diverse α-substituents of amino acids, by creating a cavity corresponding to the haptenic cyclohexyl group in the antigen-combining site, the size and the hydrophobicity of the cavity limited the diversity of the α-substituents of amino acids.[22] In our case, the α-substituents of the amino acids should be excluded from the antigen-combining site, and broader substrate specificity was achieved.

Conclusion

In this work, we have proposed a hapten design to endow antibody catalysts with two opposing qualities, such as high selectivity and broad substrate specificity. We demonstrated the generation of antibodies that enantioselectively hydrolyze a wide range of α-amino acid esters. These catalytic antibodies yield chiral *N*-Cbz protected amino acids as products, so they would be useful for the synthesis of peptides including natural and unnatural amino acids as components.

Experimental Section

General Methods (Synthesis). All oxygen- or moisture-sensitive reactions were carried out under argon. Flash chromatography was

(20) Catalytic antibodies with either *R* or *S* substrate specificity are expected to show kinetic resolution: (a) Reference 10. (b) Sinha, S. C.; Keinan, E.; Reymond, J.-L. *J. Am. Chem. Soc*. **1993**, *115*, 4893−4894. (c) Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc*. **1989**, *111*, 5961−5962. (d) Napper, A. D.; Benkovic, S. J.; Tramontano, A.; Lerner, R. A. *Science* **1987**, *237*, 1041−1043.

(21) Because the background hydrolysis (k_{uncat}) of **1f** was lower than that of the other substrates, the velocity of the L-ester hydrolysis by antibody 7G12 was 2 orders of magnitude lower than that of the other L-esters. Although ester **1f**, with a bulky substituent, was more resistant to hydrolysis, it was enantioselectively (9:1) hydrolyzed by antibody 7G12.

(22) Hirschmann, R.; Smith, A. B., III; Taylor, C. M.; Benkovic, P. A.; Taylor, S. D.; Yager, K. M.; Sprengeler, P. A.; Benkovic, S. J. *Science* **1994**, *265*, 234−237.

performed using Kisel gel (230−400 mesh) silica gel (Merck). Preparative thin-layer chromatography (TLC) was performed on Kisel gel 60 F_{254} (0.5 mm) (Merck). Preparative high-performance liquid chromatography (HPLC) was performed on a Hitachi L-6200 equipped with a L-4200 UV detector. ^{1}H NMR spectra were recorded on Bruker AM 500 (500 MHz) and AMX 600 (600 MHz) NMR spectrometers. The spectra are reported in units of ppm downfield from tetramethylsilane. Mass spectra were recorded with JEOL JMS-SX102/102A (Tandem MS) and JMS-HX/HX110A mass spectrometers.

Compound 5. To a solution of ethyl 6-hydroxyhexanoate (**4**) (2.83 g, 17.7 mmol) in CH_2Cl_2 (70 mL) was added PDC (9.95 g, 26.4 mmol) at room temperature. After 5 h of stirring, the reaction mixture was flash chromatographed (EtOAc/hexane = 1/2) to give **5** as a colorless oil (1.1276 g, 40%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.78 (t, J = 1.4 Hz, 1H), 4.14 (q, J = 7.2 Hz, 2H), 2.50−2.46 (m, 2H), 2.37−2.30 (m, 2H), 1.71−1.63 (m, 4H), 1.26 (t, J = 7.2 Hz, 3H).

Compound 6. To a solution of **5** (1.1276 g, 7.13 mmol) in EtOH (2.5 mL) were added benzylamine (1.56 mL, 14.3 mmol) and triethyl phosphite (2.44 mL, 14.2 mmol) at room temperature. After stirring at room temperature for 17.5 h, triethyl phosphite (1.0 mL, 5.8 mmol) was added and the reaction mixture was stirred at 40 °C for 6 h. The solvent was removed *in vacuo*. The residue was flash chromatographed (EtOAc/2-propanol = 20/1) to give **6** as a colorless oil (1.4837 g, 48%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35−7.23 (m, 5H), 4.21−4.09 (m, 6H), 3.97 (d, J = 13.1 Hz, 2H), 3.88 (dd, J = 13.1 Hz, 1.3 Hz, 2H), 2.85 (m, 1H), 2.30−2.25 (m, 2H), 1.83−1.53 (m, 6H), 1.34 (t, J = 7.0 Hz, 6H), 1.25 (t, J = 7.1 Hz, 3H). FABMS: m/z 386 (M^+ + H). HR-FABMS: calcd for $C_{19}H_{33}O_5NP$ (M^+ + H) 386.2132, found 386.2092.

Compound 7. A mixture of **6** (473.0 mg, 1.23 mmol) and 10% Pd−C (4.6 mg) in MeOH (3.0 mL)−formic acid (0.40 mL) was stirred at room temperature for 2.5 days under H_2. The mixture was filtered. The filtrate was concentrated *in vacuo* to give a colorless residue. The resulting residue was dissolved in THF (5.0 mL), and to this were added carbobenzoxy chloride (700 μL, 4.90 mmol) and triethylamine (1.71 mL, 12.3 mmol) at room temperature. After 3 h, 1 N HCl was added and the mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and flash chromatographed (EtOAc, TLC: R_f 0.51) to give a colorless gum (120.5 mg). The resulting gum was dissolved in CH_2Cl_2 (0.5 mL), and bromotrimethylsilane (222 μL, 1.68 mmol) was added at room temperature. After the reaction mixture was stirred at 35 °C for 2 h, the volatile solvent was removed *in vacuo* and CH_3CN (1.0 mL) and H_2O (0.2 mL) were added to the reaction mixture. After the mixture was stirred for 12 h, the solvent was removed *in vacuo* and the residue was dried. A mixture of the residue, 4-nitrobenzyl alcohol (61.2 mg, 0.40 mmol), 1*H*-tetrazole (9.7 mg, 0.14 mmol), and DCC (261.5 mg, 1.27 mmol) in CH_2Cl_2 (1.0 mL)−pyridine (0.5 mL) was stirred at 35 °C for 5 h. The reaction was concentrated *in vacuo*, diluted with CH_3CN, and filtered. The filtrate was purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 50/50, 3.0 mL/min, 254 nm, retention time 11.5 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give **7** as a colorless residue (34.5 mg, 6% from **6**). ^{1}H NMR (500 MHz, CD_3OD): δ 8.22 (d, J = 8.6 Hz, 2H), 7.62 (d, J = 8.6 Hz, 2H), 7.38−7.28 (m, 5H), 5.19 (A of ABX, dd, J_{AB} = 13.5 Hz, J_{AX} = 7.4 Hz, 1H), 5.16 (B of ABX, dd, J_{AB} = 13.5 Hz, J_{AX} = 7.4 Hz, 1H), 5.13 (A of AB, d, J = 12.6 Hz, 1H), 5.09 (B of AB, d, J = 12.6 Hz, 1H), 4.14 (q, J = 7.1 Hz, 2H), 4.06 (m, 1H), 2.33 (t, J = 7.3 Hz, 2H), 1.95−1.37 (m, 6H), 1.27 (t, J = 7.1 Hz, 3H). FABMS: m/z 509 (M^+ + H). HR-FABMS: calcd for $C_{23}H_{30}O_9N_2P$ (M^+ + H) 509.1689, found 509.1688.

Hapten 2. To a solution of **7** (15.0 mg, 0.0295 mmol) in MeOH (0.2 mL)−H_2O (0.2 mL) was added 1 N NaOH (0.1 mL, 0.1 mmol) at room temperature. After 6.5 h, the reaction was acidified with trifluoroacetic acid and purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 50/50, 3.0 mL/min, 254 nm, retention time 6.2 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give **2** as a colorless residue (14.0 mg, 99%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.22 (d, J = 8.6 Hz, 2H), 7.61 (d, J = 8.6 Hz, 2H), 7.37−7.28 (m, 5H), 5.19 (A of ABX, dd, J_{AB} = 13.5 Hz, J_{AX} = 7.7 Hz, 1H), 5.16 (B of ABX, dd, J_{AB} = 13.5 Hz, J_{AX} = 7.7 Hz, 1H), 5.14 (A of AB, d, J = 12.5 Hz, 1H), 5.08 (B of AB, d, J = 12.5 Hz, 1H), 4.07 (m, 1H), 2.32 (t, J = 7.3 Hz, 2H), 1.97−1.38 (m, 6H). FABMS: m/z 503 (M^+ + Na), 481 (M^+ + H). HR-FABMS: calcd for $C_{21}H_{25}O_9N_2$-PNa (M^+ + Na) 503.1196, found 503.1202.

Compound 8. A mixture of **2** (6.4 mg, 0.013 mmol), *N*-hydroxysuccinimide (2.5 mg, 0.022 mmol), WSC (8.3 mg, 0.043 mmol), and DMAP (0.1 mg, 0.0008 mmol) in CH_3CN (0.3 mL) was stirred at room temperature for 2 h. The mixture was purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 50/50, 3.0 mL/min, 254 nm, retention time 8.2 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give **8** as a colorless residue (6.1 mg, 79%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.23 (d, J = 8.7 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H), 7.38−7.27 (m, 5H), 5.20 (A of ABX, dd, J_{AB} = 13.4 Hz, J_{AX} = 7.5 Hz, 1H), 5.17 (B of ABX, dd, J_{AB} = 13.4 Hz, J_{AX} = 7.5 Hz, 1H), 5.13 (A of AB, d, J = 12.5 Hz, 1H), 5.09 (B of AB, d, J = 12.5 Hz, 1H), 4.08 (m, 1H), 2.85 (s, 4H), 2.66 (t, J = 7.2 Hz, 2H), 1.97−1.43 (m, 6H).

KLH-2. To a solution of **8** (2.5 mg, 0.0043 mmol) in DMF (60 μL)−200 mM Na_2HPO_4−NaH_2PO_4 (pH 7.4) (0.5 mL) was added a KLH solution in 10 mM Na_2HPO_4−NaH_2PO_4 (pH 7.4) (11.3 mg/mL, 442 μL) at room temperature. After 21 h, the mixture was purified by Sephadex G-25M (Pharmacia, PD-10)(PBS buffer) to give KLH-**2**. The concentration of KLH-**2** was determined by Bradford analysis (1.3 mg/mL). The KLH-**2** was used for immunization.

BSA-2. To a solution of **8** (3.0 mg, 0.0052 mmol) in DMF (40 μL) was added a BSA solution in 200 mM Na_2HPO_4−NaH_2PO_4 (pH 7.4) (6.0 mg in 0.5 mL) at room temperature. After 12 h, the mixture was purified by Sephadex G-25M (Pharmacia, PD-10) (PBS buffer) to give BSA-**2**. The concentration of BSA-**2** was determined by Bradford analysis (0.9−2.3 mg/mL, three fractions). The BSA-**2** was used for the ELISA experiment.

(*R*)-[1-(*N*-Carbobenzoxyamino)ethyl]phosphonic Acid. To a solution of (*R*)-(−)-(1-aminoethyl)phosphonic acid (500.8 mg, 4.00 mmol) in 4 N NaOH (3.3 mL) was added carbobenzoxy chloride (742 μL, 5.20 mmol) at 0 °C, and the reaction mixture was stirred at room temperature. After 12 h, the mixture was acidified with 2 N HCl and purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 30/70, 3.0 mL/min, 254 nm, retention time 7.7 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give (*R*)-[1-(*N*-carbobenzoxyamino)ethyl]phosphonic acid as a colorless solid (680.8 mg, 66%). ^{1}H NMR (500 MHz, CD_3OD): δ 7.41−7.31 (m, 5H), 5.14 (s, 2H), 4.01 (m, 1H), 1.39 (dd, J = 7.4 Hz, 16.2 Hz, 3H). FABMS m/z: 260 (M^+ + H). $[\alpha]_D^{26}$ −14.4° (*c* 0.9, MeOH).

(*S*)-[1-(*N*-Carbobenzoxyamino)ethyl]phosphonic Acid. $[\alpha]_D^{26}$ +14.0° (*c* 1.3, MeOH).

(*R*)-[1-(*N*-Carbobenzoxyamino)ethyl]phosphonic Acid 4-Nitrobenzyl Ester [(*R*)-3]. A mixture of (*R*)-[1-(*N*-carbobenzyloxyamino)ethyl]-phosphonic acid (83.0 mg, 0.320 mmol), 4-nitrobenzyl alcohol (57.5 mg, 0.375 mmol), 1*H*-tetrazole (4.8 mg, 0.069 mmol), and DCC (293.1 mg, 1.42 mmol) in CH_2Cl_2 (1.0 mL)−pyridine (1.0 mL) was stirred at 35 °C for 10 h. The reaction was concentrated *in vacuo*, diluted with CH_3CN and TFA, and filtered. The filtrate was purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 40/60, 3.0 mL/min, 254 nm, retention time 10.1 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give (*R*)-**3** as a colorless solid (51.8 mg, 41%). ^{1}H NMR (600 MHz, CD_3OD): δ 8.22 (d, J = 8.6 Hz, 2H), 7.63 (d, J = 8.6 Hz, 2H), 7.40−7.29 (m, 5H), 4.15 (m, 1H), 1.44 (dd, J = 7.2 Hz, 16.6 Hz, 3H). FABMS: m/z 395 (M^+ + H). HR-FABMS: calcd for $C_{17}H_{20}O_7N_2P$ (M^+ + H) 395.1008, found 395.1007. $[\alpha]_D^{26}$ +4.6° (*c* 0.6, MeOH).

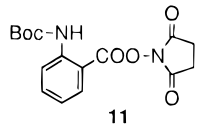
(*S*)-3. $[\alpha]_D^{26}$ −4.6° (*c* 0.7, MeOH).

2-[(*tert*-Butoxycarbonyl)amino]benzoic Acid. A mixture of anthranic acid (1.37 g, 10.0 mmol) and $(Boc)_2O$ (3.12 g, 14.3 mmol) in 0.5 N NaOH (20.0 mL)−dioxane (10.0 mL)−CH_3CN (2.0 mL) was stirred at room temperature for 8 h. After the volatile solvent was removed *in vacuo*, ice and 10% citric acid were added and the mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and

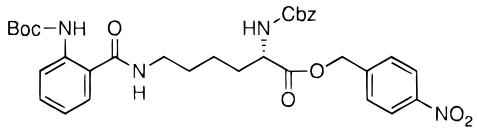
crystallized from EtOAc−hexane to give 2-[(*tert*-butoxycarbonyl)-amino]benzoic acid as colorless crystals (1.71 g, 72%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.1 (s, 1H), 8.47 (d, J = 8.0 Hz, 1H), 8.09 (dd, J = 8.0 Hz, 1.4 Hz, 1H), 7.56 (dt, J = 8.0 Hz, 1.4 Hz, 1H), 7.04 (t, J = 8.0 Hz, 1H), 1.54 (s, 9H).

Compound 11. A mixture of 2-[(*tert*-butoxycarbonyl)amino]benzoic acid (378.9 mg, 1.60 mmol), *N*-hydroxysuccinimide (225.9 mg, 1.96 mmol), WSC (565.9 mg, 2.95 mmol), and DMAP (4.7 mg, 0.04 mmol) in CH_2Cl_2 was stirred at room temperature for 1 h. To the reaction mixture was added 1% citric acid, and the mixture was extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and crystallized from EtOAc−hexane to give **11** as colorless crystals (199.7 mg, 30%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.49 (s, 1H), 8.51 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 8.0 Hz, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.07 (d, J = 8.0 Hz, 1H), 2.93 (s, 4H), 1.52 (s, 9H).

Compound L-12. A mixture of **11** (67.3 mg, 0.201 mmol), *N*α-carbobenzoxy-L-lysine (56.4 mg, 0.201 mmol), and diisopropylethylamine (0.04 mL, 0.230 mmol) in DMF (0.2 mL)−MeOH (0.2 mL)−EtOAc (0.1 mL)−H_2O (0.1 mL) was stirred at room temperature for 16 h. After the volatile solvent was removed *in vacuo*, the residue was flash chromatographed (EtOAc/hexane/H_2O = 9/1/0−8/2/0.2−5/3/1) to give a residue (122.2 mg). A mixture of this residue (122.2 mg), 4-nitrobenzyl alcohol (34.9 mg, 0.228 mmol), WSC (58.6 mg, 0.306 mmol), and DMAP (1.4 mg, 0.011 mmol) in CH_2Cl_2 (1.0 mL)−CH_3CN (0.1 mL) was stirred at room temperature for 4 h. The mixture was flash chromatographed (EtOAc/hexane = 1/1.1) to give L-**12** (35.7 mg, 28%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 10.1 (s, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.20 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.44−7.38 (m, 2H), 7.35−7.28 (m, 5H), 6.95 (t, J = 8.0 Hz, 1H), 6.37 (brs, 1H), 5.36 (brs, 1H), 5.26 (A of AB, d, J = 13.3 Hz, 1H), 5.23 (B of AB, d, J = 13.3 Hz, 1H), 5.09 (A of AB, d, J = 12.3 Hz, 1H), 5.03 (B of AB, d, J = 12.3 Hz, 1H), 4.46 (m, 1H), 3.43−3.37 (m, 2H), 1.95−1.40 (m, 6H). FABMS: m/z 635 (M^+ + H).



11



L-12

Compound L-9. A mixture of L-**12** (54.1 mg, 0.0858 mmol) and trifluoroacetic acid (0.2 mL) in CH_2Cl_2 (0.3 mL) was stirred at room temperature for 1 h. The solvent was removed *in vacuo*, and the residue was purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 60/40, 3.0 mL/min, 254 nm, retention time 10.1 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give L-**9** as a colorless gum (38.1 mg, 83%). The gum was crystallized from EtOAc−hexane to give L-**9** as colorless crystals. ^{1}H NMR (600 MHz, $CDCl_3$−CD_3OD): δ 8.21 (d, J = 8.0 Hz, 2H), 7.49 (d, J = 8.0 Hz, 2H), 7.37−7.25 (m, 6H), 7.19 (t, J = 8.0 Hz, 1H), 6.65 (d, J = 8.0 Hz, 1H), 6.64 (t, J = 8.0 Hz, 1H), 5.26 (A of AB, d, J = 13.3 Hz, 1H), 5.24 (B of AB, d, J = 13.3 Hz, 1H), 5.10 (A of AB, d, J = 12.3 Hz, 1H), 5.06 (B of AB, d, J = 12.3 Hz, 1H), 4.40 (m, 1H), 3.42−3.34 (m, 2H), 1.94−1.85 (m, 1H), 1.80−1.72 (m, 1H), 1.68−1.54 (m, 2H), 1.51−1.39 (m, 2H). FABMS: m/z 535 (M^+ + H). HR-FABMS: calcd for $C_{28}H_{31}O_7N_4$ (M^+ + H) 535.2193, found 535. 2182. The ees of L-**9** and D-**9** were determined to be >99% by HPLC (CHIRALCEL OJ-R, ϕ 4.6 × 150 nnm, CH_3CN/0.1% aqueous TFA = 55/45, 0.5 mL/min, 254 nm, retention time: L-**9** 13.7 min, D-**9** 12.8 min).

Compound L-10. A mixture of L-**12** (21.8 mg, 0.0346 mmol) and trifluoroacetic acid (0.2 mL) in CH_2Cl_2 (0.4 mL) was stirred at room temperature for 20 min. After the solvent was removed *in vacuo*, the residue was dissolved in MeOH (0.5 mL) and 1 N NaOH (0.3 mL) was added. After the mixture was stirred for 12 h, the reaction was acidified with trifluoroacetic acid and purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 55/45, 3.0 mL/min, 254 nm, retention time 5.2 min). The CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give L-**10** as a pale yellow gum. ^{1}H NMR (500 MHz, CD_3OD): δ 7.57 (d, J = 8.0 Hz, 1H), 7.40−7.30 (m, 6H), 6.97 (d, J = 8.0 Hz, 1H), 6.90 (t, J = 8.0 Hz, 1H), 5.11 (s, 2H), 4.21 (m, 1H), 3.89 (t, J = 6.9 Hz, 2H), 1.97−1.88 (m, 1H), 1.81−1.62 (m, 3H), 1.59−1.46 (m, 2H). FABMS: m/z 400 (M^+ + H). HR-FABMS: calcd for $C_{21}H_{26}O_5N_3$ (M^+ + H) 400.1872, found 400.1884.

Synthesis of *N*α-Carbobenzoxy Amino Acid 4-Nitrobenzyl Esters (1). Esters (**1a**−**h**) were prepared from either L- or D-*N*-carbobenzoxy amino acid and 4-nitrobenzyl alcohol using WSC (method A), or L- or D-*N*-carbobenzoxy amino acid and 4-nitrobenzyl bromide using triethylamine (method B). The general procedures were as follows:

Method **A:** A mixture of *N*-carbobenzoxy amino acid (1 equiv), 4-nitrobenzyl alcohol (1.1 equiv), WSC (1.3−1.6 equiv), and DMAP (0.01 equiv) in CH_2Cl_2 was stirred at room temperature for 1 h. The solvent was removed *in vacuo*, ice and 1 N HCl were added, and the mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and flash chromatographed to give a *N*-carbobenzyoxy amino acid 4-nitrobenzyl ester.

Method B: A mixture of *N*-carbobenzoxy amino acid (1 equiv), 4-nitrobenzyl bromide (1.5 equiv), and triethylamine (1.5 equiv) in EtOAc was refluxed for 2 h. The reaction was quenched with ice and 1 N HCl, and the mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and flash chromatographed to give a *N*-carbobenzoxy amino acid 4-nitrobenzyl ester.

Enantiomeric Purity of Esters 1. The enantiomeric purity of the substrate esters was determined by HPLC analysis using DAICEL CHIRALCEL OB (**1a**,**b**, hexane/2-propanol), CHIRALCEL OJ-R (**1c**,**d**, CH_3CN/0.1% aqueous TFA), and CHIRALCEL OD-R (**1e**−**g**, and D-**1h**, CH_3CN/0.1% aqueous TFA): all of the substrates, except L-**1g** (92% ee), were >99% ee. L-**1i**: $[\alpha]_D^{22}$ −12.4°, *c* 0.4, MeOH). D-**1i**: $[\alpha]_D^{22}$ +12.3° (*c* 0.2, MeOH). Isomerization of L-**1e** to D-**1e** was not detected under the assay conditions by HPLC analysis using CHIRALCEL OD-R.

***N*-Carbobenzoxy-L-alanine 4-Nitrobenzyl Ester (L-1a).** According to method A, the reaction was flash chromatographed (EtOAc/hexane = 2/3) and crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.21 (d, J = 8.0 Hz, 2H), 7.49 (d, J = 8.0 Hz, 2H), 7.37−7.30 (m, 5H), 5.26 (s, 2H), 5.24 (brd, 1H), 5.12 (s, 2H), 4.47 (m, 1H), 1.45 (d, J = 7.2 Hz, 3H). FABMS: m/z 359 (M^+ + H). HR-FABMS: calcd for $C_{18}H_{19}O_6N_2$ (M^+ + H) 359.1244, found 359.1244.

The ee was determined to be >99% by HPLC (CHIRALCEL OB, ϕ 4.6 × 250 mm, hexane/2-propanol = 10/90, 0.5 mL/min, 254 nm, retention time: L-**1a** 33.0 min, D-**1a** 75.8 min).

***N*-Carbobenzoxy-L-leucine 4-Nitrobenzyl Ester (L-1b).** According to method A, the reaction was flash chromatographed (EtOAc/hexane = 1/2) to give a colorless gum. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.22 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 8.3 Hz, 2H), 7.40−7.30 (m, 5H), 5.26 (A of AB, d, J = 13.4 Hz, 1H), 5.24 (B of AB, d, J = 13.4 Hz, 1H), 5.12 (A of AB, d, J = 11.4 Hz, 1H), 5.11 (B of AB, d, J = 11.4 Hz, 1H), 5.13−5.11 (1H), 4.46 (m, 1H), 1.74−1.52 (m, 3H), 0.95 (d, J = 7.2 Hz, 3H), 0.94 (d, J = 7.2 Hz, 3H). FABMS: m/z 401 (M^+ + H). HR-FABMS: calcd for $C_{21}H_{25}O_6N_2$ (M^+ + H) 401.1712, found 401.1713. The ee was determined to be >99% by HPLC (CHIRALCEL OB, ϕ 4.6 × 250 mm, hexane/2-propanol = 30/70, 0.5 mL/min, 254 nm, retention time: L-**1b** 20.9 min, D-**1b** 31.8 min).

***N*-Carbobenzoxy-L-norleucine 4-Nitrobenzyl Ester (L-1c).** According to method B, the reaction was flash chromatographed (EtOAc/hexane = 2/5) and crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.22 (d, J = 8.5 Hz, 2H), 7.50 (d, J = 8.5 Hz, 2H), 7.38−7.30 (m, 5H), 5.26 (s, 2H), 5.20 (brd, J = 7.9 Hz, 1H), 5.12 (s, 2H), 4.43 (m, 1H), 1.85 (m, 1H), 1.68 (m, 1H), 1.37−1.23 (m, 4H), 0.87 (t, J = 6.9 Hz, 3H). EIMS: m/z 400 (M^+). HR-EIMS: calcd for $C_{21}H_{24}O_6N_2$ (M^+) 400.1634, found

400.1639. The ee was determined to be >99% by HPLC (CHIRALCEL OJ-R, ßlc 4.6 × 150 mm, CH_3CN/0.1% aqueous TFA = 60/40, 0.5 mL/min, 2.54 nm, retention time: L-**1c** 15.7 min, D-**1c** 16.9 min).

***N*-Carbobenzoxy-L-methionine 4-Nitrobenzyl Ester (L-1d).** According to method A, the reaction was flash chromatographed (EtOAc/hexane = 2/3) to give a colorless gum. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.22 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.39−7.32 (m, 5H), 5.42 (brd, J = 7.1 Hz, 1H), 5.28 (A of AB, d, J = 12.9 Hz, 1H), 5.26 (B of AB, d, J = 12.9 Hz, 1H), 5.12 (s, 2H), 4.59 (m, 1H), 2.53 (t, J = 7.1 Hz, 2H), 2.19 (m, 1H), 2.07 (s, 3H), 2.01 (m, 1H). FABMS: m/z 419 (M^+ + H). The ee was determined to be >99% by HPLC (CHIRALCEL OJ-R, ϕ 4.6 × 150 mm, CH_3CN/0.1% aqueous TFA = 60/40, 0.5 mL/min, 254 nm, retention time: L-**1d** 14.2 min, D-**1d** 15.9 min).

***N*-Carbobenzoxy-L-phenylalanine 4-Nitrobenzyl Ester (L-1e).** According to method A, the reaction was flash chromatographed (EtOAc/hexane = 2/3) and crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.17 (d, J = 8.0 Hz, 2H), 7.38−7.23 (m, 10H), 7.08 (m, 2H), 5.23 (brd, J = 7.5 Hz, 1H), 5.19 (s, 2H), 5.09 (s, 1H), 4.92 (m, 1H), 3.12 (d, J = 6.8 Hz, 2H). FABMS: m/z 435 (M^+ + H). HR-FABMS: calcd for $C_{24}H_{23}O_6N_2$ (M^+ + H) 435.1556, found 435.1556. The ee was determined to be >99% by HPLC (CHIRALCEL OD-R, ϕ 4.6 × 250 mm, CH_3CN/0.1% aqueous TFA = 60/40, 0.5 mL/min, 254 nm, retention time: L-**1e** 39.7 min, D-**1e** 43.2 min).

***N*-Carbobenzoxy-L-valine 4-Nitrobenzyl Ester (L-1f).** According to method A, the reaction was flash chromatographed (EtOAc/hexane = 1/2) and crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.22 (d, J = 8.5 Hz, 2H), 7.51 (d, J = 8.5 Hz, 2H), 7.39−7.31 (m, 5H), 5.26 (s, 2H), 5.23 (brd, J = 8.8 Hz, 1H), 5.12 (s, 2H), 4.38 (dd, J = 4.8 Hz, 8.8 Hz, 1H), 2.20 (m, 1H), 0.98 (d, J = 6.8 Hz, 3H), 0.88 (d, J = 6.8 Hz, 3H). EIMS: m/z 386 (M^+). HR-EIMS: calcd for $C_{20}H_{22}O_6N_2$ (M^+) 386.1476, found 386.1476. The ee was determined to be >99% by HPLC (CHIRALCEL OD-R, ϕ 4.6 × 250 mm, CH_3CN/0.1% aqueous TFA = 70/30, 0.5 mL/min, 254 nm, retention time: L-**1f** 13.9 min, D-**1f** 15.8 min).

***N*-Carbobenzoxy-L-phenylglycine 4-Nitrobenzyl Ester (L-1g).** According to method B, the reaction was flash chromatographed (EtOAc/hexane = 2/5) and crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.12 (d, J = 8.5 Hz, 2H), 7.39−7.30 (m, 5H), 7.27 (d, J = 8.5 Hz, 2H), 5.77 (brd, J = 7.1 Hz, 1H), 5.44 (d, J = 7.1 Hz, 1H), 5.28 (A of AB, d, J = 13.5 Hz, 1H), 5.22 (B of AB, d, J = 13.5 Hz, 1H), 5.13 (A of AB, d, J = 12.1 Hz, 1H), 5.09 (B of AB, d, J = 12.1 Hz, 1H). FABMS: m/z 421 (M^+ + H). The ee was determined to be 92% by HPLC (CHIRALCEL OD-R, ϕ 4.6 × 250 mm, CH_3CN/0.1% aqueous TFA = 70/30, 0.5 mL/min, 254 nm, retention time: L-**1g** 19.8 min, D-**1g** 22.2 min).

***N*-Carbobenzoxy-4-hydroxyphenylglycine 4-Nitrobenzyl Ester (1h).** According to method B, the reaction was flash chromatographed (EtOAc/hexane = 1/1.25) and crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.14 (d, J = 8.5 Hz, 2H), 7.38−7.31 (m, 5H), 7.30 (d, J = 8.5 Hz, 2H), 7.22 (d, J = 8.5 Hz, 2H), 6.81 (d, J = 8.5 Hz, 2H), 5.70 (brd, J = 7.1 Hz, 1H), 5.36 (d, J = 7.1 Hz, 1H), 5.26 (A of AB, d, J = 13.9 Hz, 1H), 5.22 (B of AB, d, J = 13.9 Hz, 1H), 5.12 (A of AB, d, J = 12.0 Hz, 1H), 5.09 (B of AB, d, J = 12.0 Hz, 1H), 5.10 (s, 1H). EIMS: m/z 436 (M^+). HR-EIMS: calcd for $C_{23}H_{20}O_7N_2$ (M^+) 436.1271, found 436.1277. The ee of D-**1h** was determined to be >99% by HPLC (CHIRALCEL OD-R, ϕ 4.6 × 250 mm, CH_3CN/0.1% aqueous TFA = 45/55, 0.5 mL/min, 254 nm, retention time: L-**1h** 77.3 min, D-**1h** 81.4 min).

***N*α-Carbobenzoxy-*N*ε-(*tert*-butoxycarbonyl)-D-lysine 4-Nitrobenzyl Ester.** A mixture of *N*α-carbobenzoxy-D-lysine (194.7 mg, 0.695 mmol) and $(Boc)_2O$ (176.0 mg, 0.806 mmol) in 1 N NaOH (765 μL)−MeOH (0.1 mL) was stirred at room temperature for 10 h. The reaction was quenched with ice and 10% citric acid, and the mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give *N*α-carbobenzoxy-*N*ε-(*tert*-butoxycarbonyl)-D-lysine as a residue. A mixture of this residue, 4-nitrobenzyl bromide (231.3 mg, 1.07 mmol), and triethylamine (145 μL, 1.04 mmol) in EtOAc was refluxed for 2 h. The reaction was quenched with ice and 10% citric acid, and the mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, concentrated *in vacuo*, and flash chromatographed (EtOAc/hexane = 2/3) to give *N*α-carbobenzoxy-*N*ε-(*tert*-butoxycarbonyl)-D-lysine 4-nitrobenzyl ester (272.4 mg, 76%). The ester was crystallized from EtOAc−hexane to give colorless crystals. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.22 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 8.3 Hz, 2H), 7.38−7.30 (m, 5H), 5.40 (brd, J = 6.6 Hz, 1H), 5.26 (A of AB, d, J = 12.6 Hz, 1H), 5.25 (B of AB, d, J = 12.6 Hz, 1H), 5.12 (A of AB, d, J = 12.2 Hz, 1H), 5.10 (B of AB, d, J = 12.2 Hz, 1H), 4.55 (m, 1H), 4.41 (m, 1H), 3.12−3.07 (m, 2H), 1.88 (m, 1H), 1.72 (m, 1H), 1.60−1.31 (m, 4H), 1.42 (s, 9H). FABMS: m/z 516 (M^+ + H).

***N*α-Carbobenzoxy-D-lysine 4-Nitrobenzyl Ester (D-1i).** A mixture of *N*α-carbobenzoxy-*N*ε-(*tert*-butoxycarbonyl)-D-lysine 4-nitrobenzyl ester (52.5 mg, 0.101 mmol) and trifluoroacetic acid (0.2 mL) in $CHCl_3$ (1.0 mL) was stirred at room temperature for 4 h. The solvent was removed *in vacuo*, and the residue was crystallized from EtOAc−hexane to give D-**1i** as colorless crystals (32.6 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$−CD_3OD): δ 8.22 (d, J = 8.5 Hz, 2H), 7.51 (d, J = 8.5 Hz, 2H), 7.40−7.26 (m, 5H), 5.29 (A of AB, d, J = 13.3 Hz, 1H), 5.24 (B of AB, d, J = 13.3 Hz, 1H), 5.13 (A of AB, d, J = 12.3 Hz, 1H), 5.09 (B of AB, d, J = 12.3 Hz, 1H), 4.35 (m, 1H), 2.87 (t, J = 7.2 Hz, 2H), 1.88 (m, 1H), 1.77−1.56 (m, 3H), 1.50−1.37 (m, 2H). EIMAS: m/z 415 (M^+). HR-EIMS: calcd for $C_{21}H_{25}O_6N_3$ (M^+) 415.1743, found 415.1743.

***N*α-Carbobenzoxy-*N*ε-[*N*-(*tert*-butoxycarbonyl)glycyl]-L-lysine 4-Nitrobenzyl Ester.** A mixture of L-**1i** (31.2 mg, 0.0751 mmol), *N*-(*tert*-butoxycarbonyl)glycine (19.1 mg, 0.109 mmol), and WSC (35.1 mg, 0.183 mmol) in CH_2Cl_2 (0.5 mL) was stirred at room temperature for 1.5 h. The mixture was flash chromatographed (EtOAc) to give *N*α-carbobenzoxy-*N*ε-[*N*-(*tert*-butoxycarbonyl)glycyl]-L-lysine 4-nitrobenzyl ester (19.6 mg, 46%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.21 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 8.3 Hz, 2H), 7.37−7.29 (m, 5H), 6.18 (brs, 1H), 5.46 (brd, J = 7.8 Hz, 1H), 5.26 (d, J = 13.9 Hz, 1H), 5.25 (d, J = 13.9 Hz, 1H), 5.12 (d, J = 12.8 Hz, 1H), 5.11 (d, J = 12.8 Hz, 1H), 4.39 (m, 1H), 3.78−3.69 (m, 2H), 3.31−3.19 (m, 2H), 1.86 (m, 1H), 1.71 (m, 1H), 1.58−1.33 (m, 4H). FABMS: m/z 573 (M^+ + H).

***N*α-Carbobenzoxy-*N*ε-glycyl-L-lysine 4-Nitrobenzyl Ester (L-1j).** A mixture of *N*α-carbobenzoxy-*N*ε-[*N*-(*tert*-butoxycarbonyl)glycyl]-L-lysine 4-nitrobenzyl ester (11.6 mg, 0.0203 mmol) and trifluoroacetic acid (0.1 mL) in $CHCl_3$ (0.3 mL) was stirred at room temperature for 3.5 h. The solvent was removed *in vacuo*, and the residue was purified by HPLC (YMC A-323: C-18 reverse-phase column, ϕ 10 × 250 mm, CH_3CN/0.1% aqueous TFA = 50/50, 3.0 mL/min, 254 nm, retention time 5.7 min). CH_3CN and TFA were removed *in vacuo*, and the water was removed by lyophilization to give L-**1j** (7.4 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$−CD_3OD): δ 8.21 (d, J = 8.5 Hz, 2H), 8.14 (brd, J = 7.9 Hz, 1H), 7.51 (d, J = 8.5 Hz, 2H), 7.41−7.25 (m, 5H), 6.23 (brd, J = 7.9 Hz, 1H), 5.28 (d, J = 13.4 Hz, 1H), 5.26 (d, J = 13.4 Hz, 1H), 5.13 (d, J = 12.3 Hz, 1H), 4.35 (m, 1H), 3.60 (s, 2H), 3.21 (t, J = 6.6 Hz, 2H), 1.85 (m, 1H), 1.72 (m, 1H), 1.59−1.45 (m, 2H), 1.43−1.35 (m, 2H). EIMS: m/z 472 (M^+). HR-EIMS: calcd for $C_{23}H_{28}O_7N_4$ (M^+) 472.1957, found 472.1957.

***N*-Carbobenzoxy-L-proline 4-Nitrobenzyl Ester (*E*:*Z* = 1:1).** According to method A, the reaction was flash chromatographed (EtOAc/hexane = 2/3) to give colorless gum. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.20 (d, J = 8.2 Hz, 2H × $^1/_2$), 8.10 (d, J = 8.2 Hz, 2H × $^1/_2$), 7.45 (d, J = 8.2 Hz, 2H × $^1/_2$), 7.38−7.23 (m, 5H + 2H × $^1/_2$), 5.30−5.02 (m, 4H), 4.38 (dd, J = 3.8 Hz, 8.5 Hz, 1H × $^1/_2$), 4.43 (dd, J = 3.8 Hz, 8.5 Hz, 1H × $^1/_2$), 3.67−3.50 (m, 2H), 2.33−2.22 (m, 1H), 2.06−1.88 (m, 3H). EIMAS: m/z 384 (M^+). HR-EIMAS: calcd for $C_{20}H_{20}O_6N_2$ (M^+) 384.1321, found 384.1321.

Antibody Production. Five Balb/c mice (4-week-old females) each received an intraperitoneal injection of 50 μg of KLH-**2** and Freund's complete adjuvant on day 1. On days 11 and 21, each mouse received an intraperitoneal injection of 50 μg of KLH-**2** and Freund's incomplete adjuvant. On day 28, serum was taken from the mice and the titer was determined by ELISA. On day 60, the mouse with the highest titer was injected intravenously with 100 μg of KLH-**2** in saline (final boost). After 3 days, the spleen was removed from the mouse, and the cells (1.9×10^8) were fused with X63/Ag8653 myeloma cells (2.7×10^7) according to standard protocols.[10] The cells were plated into ten 96-well plates; each well contained 200 μL of HAT selection media

(RPMI1640, 10% fetal bovine serum, 0.1 mM hypoxanthine, 0.4 μM aminopterin, 0.016 mM thymidine) containing mouse thymus cells (6×10^5/well). The culture supernatants in wells containing macroscopic colonies were assayed by ELISA for binding to BSA-**2**. A goat antimouse IgG peroxidase conjugate was used as the secondary antibody. Colonies that initially produced antibodies that bound BSA-**2** were subcloned two or three times, after which 39 remained active. The hybridomas were grown in RPMI1640 medium containing 10% fetal bovine serum. Monoclonal antibodies (IgG) were purified from the culture supernatants of the hybridomas by salt precipitation, cation-exchange chromatography (Mono S), and affinity chromatography (Protein G), as described previously.[11] All antibodies were then concentrated and dialyzed into 50 mM Tris (pH 8.0). The antibodies were judged to be homogeneous (>95%) by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. For screening, the antibody concentration was determined by absorbance at 280 nm as $\epsilon = 1.4$ (0.1%, 1 cm), with a molecular weight of 150 000 for IgG. For the kinetics and the determination of velocities using antibodies 7G12 and 3G2 and various substrates, the active site concentrations were determined by extrapolating from Henderson plots[14] of the activity titrations.

Fluorogenic Catalytic Assays and Kinetic Measurements. Reactions were initiated by adding 40 μL of a stock solution of the ester **9** in DMSO to 360 μL of the antibody solution in 50 mM Tris (pH 8.0) at 25 °C. The increase in the fluorescence was monitored for 10 min, using a Hitachi F-4000 fluorescence spectrophotometer. The excitation and emission wavelengths were 340 and 415 nm, respectively. Hydrolysis of substrate **9** results in a 45-fold linear increase in the fluorescence under the assay conditions. The observed rate was corrected for the uncatalyzed rate of hydrolysis in the absence of antibody. The kinetic parameters, k_{cat} and K_m, were determined by a linear least-squares fitting of the Lineweaver−Burk plots, as described by the Michaelis−Menten equation. The rate constants, k_{cat}s, were obtained by dividing by the active site concentration. The background rates (k_{uncat}) were determined in the absence of antibody under otherwise identical conditions.

Active Site Concentration and Inhibition Assays (Activity Titration). The active site concentrations of antibodies 7G12 and 3G2 were determined by extrapolating from Henderson plots[14] of the activity titrations according to the fluorogenic assays with phosphonate (*R*)-**3** and substrate L-**9** for antibody 7G2 or (*S*)-**3** and substrate D-**9** for antibody 3G2: $[I]/(1 - V/V_o) = [Ab] + K_i(1 + [S]/K_m)V_o/V$, where [I] is the phosphonate concentration, V and V_o are, respectively, the velocities in the presence and absence of the phosphonate, [Ab] is the active site concentration, K_i is the inhibitor constant, [S] is the substrate concentration, and K_m is the Michaelis−Menten constant.

HPLC Catalytic Assays and Kinetic Measurements. Reactions were initiated by adding 10 μL of a stock solution of substrate **1** in DMSO to 90 μL of antibody solution in 50 mM Tris (pH 8.0) at 25 °C. Hydrolysis rates were measured by HPLC detection of 4-nitrobenzyl alcohol with a 10 μL injection of the reaction mixture. The analytical HPLC was performed on a Hitachi L-6200 equipped with a L-4000H UV detector, using a YMC ODS AM-303 analytical column eluted with CH_3CN/0.1% aqueous TFA (35:65) at a flow rate of 1.0 mL/min, with detection at 278 nm. The retention time of 4-nitrobenzyl alcohol is 6.0 min. The initial velocities were determined from the linear range of the rate. The observed rate was corrected for the uncatalyzed rate of hydrolysis in the absence of antibody. The kinetic parameters, k_{cat} and K_m, were determined by a linear least-squares fitting of the Lineweaver−Burk plots described by the Michaelis−Menten equation. The rate constants, k_{cat}s, were obtained by dividing by the active site concentration. The background rates (k_{uncat}) were determined in the absence of antibody under otherwise identical conditions.

Determination of Enantiomeric Excess of Antibodies 7G12- and 3G2-Catalyzed Reactions. Reactions were initiated by adding 5 μL of a 3 mM solution of racemic substrate **9** in DMSO to 95 μL of antibody solution in 50 mM Tris (pH 8.0) at 25 °C. Final concentrations: [racemic **9**] 150 μM and [7G12] 20 μM (active site) or [3G2] 40 μM (active site). Hydrolysis rates were measured by HPLC detection of 4-nitrobenzyl alcohol with a 5 μL injection of the reaction mixture described above. The enantiomeric excess of the product acid **10** was determined by HPLC, with either a 20 μL (7G12) or a 30 μM (3G2) injection of the reaction mixture, using a SUMICHIRAL OA-3200 column eluted with MeOH containing 0.01 M ammonium acetate at a flow rate of 1.0 mL/min, with detection at 254 nm. The retention times of L-**10** and D-**10** are 15.0 and 13.0 min, respectively. In the 7G12-catalyzed reaction, after 137 min, the hydrolysis generated 59 μM of a 96% ee of L-**10** without correction for the background reaction. In the 3G2-catalyzed reaction, after 177 min, the hydrolysis generated 34 μM of a 94% ee of D-**10** without correction for the background reaction.

Competitive ELISA. Competitive ELISA was performed by using BSA-**2** and inhibitors, (*R*)-**3** and (*S*)-**3**, as described previously.[15,16,18] The K_d was determined by a Klotz plot.[15]

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Supporting Information Available: Figures of the fluorescence assays, kinetics, and inhibition of the antibody-catalyzed reactions and HPLC charts of the determination of the enantiomeric excess of the hydrolysis product **10** (7 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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