Esterolytic Antibodies Induced to Haptens with a 1,2-Amino Alcohol Functionality

Hiroaki Suga,† Oguz Ersoy,† Takeshi Tsumuraya,†,‡ Jung Lee,† Anthony J. Sinskey,§ and Satoru Masamune*,†

Contribution from the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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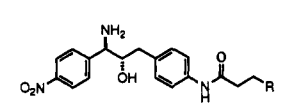
Abstract: Three structurally related haptens **1–3** were designed and synthesized with the goal of generating antibodies for the hydrolysis of ester **4a** and amide **4b**. These haptens contain a 1,2-amino alcohol functionality which replaces the ester/amide moiety of the substrates. A number of catalytic antibodies were generated, and the Michaelis–Menten kinetics constants of three representative catalytic antibodies induced to each of haptens **1–3** were determined. These catalytic antibodies accelerated the hydrolysis of ester **4a** with $k_{cat}/k_{un} = \sim 3 \times 10^3$, and their catalytic activities were effectively inhibited by the addition of their respective haptens. To evaluate the structural influences of the hapten on antibody binding and specificity as well as catalytic activity, a total of 18 antibodies including the above catalytic antibodies and three representative noncatalytic antibodies from each group were selected, and their dissociation constants with their respective haptens, amide **4b**, and products were determined. The studies have shown that (1) the structural variations among the three haptens induce no significant changes in catalytic activity of antibodies while they slightly influence the antibody binding and specificity for the substrate and products and (2) a high probability (reaching nearly 50%) of finding catalytic activity among the monoclonal antibodies raised to hapten **1** is found, suggesting that the induction of a charged complementary amino acid residue in close proximity to the reaction site may be important to generation of catalytic antibodies.

Introduction

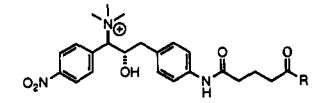
In 1986 two monumental reports appeared on antibody catalysis and laid a solid foundation for the subsequent development of this field.[1] A large number of catalytic antibodies have been prepared using haptens which are analogous to the putative transition states of the chosen reactions in terms of charge distribution and geometry.[2] Thus, ester hydrolysis catalyzed by antibodies raised against negatively charged, tetrahedral phosphonate haptens has constituted a major initial success in this area.[1,3-5] However, the rate accelerations achieved by catalytic antibodies are in most cases several orders of magnitude lower than those realized by their natural enzyme counterparts. Furthermore, efforts to prepare amidase-like antibodies, an obvious and important goal, have thus far met with little success owing largely to the greater strength of the amide bond.[4]

Aspartic proteases are a well-defined class of hydrolytic enzymes, and an examination of their reaction mechanism might serve as a good guide for hapten design and subsequently amidase-like antibody production.[6] The aspartic proteases carry two essential aspartate residues, one in the neutral form and the other in the ionized form. These residues catalyze amide hydrolysis supposedly through at least three distinctive steps: (step 1) One essential, ionized aspartate accepts a proton from a water molecule to form an hydroxide ion for subsequent attack at the amide carbonyl group, (step 2) the other neutral aspartate residue stabilizes the resulting tetrahedral oxyanion (transition state), and (step 3) the proton accepted in step 1 is used to neutralize the leaving RNH^- group. Therefore, any hapten design based on this mechanism should incorporate acidic and basic functionalities which are expected to induce complementary basic and acidic residues in the antibody combining sites.[7] It is noted that the negatively charged phosphonate hapten mentioned above apparently serves to generate the essential acidic residue (step 2) in catalytic antibodies, but the problems associated with steps 1 and 3 had received little attention at the time our investigation was initiated.

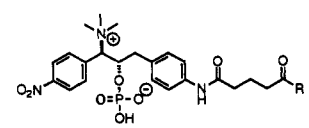
As our entry into the field of catalytic antibodies we selected and synthesized haptens **1–3**, which each contain a 1,2-amino



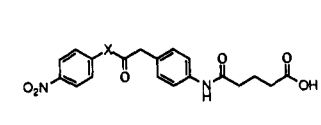
1a R = SH
1b R = CH_2COOH



2a R = *N*-hydroxysuccinimide
2b R = OH



3a R = *N*-hydroxysuccinimide
3b R = OH



4a X = O
4b X = NH

† Department of Chemistry.
‡ Present address: Kao Institute for Fundamental Research, Tochigi, Japan.
§ Department of Biology.
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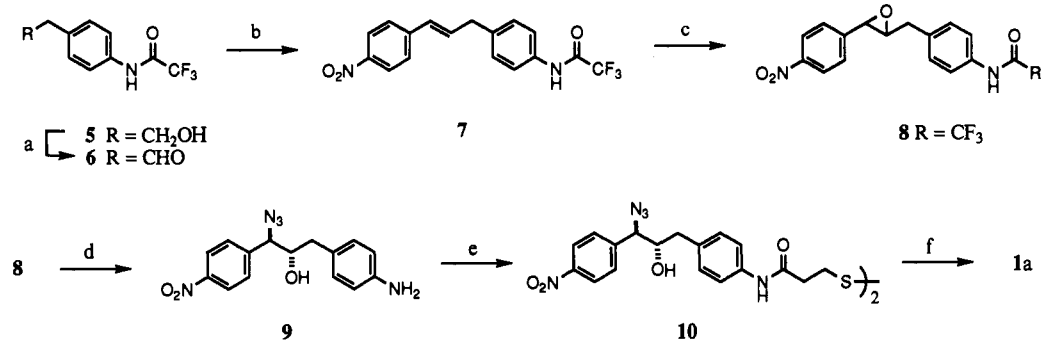
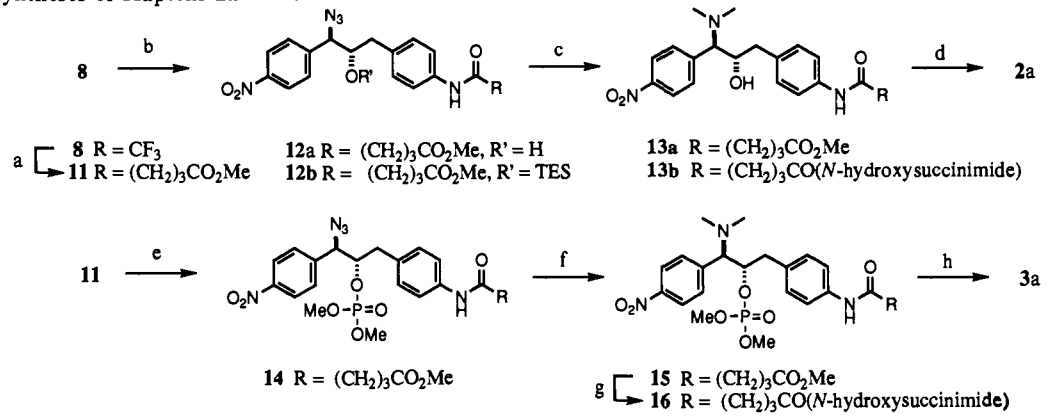
Scheme 1. Synthesis of Hapten **1a**[a]



[a] (a) Dess–Martin periodinane, CH_2Cl_2, 86%; (b) *p*-$NO_2PhCH_2P(O)(OEt)_2$, NaH, 15-crown-5, THF, 62%; (c) *m*-CPBA, CH_2Cl_2, 99%; (d) (i) NaN_3, acetone/H_2O (1/1), (ii) K_2CO_3, MeOH/H_2O (1/1), 62%; (e) 3,3′-dithiodipropionic acid, bis(succinimido) ester CH_2Cl_2, 55%; (f) (i) Ph_3P, THF, then H_2O, 70%; (ii) DTT, cat. Et_3N, CH_2Cl_2, 77%.

Scheme 2. Syntheses of Haptens **2a** and **3a**[a]



[a] (a) (i) $NaBH_4$, EtOH, (ii) $ClCO(CH_2)_3CO_2Me$, Et_3N, CH_2Cl_2, 84%; (b) (i) NaN_3, NH_4Cl, acetone/H_2O (1/1), (ii) TESOTf, 2,6-lutidine, CH_2Cl_2, 88%; (c) (i) Ph_3P, THF, then H_2O, (ii) 37% CH_2O in water, $NaCNBH_3$, MeCN, (iii) 5% HF in MeCN/H_2O, 51%; (d) (i) LiOH, *t*-BuOH/H_2O (2/1), (ii) EDC, *N*-hydroxysuccinimide, CH_2Cl_2, 67%, (iii) MeI, acetone, 42%; (e) (i) $(MeO)_2PN(Et)_2$, 1*H*-tetrazole, THF, (ii) *m*-CPBA, CH_2Cl_2, 80%; (f) (i) $HS(CH_2)_3SH$, Et_3N, MeOH, (ii) 37% CH_2O in water, $NaCNBH_3$, MeCN, 45%; (g) (i) LiOH, *t*-BuOH/H_2O (2/1.5), (ii) EDC, *N*-hydroxysuccinimide, CH_2Cl_2, 65%; (h) (i) MeI, acetone, 54%, (ii) TMSI, MeCN, 70%.

alcohol functionality replacing the amide/ester moiety of the substrate, with the goal of generating antibodies to hydrolyze ester **4a** and amide **4b**. In order to critically evaluate our hapten designs and begin to understand rules for creating catalytic sites, our studies have been extensive and have encompassed complete sets of monoclonal antibodies raised to each of the haptens **1–3**. In addition to determining kinetic parameters of several catalytic antibodies generated, we have also measured the affinities of both catalytic and noncatalytic antibodies for their respective haptens, substrates, and products. This approach has allowed us to evaluate the structural influences of each hapten on the properties of entire sets of antibodies. This article summarizes the results and provides information that is useful for the future development of catalytic antibody technology.

Results

Linker Selection, Hapten Synthesis, and Protein Conjugation. To obtain an immune response it is necessary to couple the hapten to a carrier protein, and an appropriate linker with an activated group must be selected for this purpose. A conventional *N*-hydroxysuccinimide-activated ester of a glutaric acid linker was chosen to couple haptens **2** and **3** to lysine residues of the carrier protein.[8] However, since the primary amino group of hapten **1** could undergo self-coupling with this activated ester, a different linker bearing a free sulfhydryl group was selected. In this way, hapten **1** could be selectively coupled to maleimide-modified lysine residues of carrier proteins without temporary protection of the primary amine.[9]

Epoxide **8**, a common intermediate for syntheses of the haptens **1–3**, was prepared in three steps from alcohol **5** (Scheme 1). The oxidation of alcohol **5** was problematic; conventional methods such as Swern, PDC, and Collins oxidations provided low yields of aldehyde **6** with a substantial amount of byproducts. However, the Dess–Martin periodinane oxidation[10] proceeded well, the formation of the side reactions presumably being suppressed under the mild acidic conditions employed. Coupling of **6** with diethyl (*p*-nitrobenzyl)phosphonate, readily derived from *p*-nitrobenzyl bromide with triethyl phosphite,[11] led to the construction of the main skeleton of the haptens, and the oxidation of olefin **7** by *m*-chloroperoxybenzoic acid (*m*-CPBA) yielded (racemic) epoxide **8**.

Hapten **1a** was obtained in four steps from epoxide **8** (Scheme 1). The epoxy-ring opening with sodium azide and then treatment with potassium carbonate afforded azido alcohol **9** regioselectively (in the ratio of 94:6), and this was selectively coupled with 3,3′-dithiodipropionic acid with the aid of *N*-hydroxysuccinimide to afford azido-alcohol **10**. Reduction of the azide by triphenylphosphine (PPh_3)[12] followed by treatment of dithiothreitol (DTT) provided hapten **1a** containing a free sulfhydryl group.

Hapten **2a** was prepared in five steps from **8** (Scheme 2). Removal of the *N*-trifluoroacetate protecting group with sodium borohydride followed by coupling of the resulting aniline to the

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Table 1. Dissociation Constants and Michaelis–Menten Parameters of Antibodies

A. Hapten **1**

antibody[a]	K_d of hapten **1** (10^{-5} M)	K_d of amide **4b** (10^{-4} M)	K_d of PNP (10^{-2} M)	k_{cat} (min^{-1})	K_m (μM)	K_i of hapten **1** (μM)	k_{cat}/k_{un} (10^3)
H6-32*	0.22	4.1	2.3	0.71	850	360	2.4
H6-34*	0.33	7.4	6.3	0.31	400	11	1.0
H6-39*	0.71	14	9.0	0.72	880	490	2.4
H6-26	0.38	4.5	1.6				
H6-7	0.43	2.2	8.8				
H6-73	6.5	34	16				

B. Hapten **2**

antibody	K_d of hapten **2** (10^{-5} M)	K_d of amide **4b** (10^{-4} M)	K_d of PNP (10^{-2} M)	k_{cat} (min^{-1})	K_m (μM)	K_i of hapten **2** (μM)	k_{cat}/k_{un} (10^3)
H5-38*	1.2	2.0	9.0	1.0	870	5	3.3
H5-25*	0.17	4.1	9.4	0.79	1400	5	2.7
H5-67*	1.6	2.1	5.5	0.48	970	4	1.6
H5-1	0.70	3.2	5.1				
H5-41	2.8	7.4	5.7				
H5-90	1.4	3.5	4.6				

C. Hapten **3**

antibody	K_d of hapten **3** (10^{-5} M)	K_d of amide **4b** (10^{-4} M)	K_d of PNP[b] (10^{-2} M)	k_{cat} (min^{-1})	K_m (μM)	K_i of hapten **3** (μM)	k_{cat}/k_{un} (10^3)
H7-59*	0.19	38	12	0.49	1100	230	1.6
H7-26*	7.3	1.2	5.3	0.39	480	190	1.3
H7-21*	0.042	22	4.9	0.36	250	17	1.2
H7-22	0.15	14	7.3				
H7-69	5.2	32	32				
H7-58	0.037	35	9.4				

[a] *denotes catalytic antibodies. [b] K_ds of antibodies for the product acid are as follows from top to bottom: 8.4, 1.8, 2.3, 2.1, 1.4, 0.89 (10^{-2} M).

glutaric acid linker afforded **11**. The conversion of epoxide **11** to azido alcohol **12a** and subsequent protection of the hydroxy group with triethylsilyl (TES) triflate provided **12b**. Reduction of the azide by PPh_3 and reductive methylation[13] of the resulting amine followed by removal of the TES group provided *N,N*-dimethylamino alcohol **13a**. Hydrolysis of the methyl ester in the linker, conversion of the resulting acid to the *N*-hydroxysuccinimide ester **13b**, and finally treatment with methyl iodide completed the synthesis of hapten **2a**.

Hapten **3a** was similarly synthesized from **11** (Scheme 2). After the epoxy-ring opening of **11**, treatment of the alcohol with *O,O*-dimethyl *N,N*-diethylphosphoramidite[14] and subsequent oxidation of the intermediate phosphite provided phosphate triester **14**. Reduction of the azide by propane-1,3-dithiol[15] and reductive methylation of the resulting amine afforded **15**. Conversion of the methyl ester to *N*-hydroxysuccinimide ester **16** and treatment with methyl iodide and then with trimethylsilyl iodide provided the zwitterionic hapten **3a**.

Prior to coupling with hapten **1a**, the carrier proteins bovine serum albumin (BSA) and bovine plasma fibrinogen (Fib) were individually modified with the *N*-hydroxysuccinimide ester of 3-maleimidepropionic acid.[9c] The reaction of the resulting maleimide–BSA and maleimide–Fib conjugates with hapten **1a** afforded the corresponding BSA–**1** and Fib–**1** conjugates, respectively. Although BSA–**1** remained in buffer solution during its preparation, Fib–**1** precipitated. Therefore, we decided to use BSA–**1** for screening (see below) and Fib–**1** for immunization. On the other hand, the coupling of hapten **2a** and **3a** containing *N*-hydroxysuccinimide activated esters with BSA and Fib both yielded solutions of BSA– and Fib–hapten conjugates which were used for immunization and screening, respectively.

For inhibition studies, haptens containing a free glutaric acid linker were chosen due to their enhanced water solubility. Reduction of the azide of **12a** followed by hydrolysis of the methyl ester provided inhibitor **1b**. The quaternization of amine **13** followed by hydrolysis of the methyl ester afforded inhibitor **2b**. Inhibitor **3b** was similarly synthesized from **15** by quaternization, deprotection of the dimethyl phosphate, and hydrolysis of the methyl ester. All inhibitors were soluble in the wide range of buffer solutions used in the assays.

Antibody Production. Four Balb/c mice were immunized with the protein–hapten conjugate Fib–**1**. After two injections of this conjugate, serum immunoglobulin G (IgG) was titrated against the BSA–**1** conjugate by means of an enzyme-linked immunosorbent assay (ELISA). The mouse with the highest titer (1:12 800) was subsequently boosted, and 3 days later, its spleen was taken for the preparation of hybridomas.[16] All IgG-producing hybridomas that bound to the BSA–**1** conjugate were subcloned two or three times, to yield a total of 34 cell lines producing monoclonal antibodies. In the same manner, immunization with BSA–**2** and BSA–**3** produced 48 and 20 monoclonal cell lines, respectively. All clones were individually propagated in ascites to obtain milligram quantities of monoclonal antibodies, which were purified by salt precipitation, anion exchange, and affinity chromatography.

Antibody Dissociation Constants with Hapten, Substrate, and Products. To survey the general trend of antibody binding and specificity, the dissociation constants (K_d) of representative catalytic and noncatalytic antibodies to their respective haptens, amide **4b**, *p*-nitrophenol (PNP), and the product acid, were determined by a competition ELISA procedure.[17] The K_ds

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(17) Competition ELISA, which was originally described in a report (Friguet, B.; Chaffotte, A. F.; Djaudi-ohaniance, C.; Goldberg, M. E. *Immunol. Methods* **1985**, *77*, 305), was performed under modified conditions. In our procedure, the observed K_d is not necessarily the absolute K_d of an antibody with an inhibitor because of the short equilibration time (30 min, see the Experimental Section). However, K_ds of most antibodies that were determined for the respective inhibitors after the equilibration for 1 h are similar to those obtained after 30 min. Since the K_ds were measured under identical conditions in all experiments, these values may be used for comparison. The antibody binding to substrate and products (PNP and the product acid) indicates the degree of the product inhibition for this antibody under kinetic assay conditions (see the Discussion).

obtained are shown in Table 1. For antibodies raised to haptens **1** and **2**, no inhibition of the antibody–hapten binding was observed in the presence of 20 mM product acid (the maximum concentration attainable in aqueous solution). In contrast, antibodies elicited to hapten **3** had K_ds for the product acid in a similar range to that for PNP (see footnote b of Table 1).

Esterolytic Assay. Antibodies (10 μM) were initially screened for their ability to catalyze the hydrolysis of ester **4a** in a buffered solution (50 mM Mes, 80 μM NaCl (pH 6.2), and 50 mM Tris·HCl, 80 μM NaCl (pH 7.8)) containing 5% of organic cosolvent (4.8% acetonitrile and 0.2% dimethyl sulfoxide). The production of the *p*-nitrophenolate ion (absorbance observed at 405 nm) was monitored. The number of antibodies catalyzing the hydrolysis reaction was *16* out of the 34 monoclonal antibodies raised to hapten **1**, *7* out of the 48 antibodies raised to hapten **2**, and *4* out of the 20 antibodies raised to hapten **3**.

Michaelis–Menten Kinetics. Most of the catalytic antibodies (eight from hapten **1**, and all of them from haptens **2** and **3**) were further characterized and found to obey Michaelis–Menten kinetics for the hydrolysis of **4a**. The first-order rate constants (k_{cat}) and Michaelis constants (K_m) of three representative catalytic antibodies (denoted by asterisk) from each group of antibodies raised to haptens **1–3** are shown in Table 1. The inhibition constants (K_i) of the catalytic antibodies for the respective inhibitors **1b–3b** were also determined and are listed in Table 1. All assays were performed at 25 °C in 50 mM Tris·HCl, 80 μM NaCl (pH 7.8) containing 5% of organic cosolvent (4.8% acetonitrile and 0.2% dimethyl sulfoxide).

Discussion

Hapten Design. In an effort to try to understand rules for generating catalytic antibodies from haptens, we designed three structurally related haptens (**1–3**), all of which contain a 1,2-amino alcohol functionality. The design is based on the reaction mechanism described above for the aspartic protease-catalyzed amide hydrolysis. Haptens **1** and **2** have the following features: (1) The protonated primary amine in **1** and the quaternized ammonium salt in **2** act as positive charges to generate a basic residue in the antibody combining site,[18] and also an empty cavity for the incoming water molecule (see steps 1 and 3 described in Introduction). (2) The secondary hydroxy groups in **1** and **2** represent the tetrahedral geometry required to stabilize the transition state in step 2.[19] In addition to feature 2, hapten **3** carries an additional negatively charged phosphate group attached to the hydroxyl moiety to induce an additional acidic residue to further stabilize the oxyanion transition state. Although it has been reported that some catalytic antibodies were relatively flexible to substrate variations,[20] we were concerned at the outset that the functional groups in haptens **2** and **3** would place the catalytic residues too far from the reaction site (misfit). Nonetheless, it is important to learn the extent to which the structural mismatch between hapten and substrate influences both catalytic activity and binding ability to the substrate and the products.

During the course of these studies, Janda *et al.* described a significant investigation which used a set of different antigens to prepare catalytic antibodies capable of hydrolyzing a phenyl benzoate ester.[21] Esterolytic antibodies were successfully generated when the hapten incorporated either an *N*-methylpyridinium salt or a benzoate as a charged residue, and a secondary alcohol to represent a neutral tetrahedral framework of the transition state. When the charged residue was replaced by a neutral group (pyridine or toluene), no catalytically active antibodies were isolated. Unfortunately, the incorporation of the charge into an adjacent aromatic ring system limits the use of this design to substrates containing benzoic acid derivatives. In contrast, the straight replacement of the ester/amide moiety in substrate **4a**/**4b** with the simple 1,2-amino alcohol functionality provides the structures of haptens **1–3**. The application of our hapten design should not, therefore, be limited to the hydrolysis of substrate **4** but can be extended to that of many other substrates. For this reason, we continued the studies on haptens **1–3**, a number of monoclonal antibodies were generated, and their affinities and catalytic profiles evaluated in the context of their hapten designs.

Antibody Binding and Specificity. To evaluate the structural influence of the hapten on antibody binding and specificity, a total of 18 antibodies were examined. Three representative catalytic antibodies and three noncatalytic antibodies were chosen from each group of antibodies induced to haptens **1–3**, and their dissociation constants (K_d) with their respective haptens, amide **4b**, and *p*-nitrophenol (PNP), and the product acid, were determined (Table 1).

As expected, the K_d values of the antibodies with their respective haptens indicated tight binding. These K_ds lie in the range 10^{-7}–10^{-5} M, which is typically observed for antibody–antigen binding. Next, the affinities for substrate were evaluated by the measurement of K_d using amide substrate **4b** rather than ester substrate **4a**, as the rapid hydrolysis of ester **4a** by the catalytic antibodies prevented accurate measurements of the K_ds in these cases. However, we believe that the K_ds with amide **4b** should approximate those with ester **4a**.[22] Most of the antibodies elicited to haptens **1** and **2** have K_ds of roughly 10^{-3}–10^{-4} M for amide **4b**, while the antibodies generated by hapten **3** generally display about 10-fold lower affinities, supposedly due to the large structural and electronic misfit between hapten **3** and amide **4b**. On the other hand, no large difference in the affinity for **4b** between catalytic antibodies and noncatalytic antibodies within each group were found.

Finally, dissociation constants of the antibodies were measured for the two products, *p*-nitrophenol (PNP) and the product acid. These constants have much to do with the degree of product inhibition during hydrolysis, which has often limited the number of turnovers in antibody catalysis.[4,5a] The affinities of antibodies in all three groups to PNP were found to be relatively weak, lying in the range 10^{-2}–10^{-1} M, unlike some previously reported antibodies.[4b,5a] In an effort to measure the K_ds for the product acid, we found that the affinities of antibodies induced to haptens **1** and **2** were too weak to be determined within the solubility limit of this acid (20 mM), while antibodies elicited by hapten **3** weakly associated with the acid and had K_ds in the 10^{-2}–10^{-1} M range.[23] It should be noted that the antibody K_ds with PNP and the product acid were generally 10^2–10^3 times higher than those with substrate **4b**, indicating that significant product inhibition was not occurring during the hydrolysis of **4a**. Also, the affinities of the catalytic antibodies to these products were again similar to those of the noncatalytic antibodies.

The above studies show that the K_d values of the antibodies generated by one hapten are generally similar to each other regardless of whether they are catalytic or noncatalytic. This result suggests that the lack of antibody catalytic activity is

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(21) Janda, K. D.; Weinhouse, M. I.; Schloeder, D. M.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 1274. (b) Janda, K. D.; Weinhouse, M. I.; Danon, T.; Pacelli, K. A.; Schloeder, D. M. *Ibid.* **1991**, *113*, 5427. (c) Janda, K. D. *Biotechnol. Prog.* **1990**, *6*, 178.

(22) In fact, the K_ds of noncatalytic antibodies with amide **4b** were found to be almost identical to those with ester **4a**; for example, the K_ds of noncatalytic antibodies H5-1, H5-41, and H5-90 with **4a** were determined to be 1.2×10^{-4}, 3.2×10^{-4}, 0.97×10^{-4} M, respectively.

(23) This is likely due to the interaction of an acidic residue, induced by the phosphate group of hapten **3a**, with the carboxylate ion of the product acid. Such interaction has been seen in other catalytic antibodies generated by a phosphonate hapten. See refs 4b and 5d.

probably due neither to insufficient substrate binding nor to product inhibition, but rather to an inappropriate placement of active site residues in the antibody combining site. This observation is consistent with Janda's findings,[21] indicating that this conclusion may be generally valid in antibody catalysis.

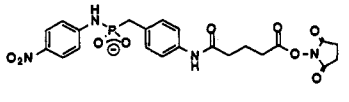
Esterolytic Activity of Antibodies. The initial screening for the esterolytic catalysis identified *16* catalytic antibodies out of the 34 monoclonal antibodies to hapten **1**, *7* out of the 48 to hapten **2**, and *4* out of the 20 to hapten **3**. It is significant that all three of the haptens based on the 1,2-amino alcohol design were able to induce esterolytic activity in antibodies, although none of the antibodies isolated hydrolyzed the amide bond of **4b** with a significant rate acceleration. The Michaelis–Menten parameters (k_{cat}, K_m, and K_i in Table 1) for three representative catalytic antibodies derived from each of the three haptens revealed that all of the catalytic antibodies could hydrolyze ester **4a** with a comparable rate acceleration, k_{cat}/k_{un} lying between 1×10^3 and 3.3×10^3. It is interesting to note that, although structural variations in haptens **1–3** alter the antibody specificities and affinities as shown in Table 1, no significant changes in catalytic rate are induced.

The noticeable difference among the three groups of antibodies raised by haptens **1–3** lies, however, in the probability of finding catalytic activity in the isolated monoclonal antibodies. This probability was remarkably high when antibodies were elicited by hapten **1**, reaching nearly 50%, whereas relatively low probabilities were observed with haptens **2** and **3**. We speculate that haptens **2** and **3** with a sterically demanding NMe_3^+ group (as compared to a NH_3^+ group) elicit antibodies in which the complementary amino acid residue tends to be generated, in the transition state of hydrolysis, away from the location of the carbonyl group of **4a**. The close proximity of the amino acid residue to the carbonyl group appears to be important and can be more readily achieved with the NH_3^+ group. Furthermore, the similar maximum rate accelerations observed with antibodies raised to haptens **1–3** deserve some comment. The catalytic efficiency was not enhanced by the introduction of an additional negatively charged phosphate group in hapten **3**. Although the phosphate group could induce an appropriate charge, it may be too large to permit the induction of a catalytic residue in close proximity to the transition-state oxyanion. This interpretation, if correct, would lead to the tentative conclusion that the rate enhancement attainable by antibodies raised to a singly charged 1,2-amino alcohol functionality would be in the range of 10^3 as observed with haptens **1–3**. The present studies have provided insight valuable to future hapten design; it is clear that the appropriate positioning of a zwitterionic charge and the size of the inducing functionality are critical. Work is being pursued with haptens newly designed on the basis of this knowledge.

Remarks. In the context of future hapten designs, it is appropriate to outline briefly a heterologous immunization strategy, which is considered to be an alternative to the use of a zwitterionic hapten. We have recently applied the heterologous immunization protocol,[24] wherein an animal is immunized with two cross reactive haptens, hapten **2** and a phosphonamidate hapten (i),[25] to generate catalytic antibodies. This method not only has successfully enhanced the catalytic efficiency of antibodies but also has increased the probability of encountering catalytically active antibodies relative to the procedure employing only hapten **2** reported herein. A heterologous immunization protocol employing hapten **1** and the phosphonamidate hapten is likely to induce catalytic antibodies with much higher probability, which in turn, provides a higher chance of isolating efficient catalysts for the cleavage of amide **4b**. In more general terms, the combined strategy of using synthetic haptens, each containing a different charge, in conjunction with the heterologous immunization protocol, should provide a powerful tool for generating antibodies with a wide variety of different catalytic specificities and activities.

(24) Suga, H.; Ersoy, O.; Williams, S. F.; Margolies, M. N.; Sinskey, A. J.; Masamune, S. Submitted for publication.

(25) The structure of the phosphonamidate hapten[4a] utilized in the heterologous immunization protocol is



(i) phosphonamidate hapten

Experimental Section

General Methods (Synthesis). All reactions were carried out in oven-dried glassware under an atmosphere of argon. Reactions containing water were run under an atmosphere of air. Column chromatography was performed using 230–400 mesh silica gel (Merck). Proton (^{1}H NMR) and phosphorus (^{31}P NMR) magnetic resonance spectra were recorded at 300 and 60 MHz on a Varian XL-300 spectrometer, respectively. The spectra were referenced to $CDCl_3$ (7.24 ppm), acetone-d_6 (2.04 ppm), D_2O (4.67 ppm), or CD_3OD (4.78 ppm). Data are reported as follows: chemical shift [multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (Hz), integration]. Infrared (IR) spectra were recorded on a Perkin-Elmer 283B spectrometer. Melting points were not corrected. High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT 8200 spectrometer. Elemental analyses were performed by Desert Analytics (Tucson, AZ).

2-[4-[(Trifluoroacetyl)amino]phenyl]ethan-1-ol (5). To a stirred solution of 4-aminophenethyl alcohol (8.00 g, 58.3 mmol) and triethylamine (9.8 mL, 70.0 mmol) in CH_2Cl_2 (100 mL) was slowly added trifluoroacetic anhydride (8.60 mL, 61.2 mmol) at 0 °C, and the mixture was allowed to warm to room temperature and was stirred for 1 h. The solution was washed with saturated aqueous $NaHCO_3$ (30 mL) followed by brine (30 mL), and the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue crystallized upon trituration with $CHCl_3$, and the crystals, after filtration, were recrystallized from $CHCl_3$ to afford **5** in 60% yield (8.16 g, 35.0 mmol) as colorless crystals: mp 129–131 °C; IR ($CHCl_3$) 3420, 1730, 1600, 1280, 1140 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.44 (dd, J = 10.5 Hz, 2H), 7.14 (d, J = 10.5 Hz, 2H), 3.71 (t, J = 7.1 Hz, 2H), 3.20 (s, 1H), 2.75 (t, J = 7.1 Hz, 2H). Anal. Calcd for $C_{10}H_{10}F_3NO_2$: C, 51.51; H, 4.32; N, 6.01. Found: C, 51.63; H, 4.32; N, 6.04.

2-[4-[(Trifluoroacetyl)amino]phenyl]acetaldehyde (6). To a stirred suspension of **5** (4.66 g, 20 mmol) in CH_2Cl_2 (200 mL) at –78 °C was added a solution of Dess–Martin periodinane (10.0 g, 23.6 mmol) in CH_2Cl_2 (50 mL), and the resulting suspension was allowed to warm to room temperature over a 1-h period with vigorous stirring. After 2 h, the reaction mixture became an orange clear solution, and the solution was stirred for an additional 1 h. The resulting solution was poured into a vigorously stirred saturated aqueous solution of $NaHCO_3$ and $Na_2S_2O_3$ (1/1, 100 mL), and the organic layer was washed with brine (50 mL), dried over $MgSO_4$, and concentrated. The residue was chromatographed (silica gel, 70/30 hexane/Et_2O) to give a yellow solid, and the solid was recrystallized from 50/50 $CHCl_3$/hexane to afford **6** in 84% yield (3.90 g, 16.8 mmol) as colorless crystals: mp 112–114 °C; IR ($CHCl_3$) 3420, 2820, 1720, 1600, 1140 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.72 (t, J = 1.8 Hz, 1H), 7.88 (brs, 1H), 7.54 (d, J = 8.7 Hz, 2H), 7.22 (d, J = 8.7 Hz, 2H), 3.68 (d, J = 1.8 Hz, 2H). Anal. Calcd for $C_{10}H_8F_3NO_2$: C, 51.96; H, 3.49; N, 6.06. Found: C, 52.28; H, 3.44; N, 5.84.

3-[4-[(Trifluoroacetyl)amino]phenyl]-1-(4-nitrophenyl)-(*E*)-prop-1-ene (7). To a stirred suspension of sodium hydride (80% in oil, 270 mg, 9.00 mmol) and a catalytic amount of 15-crown-5 (90 μL, 0.45 mmol) in THF (25 mL) at 0 °C was slowly added a solution of diethyl (*p*-nitrobenzyl)phosphonate (2.56 g, 9.73 mmol) in THF (25 mL) over a 3-min period. A rapid evolution of hydrogen started immediately, and the solution colored wine red during 30 min. To this was added a solution of **6** (1.50 g, 6.49 mmol) in THF (25 mL) at 0 °C with stirring, and the resulting mixture was maintained at 0 °C for 1 h to give a black precipitate. The reaction was quenched with saturated aqueous $NaHCO_3$, and the suspension was stirred until it became a clear yellow solution. The resulting solution was washed with EtOAc (3 × 25 mL), and the combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, and concentrated. Purification of the residue through a short column of silica gel (70/30 hexane/EtOAc) provided a solid which was recrystallized from 50/50 hexane/Et_2O to afford **7** in 62% yield (1.41 g, 4.03 mmol) as colorless crystals: mp 115–117 °C; IR ($CHCl_3$) 3420, 1720, 1600,

1335, 1140 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.14 (d, J = 9.0 Hz, 2H), 7.53 (d, J = 9.0 Hz, 2H), 7.45 (d, J = 8.7 Hz, 2H), 7.25 (d, J = 8.7 Hz, 2H), 6.53 (dt, J = 5.7, 15.9 Hz, 1H), 6.47 (d, J = 15.8 Hz, 1H), 3.58 (d, J = 5.7 Hz, 2H). Anal. Calcd for $C_{17}H_{13}F_3N_2O_3$: C, 58.29; H, 3.74; N, 8.00. Found: C, 58.38; H, 3.80; N, 7.83.

3-[4-[(Trifluoroacetyl)amino]phenyl]-1-(4-nitrophenyl)-1,2-(*E*)-epoxypropane (8). A mixture of **7** (996.1 mg, 2.76 mmol) and *m*-CPBA (1.19 g, 5.52 mmol) in CH_2Cl_2 (40 mL) was heated at reflux under an argon atmosphere for 24 h. After being cooled to room temperature, the solution was washed with saturated aqueous $NaHCO_3$ (20 mL) and brine (20 mL), and the combined organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography (silica gel, 50/50 hexane/Et_2O) to provide **8** (918 mg, 2.73 mmol) in 99% yield as colorless crystals: mp 126–128 °C; IR ($CHCl_3$) 3420, 1730, 1600, 1340, 1140 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.17 (d, J = 8.7 Hz, 2H), 7.85 (brs, 1H), 7.53 (d, J = 8.7 Hz, 2H), 7.39 (d, J = 8.7 Hz, 2H), 7.30 (d, J = 8.7 Hz, 2H), 3.74 (d, J = 1.8 Hz, 1H), 3.15 (ddd, J = 1.8, 4.2, 5.7 Hz, 1H), 3.11 (dd, J = 4.2, 14.6 Hz, 1H), 3.00 (dd, J = 5.7, 14.6 Hz, 1H). Anal. Calcd for $C_{17}H_{13}F_3N_2O_4$: C, 55.74; H, 3.58; N, 7.64. Found: C, 55.88; H, 3.56; N, 7.53.

3-(4-Aminophenyl)-1-(4-nitrophenyl)-1-azidopropan-2-ol (9). A solution of **8** (500 mg, 1.37 mmol), sodium azide (910 mg, 14.0 mmol), and NH_4Cl (249 mg, 14.0 mmol) in acetone/water (10 mL/5 mL) was heated at 70 °C for 48 h. The acetone was removed under reduced pressure, and the resulting aqueous residue was dissolved in MeOH (5 mL) and water (5 mL). After the addition of K_2CO_3 (553 mg, 4.00 mmol), the orange solution was stirred at room temperature overnight. The methanol was removed on a rotary evaporator, and the aqueous residue was extracted with EtOAc (3 × 5 mL). The organic extracts were washed with brine (5 mL), dried over $MgSO_4$, and concentrated. Purification of the residue by chromatography (silica gel, 50/50 hexane/EtOAc) provided **9** in 62% yield (264 mg, 0.84 mmol) as orange crystals: mp 137–139 °C; IR ($CHCl_3$) 3540, 3400, 2090, 1600, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.24 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 2H), 6.63 (d, J = 8.4 Hz, 2H), 4.63 (d, J = 5.4 Hz, 1H), 4.00 (ddd, J = 4.2, 5.4, 9.0 Hz, 1H), 3.6 (brs, 1H), 2.71 (dd, J = 4.2, 13.8 Hz, 1H), 2.56 (dd, J = 9.0, 13.8 Hz, 1H), 1.7 (brs, 2H). Anal. Calcd for $C_{15}H_{15}N_5O_3$: C, 57.50; H, 4.83; N, 22.35. Found: C, 57.65; H, 4.90; N, 22.18.

3,3′-Dithiodipropionic Acid, Bis[[4-[3-(4-nitrophenyl)-3-azido-2-hydroxypropyl]phenyl]amide] (10). A solution of **9** (264 mg, 0.844 mmol) and 3,3′-dithiodipropionic acid, bis(succinimido) ester (155 mg, 0.348 mmol) in CH_2Cl_2 (15 mL) was heated at reflux for 48 h, and the resulting solution was washed with brine (2 × 5 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, 40/60 hexane/EtOAc) to afford **10** in 55% yield (170 mg, 0.212 mmol) as a yellow amorphous solid: mp 75–80 °C (dec); IR ($CHCl_3$) 3540, 3420, 2090, 1740, 1345, 1600, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.13 (d, J = 8.7 Hz, 2H), 7.48 (d, J = 8.7 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 6.99 (d, J = 8.4 Hz, 2H), 4.54 (d, J = 5.7 Hz, 1H), 3.99 (ddd, J = 5.3, 6.3, 8.7 Hz, 1H), 2.98 (dd, J = 6.3, 9.5 Hz, 1H), 2.94 (t, J = 6.6 Hz, 2H), 2.76 (s, 1H), 2.65 (t, J = 6.6 Hz, 2H), 2.54 (dd, J = 8.7, 9.5 Hz, 1H).

3,3′-Dithiodipropionic Acid, Bis[[4-[3-(4-nitrophenyl)-3-amino-2-hydroxypropyl]phenyl]amide] (dithio-1a). A solution of **10** (49.4 mg, 0.0617 mmol) and triphenylphosphine (38.5 mg, 0.145 mmol) in THF (3 mL) was stirred 2 days at room temperature, and to this was added water (3.6 μL, 0.20 mmol). After stirring another 2 days, the solvent was removed under reduced pressure, and the residue was passed through a short column of silca gel (EtOAc) to provide dithio-**1a** in 70% yield (32.3 mg, 0.0432 mmol) as a yellow oil: IR ($CHCl_3$) 3420, 3300, 2920, 1670, 1600, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.09 (d, J = 8.7 Hz, 2H), 7.48 (d, J = 8.7 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 7.14 (d, J = 8.4 Hz, 2H), 3.03 (t, J = 6.6 Hz, 2H), 2.91 (d, J = 5.4 Hz, 1H), 2.80 (d, J = 2.7 Hz, 1H), 2.73 (t, J = 6.6 Hz, 2H), 2.26 (dt, J = 2.7, 5.4 Hz, 1H), 1.65 (brs, 2H).

3-[4-[(3-Mercapto-1-oxopropenyl)amino]phenyl]-1-(4-nitrophenyl)-1-aminopropan-2-ol (1a). To a solution of dithio-**1a** (26.0 mg, 0.0347 mmol) and a catalytic amount of triethylamine (1.4 μL, 0.014 mmol) in CH_2Cl_2 (1.5 mL) was added dithioerythritol (5.9 mg, 0.0382 mmol) at room temperature, and the mixture was stirred 4 h. The volatile material was removed under reduced pressure, and the residue was chromatographed (silica gel, EtOAc) to furnish **1a** in 77% yield (20.0 mg, 0.0533 mmol) as a yellow oil: IR ($CHCl_3$) 3420, 2920, 2680, 1590, 1510, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.13 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 8.4 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 2.96 (ABq, J = 5.4 Hz, 2H), 2.88 (dt, J = 6.5, 8.4 Hz, 1H), 2.81 (d, J = 2.4 Hz, 1H), 2.67 (t, J = 6.5 Hz, 2H), 2.77 (brm, 1H), 1.69 (t, J = 8.4 Hz, 1H), 1.5 (br s, 3H).

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-1-(4-nitrophenyl)-1,2-(*E*)-epoxypropane (11). To a solution of **8** (1.00 g, 2.73 mmol) in EtOH (25 mL) was added sodium borohydride (513 mg, 13.5 mmol) at 0 °C, and the mixture was stirred for 1 h. The reaction was quenched by addition of acetic acid, and the resulting white precipitate was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was diluted with EtOAc. The solution was washed with saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL), dried over $MgSO_4$, and concentrated. The crude residue was dissolved in CH_2Cl_2 (20 mL), and to this solution was added triethylamine (0.46 mL, 2.37 mmol) followed by 5-methoxy-5-oxopentanoyl chloride (641 mg, 2.73 mmol) at room temperature with stirring. After 30 min, the solution was washed with saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL), dried ($MgSO_4$), and concentrated. The residue was crystallized from 50/50 $CHCl_3$/hexane to afford **11** in 84% yield (910 mg, 2.28 mmol) as colorless crystals: mp 133–135 °C; IR ($CHCl_3$) 3420, 1730, 1600, 1340, 1140 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.16 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.36 (brs, 1H), 7.21 (d, J = 8.4 Hz, 2H), 3.72 (d, J = 1.8 Hz, 1H), 3.67 (s, 3H), 3.12 (dt, J = 1.8, 5.7 Hz, 1H), 2.99 (d, J = 5.7 Hz, 2H), 2.43 (t, J = 7.2 Hz, 2H), 2.41 (t, J = 7.2 Hz, 2H), 2.03 (quint, J = 7.2 Hz, 2H). Anal. Calcd for $C_{21}H_{22}N_2O_6$: C, 63.31; H, 5.57; N, 7.03. Found: C, 63.26; H, 5.43; N, 6.96.

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-1-(4-nitrophenyl)-1-azidopropan-2-ol (12a). The mixture of **11** (892 mg, 2.24 mmol), sodium azide (1.46 g, 22.4 mmol), and NH_4Cl (1.23 g, 23.0 mmol) in acetone/water (20 mL/10 mL) was heated at 70 °C for 48 h. The acetone was removed on a rotary evaporator, and the resulting aqueous residue was extracted with EtOAc (3 × 10 mL). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL), and the combined organic layers were dried over $MgSO_4$ and concentrated. Purification of the residue by chromatography (silica gel, 50/50 hexane/EtOAc) provided **12a** in 89% yield (878 mg, 1.99 mmol) as a yellow oil: IR ($CHCl_3$) 3430, 2950, 1720, 1680, 1590, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.21 (d, J = 9.0 Hz, 2H), 7.69 (s, 1H), 7.53 (d, J = 9.0 Hz, 2H), 7.37 (d, J = 8.4 Hz, 2H), 7.06 (d, J = 8.4 Hz, 2H), 4.61 (d, J = 6.0 Hz, 1H), 3.99 (m, 1H), 3.63 (s, 3H), 2.74 (dd, J = 3.3, 13.8 Hz, 1H), 2.90 (dd, J = 9.0, 13.8 Hz, 1H), 2.49 (brs, 1H), 2.37 (t, J = 7.5 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 1.96 (quin, J = 7.5 Hz, 2H); HRMS $[M]^+$ calcd 441.1648, found 441.1645.

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-2-[(triethylsilyl)oxy]-1-(4-nitrophenyl)propylazide (12b). To a solution of **12a** (316 mg, 0.716 mmol) and 2,6-lutidine (108 μL, 0.931 mmol) in CH_2Cl_2 (8 mL) at −78 °C was added triethylsilyl trifluoromethanesulfonate (194 μL, 0.859 mmol) with stirring, and the mixture was allowed to warm to room temperature over a 4-h period. The solution was washed with saturated aqueous $NaHCO_3$ (5 mL) followed by brine (5 mL), and the combined organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel, 70/30 hexane/EtOAc) to afford **12b** in 99% yield (395 mg, 0.71 mmol) as a yellow oil: IR ($CHCl_3$) 3540, 3420, 2940, 2080, 1720, 1680, 1590, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.19 (d, J = 8.7 Hz, 2H), 7.66 (brs, 1H), 7.48 (d, J = 8.7 Hz, 2H), 7.42 (d, J = 8.7 Hz, 2H), 7.01 (d, J = 8.7 Hz, 2H), 4.45 (d, J = 4.5 Hz, 1H), 4.13 (ddd, J = 4.5, 4.8, 6.9 Hz, 1H), 3.65 (s, 3H), 2.75 (dd, J = 6.9, 14.1 Hz, 1H), 2.52 (dd, J = 4.8, 14.1 Hz, 1H), 2.41 (t, J = 7.5 Hz, 2H), 2.40 (t, J = 7.5 Hz, 2H), 2.01 (quint, J = 7.5 Hz, 2H), 0.83 (t, J = 8.0 Hz, 9H), 0.39 (q, J = 8.0 Hz, 6H).

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-2-[(triethylsilyl)oxy]-1-(4-nitrophenyl)-*N,N*-dimethylpropylamine (12c). A solution of **12b** (395 mg, 0.710 mmol) and triphenylphosphine (223 mg, 0.852 mmol) in THF (5 mL) was stirred 2 days at room temperature, and to this was added water (19 μL, 1.06 mmol). After stirring another 2 days, the solvent was removed under reduced pressure, and the residue was passed through a short column of silica gel (50/50 hexane/EtOAc) to give the corresponding amine contaminated with triphenylphosphine oxide. To the product, dissolved in acetonitrile (5 mL), was added 37% paraformaldehyde in water (0.6 mL, 7.0 mmol) with stirring. After 10 min, sodium cyanoborohydride (352 mg, 5.60 mmol) was added at room temperature, and the suspension was stirred for 2 h. The reaction was quenched by addition of acetic acid, and the volatile solvents were removed on a rotary evaporator. The residue was diluted with EtOAc (10 mL), and the solution was washed with saturated aqueous $NaHCO_3$ (5 mL) followed by brine

(5 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (silica gel, 50/50 hexane/EtOAc) to provide **12c** (198 mg, 0.354 mmol) as a yellow oil. The total yield from **12b** was 51%: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.14 (d, J = 9.0 Hz, 2H), 7.43 (d, J = 9.0 Hz, 2H), 7.40 (d, J = 8.7 Hz, 2H), 7.06 (d, J = 8.7 Hz, 2H), 4.42 (q, J = 5.4 Hz, 1H), 3.65 (s, 3H), 3.11 (d, J = 5.4 Hz, 1H), 2.70 (ABq, J = 5.4 Hz, 1H), 2.42 (t, J = 7.5 Hz, 2H), 2.41 (t, J = 7.5 Hz, 2H), 2.08 (s, 6H), 2.01 (quint, J = 7.5 Hz, 2H), 0.78 (t, J = 7.8 Hz, 9H), 0.37 (q, J = 7.8 Hz, 6H); HRMS $[M]^+$ calcd 558.1999, found 558.1996.

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-1-(4-nitrophenyl)-2-hydroxy-*N,N*-dimethylpropylamine (13a). To a solution of **12c** (150 mg, 0.268 mmol) in acetonitrile (5 mL) was added 1.1 mL of 5% hydrogen fluoride in acetonitrile/water (85/15) at room temperature, and the mixture was stirred overnight. The resulting solution was neutralized by addition of saturated aqueous $NaHCO_3$ (2 mL), and the volatile solvents were removed on a rotary evaporator. The aqueous residue was extracted with EtOAc (3 × 3 mL), and the combined extracts were washed with brine (3 mL). The organic layers were dried over $MgSO_4$ and concentrated, and the residue was chromatographed (silica gel, EtOAc) to afford **13a** in 100% yield (118 mg, 0.268 mmol): IR ($CHCl_3$) 3430, 2950, 2780, 1720, 1680, 1590, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.17 (d, J = 8.7 Hz, 2H), 8.72 (brs, 1H), 7.42 (d, J = 8.7 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 8.4 Hz, 2H), 4.34 (dt, J = 4.0, 8.7 Hz, 1H), 3.63 (s, 3H), 3.11 (d, J = 4.5 Hz, 1H), 2.7 (brs, 1H), 2.48 (dd, J = 3.9, 13.8 Hz, 1H), 2.38 (t, J = 7.5 Hz, 2H), 2.35 (t, J = 7.5 Hz, 2H), 2.24 (dd, J = 8.7, 13.8 Hz, 1H), 2.18 (s, 6H), 1.98 (quint, J = 7.5 Hz, 2H); HRMS $[M - OCH_3]^+$ calcd 412.1872, found 412.1875.

3-[4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]phenyl]-2-hydroxy-1-(4-nitrophenyl)-*N,N*-dimethylpropylamine (13b). A solution of **13a** (20 mg, 0.045 mmol) and lithium hydroxide monohydrate (4.0 mg, 0.09 mmol) in *t*-BuOH (0.6 mL) and H_2O (0.3 mL) was stirred 24 h at room temperature, and the solvents were removed by freeze drying. The residue was extracted with EtOAc (5 mL), and the extract, after filtration, was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (3 mL), and to this solution was added EDC (13.4 mg, 0.070 mmol) and *N*-hydroxysuccinimide (9.2 mg, 0.080 mmol) at room temperature. After stirring 3 h, the insoluble materials were filtered off and washed with CH_2Cl_2 (3 mL), and the combined filtrates were washed with brine (3 × 2 mL). The combined organic layers were dried over $MgSO_4$ and concentrated to provide **13b** in 67% yield (16 mg, 0.030 mmol) as a yellow oil. The product was used for the next step without further purification: IR (CH_2Cl_2) 3430, 2950, 2780, 1820, 1790, 1740, 1680, 1590, 1340 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.17 (d, J = 8.1 Hz, 2H), 8.02 (s, 1H), 7.47 (d, J = 8.1 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 8.4 Hz, 2H), 4.35 (dt, J = 4.5, 8.4 Hz, 1H), 3.11 (d, J = 4.5 Hz, 1H), 2.84 (brs, 4H), 2.67 (t, J = 6.9 Hz, 2H), 2.48 (dd, J = 3.9, 14.0 Hz, 1H), 2.41 (t, J = 6.9 Hz, 2H), 2.24 (dd, J = 5.4, 14.0 Hz, 1H), 2.19 (s, 6H), 2.13 (quint, J = 6.9 Hz, 2H).

[3-[4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]phenyl]-2-hydroxy-1-(4-nitrophenyl)propyl]trimethylammonium iodide (2a). To a solution of **13b** (16 mg, 0.030 mmol) in acetone (1 mL) was added methyl iodide (19 μL, 0.030 mmol) at room temperature, and the mixture was stirred overnight. The solvent was removed on a rotary evaporator, and the residue was vigorously stirred with CH_2Cl_2 (1 mL) for 5 min. A yellow viscous oil remained undissolved and was isolated by decantation (8.5 mg, 0.017 mmol, 42% yield). ^{1}H NMR (in acetone-d_6) analysis demonstrated that the oil **2a** was >90% purity, and the product was used for conjugation with carrier proteins without further purification: mp 225-230 °C (dec); ^{1}H NMR (acetone-d_6, 300 MHz) δ 9.11 (s, 1H), 8.48 (d, J = 7.8 Hz, 1H), 8.34 (d, J = 7.8 Hz, 1H), 8.30 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.45 (d, J = 8.7 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 5.47 (brs, 1H), 5.08 (brm, 1H), 3.44 (s, 9H), 2.83 (s, 4H), 2.68 (t, J = 8.4 Hz, 2H), 2.58 (dd, J = 3.9, 16.0 Hz, 1H), 2.48 (t, J = 8.4 Hz, 2H), 2.39 (dd, J = 5.4, 16.0 Hz, 1H), 2.05 (quint, J = 8.4 Hz, 2H); ^{1}H NMR (D_2O, 300 MHz) δ 8.14–8.30 (m, 3H), 7.77 (s, 1H), 7.45 (d, J = 7.8 Hz, 1H), 7.19 (d, J = 8.1 Hz, 2H), 6.96 (d, J = 8.1 Hz, 2H), 4.88 (dt, J = 1.5, 5.7 Hz, 1H), 4.25 (d, J = 1.5 Hz, 1H), 3.05 (s, 9H), 2.78 (s, 4H), 2.68 (t, J = 7.2 Hz, 2H), 2.48–2.22 (m, 2H), 2.39 (t, J = 7.2 Hz, 2H), 1.95 (quint, J = 7.2 Hz, 2H).

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-2-(*O,O*-dimethylphosphoryl)-1-(4-nitrophenyl)propylazide (14). To a solution of **11** (120 mg, 0.27 mmol) and dimethyl *N,N*-diethylphosphoramidate (89 μL, 0.49 mmol) in THF (5mL) was added 1*H*-tetrazole (114 mg, 1.36 mmol) at room temperature under an argon atmosphere. The mixture was stirred for 15 min and then cooled to –78 °C, and to this was added a solution of *m*-CPBA (99 mg, 0.49 mmol) in CH_2Cl_2 (6 mL). After 10 min, 5% aqueous $KHSO_4$ (14 mL) was added, and the mixture was stirred 10 min. The organic layer was washed with saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL) and dried over Na_2SO_4. The concentrated residue was purified by column chromatography (silica gel, 25/75 hexane/EtOAc) to provide **14** in 80% yield (120 mg, 0.216 mol) as a colorless oil: IR (CH_2Cl_2) 3430, 2100, 1730, 1690, 1595, 1515, 1345 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.22 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.5 Hz, 2H), 7.07 (d, J = 8.5 Hz, 2H), 5.08 (d, J = 4.6 Hz, 1H), 4.79 (m, 1H), 3.59 (s, 3H), 3.56 (d, J = 11.3 Hz, 3H), 3.34 (d, J = 11.3 Hz, 3H), 3.02 (m, 1H), 2.81 (m, 1H), 2.37 (t, J = 7.5 Hz, 2H), 2.34 (t, J = 7.4 Hz, 2H), 1.96 (quint, J = 7.5 Hz, 2H); ^{31}P NMR ($CDCl_3$, 60 MHz) δ –4.01.

3-[4-[(5-Methoxy-1,5-dioxopentyl)amino]phenyl]-2-(*O,O*-dimethylphosphoryl)-1-(4-nitrophenyl)-*N,N*-dimethylpropylamine (15). To a solution of **14** (410 mg, 0.75 mmol) in methanol (7 mL) was added propane-1,3-dithiol (262 μL, 2.61 mmol) and triethylamine (364 μL, 2.61 mmol) at room temperature, and the mixture was stirred 20 h. The resulting solution was concentrated under reduced pressure, and the residue was purified by column chromatography (silica gel, 50/50 $CHCl_3$/EtOH followed by EtOH) to afford an oily residue. The residue was dissolved in acetonitrile (10 mL), and to this was added 37% paraformaldehyde in water (650 μL, 7.5 mmol). The mixture was stirred for 20 min, $NaCNBH_3$ (377 mg, 5.99 mmol) was added, and the resulting solution was vigorously stirred 2 h at room temperature. The reaction was then quenched by the addition of glacial acetic acid (1 mL) and allowed to stir for another 20 min. The resulting mixture was diluted with EtOAc (10 mL) and washed with saturated aqueous $NaHCO_3$ (5 mL) followed by brine (5 mL), and the organic layer was dried over $MgSO_4$ and concentrated. The residue was chromatographed on silica gel (EtOAc) to furnish **15** (183 mg, 0.338 mmol). The total yield from **14** was 45%: IR (CH_2Cl_2) 3420, 2940, 1730, 1690, 1595, 1515, 1405, 1345 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.18 (d, J = 8.7 Hz, 2H), 7.53 (s, 1H), 7.46 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.7 Hz, 2H), 7.22 (d, J = 8.1 Hz, 2H), 5.21 (m, 1H), 3.67 (s, 3H), 3.51 (d, J = 11.2 Hz), 3.34 (m, 1H), 3.32 (d, J = 11.2 Hz, 3H), 3.03 (m, 2H), 2.34 (m, 4H), 2.11 (s, 6H), 2.03 (m, 2H); ^{31}P NMR ($CDCl_3$, 60 MHz) δ –22.5.

3-[4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]phenyl]-2-(*O,O*-dimethylphosphoryl)-1-(4-nitrophenyl)-*N,N*-dimethylpropylamine (16). To a solution of **15** (99 mg, 0.18 mmol) in *t*-BuOH (2 mL) and water (1.5 mL) was added lithium hydroxide monohydrate (16 mg, 0.36 mmol), and the mixture was stirred 5 h at room temperature. The resulting solution was concentrated under reduced pressure, and the residue was passed through a short column (silica gel) eluting with EtOH to provide a yellow oil. This oil was stirred with EDC (51 mg, 0.27 mmol) and *N*-hydroxysuccinimide (39 mg, 0.32 mmol) in CH_2Cl_2 (7 mL) for 12 h at room temperature, and the resulting solution was washed with brine (5 mL), dried over $MgSO_4$, and concentrated. The resulting residue was purified by column chromatography (silica gel, EtOH) to afford **16** (74 mg, 0.234 mmol) as a yellow solid. The total yield from **15** was 65%: mp 78–80 °C; IR (CH_2Cl_2) 3420, 3370, 2940, 1820, 1790, 1740, 1590, 1600, 1515, 1345 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.33 (s, 1H), 8.13 (d, J = 8.5 Hz, 2H), 7.45 (d, J = 8.5 Hz, 2H), 7.36 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.2 Hz, 2H), 5.15 (m, 1H), 3.44 (d, J = 11.3 Hz, 3H), 3.30 (d, J = 11.2 Hz, 3H), 2.98 (m, 2H), 2.80 (brs, 4H), 2.65 (t, J = 6.9 Hz, 2H), 2.41 (t, J = 6.9 Hz, 2H), 2.11 (quint, J = 6.9 Hz, 2H), 2.07 (s, 6H); ^{31}P NMR ($CDCl_3$) δ –22.5.

[3-[4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]phenyl]-2-phosphoryl-1-(4-nitrophenyl)propyl]trimethylammonium iodide (3a). To a solution of **16** (16 mg, 0.025 mmol) in acetone (1 mL) was added methyl iodide (16 μL, 0.025 mmol) at room temperature, and additional 32-μL portions (0.05 mmol) of the same reagent were added each day for 6 days. At day 7 the solvent was removed on a rotary evaporator, and the residue was vigorously stirred with CH_2Cl_2 (1 mL) for 5 min. The CH_2Cl_2 solution was decanted, and the remaining insoluble yellow viscous oil was dissolved in acetonitrile (1 mL). To this was added trimethylsilyl iodide (13 μL) at 0 °C, and the mixture was stirred 2 h. The solvent was removed under reduced pressure to afford **3a** in 44% yield (7.0 mg, 0.11 mmol) as a colorless solid. The product was used for conjugation with carrier proteins without further purification: mp 217–222 °C (dec); ^{1}H NMR (CD_3OD, 250 MHz) δ 8.38 (m, 2H), 8.25 (brd, 2H), 7.55 (brd, 2H), 7.50 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.4 Hz), 5.57 (m, 1H), 4.45 (brs, 1H), 3.34 (m, 1H), 3.23 (s, 9H), 2.84 (brs, 4H), 2.75 (t, J = 8.4 Hz, 2H), 2.52 (t, J = 8.4 Hz, 2H), 2.48 (m, 1H), 2.12 (quint, J = 8.4 Hz, 2H); ^{31}P NMR (CD_3OD) δ –0.27.

Preparation of Antigen. Hapten **1a** (2–4 mg) was dissolved in 0.25 mL of DMF and diluted with 0.25 mL of water. The resulting solution

was slowly added to a solution of maleimide–BSA or maleimide–Fib[9c] (~5 mg) in 0.5 mL of 0.2 M potassium phosphate buffer (KPi) at pH 7.2, and the mixture was gently stirred 2 h at room temperature. The resulting solution (BSA–**1**) or suspension (Fib–**1**) was dialyzed twice against KPi (1 L) at 4 °C. The concentration of BSA–**1** was determined by Bradford analysis (2–4 mg/mL of the conjugate was generally obtained), and the number of hapten molecules per BSA was estimated to be more than 10 molecules by measuring the absorbance at 250 nm (λ_{max} of **1a**). The BSA–**1** conjugate was used for ELISA experiments, and the suspension of the conjugate Fib–**1** was used for immunization (assuming that it contained ~5 mg/mL of protein).

The protein–hapten conjugates of **2a** and **3a** were prepared in a similar manner. To a solution of the hapten (2–4 mg) in KPi (0.5 mL) was slowly added a solution of BSA or Fib (5 mg) in KPi (5 mL), and the mixture was stirred 2 h at room temperature. After dialysis of the mixture against KPi, its concentration (3–5 mg/mL) and number of hapten molecules per protein (>10 hapten molecules/protein) were determined in the same manner as described above, and the BSA–hapten and the Fib–hapten conjugates were used for immunization and ELISA, respectively.

Antibody Production and Purification. Four Balb/c mice each received a subcutaneous injection of 100 μg of the appropriate protein–hapten conjugate emulsified in RIBI adjuvant (MPL and TDM emulsion) on days 1 and 14 (boost #1). On day 21, serum was taken from the animals, and the titer was determined by ELISA. On day 35, the mouse with the highest titer received a second subcutaneous boost (boost #2) with the same protein–hapten conjugate.

Three days after the last boost (boost #2), the spleen was taken from the animal and the cells were fused with 5×10^7 653/HGPRT$^-$ myeloma cells to prepare hybridomas. Cells were plated into six 96-well cell culture plates; each well containing 100 μL of HAT-DMEM (hypoxanthine, aminopterin, thymidine-Dulbecco's minimal essential medium) with 20% of fetal bovine serum and 1% of hybridoma enhancing supplement (Sigma). After 2 weeks, the plates were analyzed by ELISA for binding to the appropriate protein–hapten conjugate, and all colonies that displayed tight binding were subcloned two or three times to produce monoclonal cell lines. Each of these cell lines were individually injected into pristane-primed Balb/c mice to generate ascitic fluid. The harvested ascitic fluid was treated with a saturated ammonium sulfate solution (AS) to give a final concentration of 45% AS, and the precipitated antibodies were dissolved in and dialyzed against 50 mM Tris·HCl, pH 7.8.

Antibodies (IgG) were purified by DEAE-Sephacel anion-exchange chromatography (salt gradient from 0 to 500 mM NaCl in 50 mM Tris·HCl, pH 7.8) and protein G-Sepharose affinity chromatography (antibodies were loaded on in 20 mM potassium phosphate, pH 7.2, and eluted with 100 mM glycine·HCl, pH 2.7). The purity of the final antibody solution was greater than 95% by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Antibody Affinity Measurement. An ELISA was performed to identify positive hybridoma clones producing IgG molecules which specifically bind to the hapten. Each well of a 96-well polyvinyl assay plate (ELISA plate) was coated with 100 μL of the appropriate protein–hapten conjugate (2 μg/mL) in 50 mM Na_2CO_3 (pH 9.0) and incubated at 4 °C overnight or at room temperature for 2 h. The plate was washed with phosphate buffer salt (PBS, 10 mM, pH 7.2 phosphate, 150 mM NaCl) containing 5% Tween 20 (PBS Tween) three times followed by water (washing protocol). The hybridoma supernatant from each well of the 96-well cell culture plates was added to the ELISA plates and incubated for 1 h, the supernatant was removed, and the plates were washed according to the washing protocol. A solution of goat anti-mouse IgG-horseradish peroxidase (Bio-Rad) in PBS Tween was added to each well, the plates were incubated for 1 h, and the washing protocol was repeated. Finally, each well was treated with 100 μL of a solution of 1,2-phenylenediamine (0.4 mg/mL) in 0.1 M sodium citrate (pH 5.0) containing 0.1% hydrogen peroxide, the yellow color was allowed to develop for approximately 5 min, and the addition of 20 μL of 1.0 M H_2SO_4 was used to end the reaction. Positive clones were identified by measuring the absorbance at 450 nm using a microtiter plate reader or more simply by visual identification through comparison with the negative controls.

Titration of antibodies in animal serum was carried out as follows. The protein–hapten coated ELISA plate was prepared as described above, and to each well was added 100 μL of PBS. The serum was diluted 200-fold in PBS, and 100 μL was added to the first well (A1) of the plate. The resulting solution was serially diluted 2-fold from well A1 to well A8 (from 1:400 to 1:51 200 dilution), and the plate was incubated for 1 h. The ELISA procedure was then applied as described above, and the absorbance at 450 nm was measured. A plot of the log of the serum dilution vs absorbance provided a sigmoidal curve, and the titer was defined as the serum dilution at which the absorbance became comparable to that of the background.

A competition ELISA protocol was followed to determine dissociation constants (K_ds) of inhibitors (haptens **1b–3b**, substrates **4a** and **4b**, PNP, and the product acid) for each antibody. Prior to carrying out the competition ELISA, the optimum antibody concentration and the incubation time on the ELISA plate (precoated with 2 μg/mL of the protein–hapten conjugate and then blocked with BSA) were determined to establish a reproducible titration curve. This is critical for detecting a weak antibody affinity to an inhibitor. The minimum antibody concentration is defined as the concentration at which ca. 70% of the antibody (reflecting on the absorbance) binds to the protein–hapten conjugate. In our experiments, the concentration and the incubation time were generally in the range 0.25–1.0 μg/mL and 10–20 min, respectively. Competition ELISA experiments were performed as follows. A 60-μL solution of each inhibitor (in 50 mM Tris·HCl, 80 μM NaCl, pH 7.8) was serially diluted 2-fold across a BSA-blocked ELISA plate (from well A1 to well A12), containing 60 μL of buffer per well. To each well was then added 60 μL of an antibody solution (the final concentration was preadjusted), and the mixture was equilibrated for 30 min at 25 °C. The resulting antibody-inhibitor solution (100 μL) from each well was transferred to the corresponding well of the ELISA plate with a multipipeter and incubated for the preadjusted time (10–20 min). After washing the plate, the ELISA protocol was followed and a titration curve (the sigmoidal plot of the log of the antibody concentration vs absorbance) was obtained. The results were analyzed by a Klotz plot (a double-reciprocal plot of the inhibitor concentration vs the ratio of inhibitor-bound antibody to the total added antibody), the slope of the line yielding the K_d for the inhibitor. All assays were repeated three times.

Kinetic Measurements. Each purified monoclonal antibody solution was dialyzed against the assay buffer, and the concentration was determined by measurement of the absorbance at 280 nm. Assays were performed in a microtiter plate reader (Bio-Tek Instruments) equipped with the Delta Soft II computer program (Biometalics, Inc.). All assays were performed at least in duplicate.

Initial screening for catalysis by antibodies was performed at 25 °C in buffer solution (50 mM Mes, 80 μM NaCl, pH 6.2, or 50 mM Tris·HCl, 80 μM NaCl, pH 7.8) with 5% of organic cosolvents (4.8% acetonitrile and 0.2% dimethyl sulfoxide), containing 1.0 mM ester **3a** and 10 μM antibody. The rates were determined by measuring the initial change in linear absorbance at 405 nm reflecting on *p*-nitrophenolate release. The background hydrolysis rate of the substrate (in the absence of antibody) was also measured each time. Antibodies found to be capable of catalyzing the hydrolysis were further studied.

The antibody-catalyzed hydrolysis rates of ester **3a** in the presence of 5–10 μM antibody were measured as a function of substrate concentration under the conditions used in the aforementioned screening. The substrate concentration was varied from 0.25 to 3.0 mM (the solubility limit of **3a**) to obtain eight data points, and the observed rates were corrected for the uncatalyzed rate of hydrolysis (in the absence of antibody). The corrected rates were analyzed by a Lineweaver–Burk plot to determine the Michaelis–Menten parameters (k_{cat} and K_m). Background hydrolysis rate constants (k_{un}) of **3a** at each pH were determined by extrapolation to zero-buffer concentration.

Inhibition constants (K_i) were determined in the standard fashion. The antibody-catalyzed hydrolysis rates as a function of both the inhibitor concentration (fixed substrate concentration) and the substrate concentration in the presence of a fixed concentration of inhibitor were measured at pH 7.8 to provide a Dixon plot and a Lineweaver–Burk plot, respectively. Analyses of these afforded K_i values of the respective inhibitors for the antibodies.

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