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The Potential Application of Catalytic Antibodies to Protecting Group Removal: Catalytic Antibodies with Broad Substrate Tolerance

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Abstract: A catalytic antibody was developed to selectively cleave the alcohol ester of 4-nitrophenylacetyl moiety while also tolerating a wide variety of structural variation on the alcohol portion of the molecule. The basis to the success of this study was that antibody epitope recognition was directed toward only key elements contained within the 4-nitrophenylacetyl group and not the entire haptenic molecule. This study offers the potential application of catalytic antibodies as practical reagents for the selective deprotection of complex multifunctionalized molecules possessing class similar protecting groups. Such a chemoabzymatic approach could eventually minimize synthetic complications which can arise from functional group protection in the synthesis of complex natural products.

Introduction

Efficient protection and deprotection of functional groups is tantamount in any successful organic synthetic approach. In polyfunctionalized molecules, an optimal protecting group scheme requires the introduction of a minimal number of new functional groups so as to avoid subsequent synthetic complications. One tactic to achieve efficient functional group protection would be to utilize structurally similar protecting groups; however, selective removal of class similar protecting groups is extremely difficult by chemical methods and has only recently been explored enzymatically.[1] Consequently, we have investigated the potential of utilizing catalytic antibodies as chemoselective deprotecting reagents.[2,3]

Catalytic antibodies have been proposed and briefly analyzed in protective group removal; however, they have yet to be generalized.[4] In the cases described, catalytic antibody epitope recognition has been unidimensional; moreover, the immune response has been driven to be homogeneous to the immunizing molecule. Such narrow specificity coupled with the cost and time of generating catalytic antibodies has dampened their applicability. To expand the window for catalytic antibodies in organic synthesis, we have devised a singular haptenic approach which tolerates structural variation in the molecule of interest while also providing a measure of the fidelity for the selective removal of class similar protecting groups.

In this study, catalytic antibodies were developed for the selective removal of the ester protecting group. The choice of such a target was two-prong in nature. First, the hydroxyl moiety can be found in a number of natural products and drugs;[5] also, because of its nucleophilic properties, its protection and ultimately its unmasking are critical in virtually all synthetic

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1995.

(1) Waldmann, H.; Sebastian, D. *Chem. Rev.* **1994**, *94*, 911.

(2) For some general reviews on catalytic antibodies, see: (a) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391. (b) Janda, K. D.; Chen, J. Y.-C. In *The pharmacology of monoclonal antibodies*; Rosenberg, M., Moore, G. P., Eds.; Springer-Verlag: New York, 1994; pp 209–242.

(3) For some examples in this area, see: (a) Li, T.; Janda, K. D.; Ashley, J. A.; Lerner, R. A. *Science* **1994**, *264*, 1289. (b) Hsieh, L. C.; Yonkovich, S.; Kochersperger, L.; Schultz, P. G. *Ibid.* **1993**, *260*, 337.

(4) (a) Iverson, B. L.; Cameron, K. E.; Jahangiri, G. K.; Pasternak, D. S. *J. Am. Chem. Soc.* **1990**, *112*, 5320. (b) Iwabuchi, Y.; Miyashita, H.; Tanimura, R.; Kinoshita, K.; Kikuchi, M.; Fujii, I. *J. Am. Chem. Soc.* **1994**, *116*, 771.

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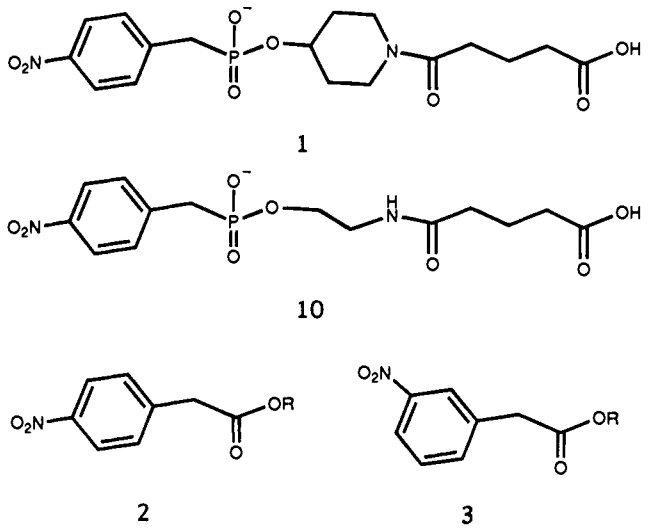


Figure 1. Structures of hapten **1** and generalized substrates used in the catalytic antibody investigations.

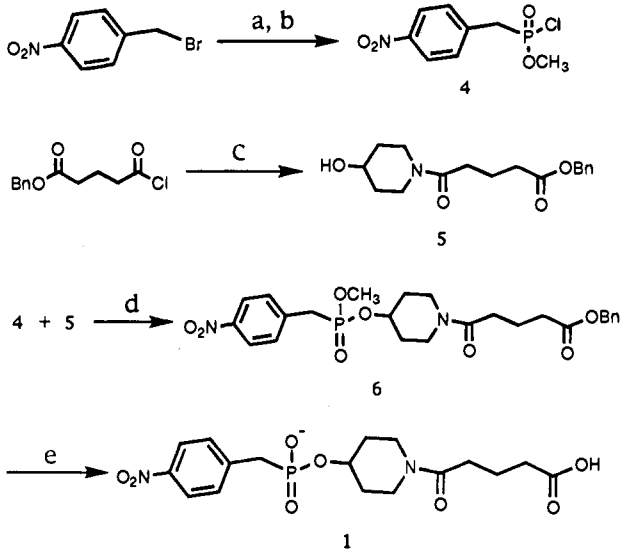
endeavors. Second, esterolytic cleavage utilizing catalytic antibodies is relatively well-established,[6] including a strong basis for their mechanism of action.[7]

Results and Discussion

As a first step toward antibody-catalyzed protecting group removal, we studied the selective cleavage of the 4-nitrophenylacetyl group in the presence of a 3-nitrophenylacetyl group. Although 4-nitrophenylacetyl has not been utilized as an alcohol protecting group, its overall chemical stability satisfies most synthetic manipulations. For example, it is stable toward a variety of conditions including acidic, basic, and nucleophilic treatments. It is also stable toward hydride reductions and oxidations.

To elicit catalytic antibodies which could selectively cleave the 4-nitrophenylacetyl appendage, a transition state analog haptenic approach employing the phosphonate moiety was adopted.[8] Specifically, phosphonate **1** was expected to induce antibodies for the hydrolysis of generalized ester substrates such as **2** (Figure 1). The key features of **1** are that it provides imbalanced immunodominate recognition, this via its strongly immunogenic nitro moiety,[9] and a nonspecificity element which is encompassed in the 4-hydroxypiperidine functionality. It was anticipated that the combination of these two elements would provide antibody catalysts which were selective for the hydrolysis of the 4-nitrophenylacetyl group while also being relatively "relaxed" in the structural-scaffolding requirements benzylic to this moiety.[10]

Scheme 1. Synthesis of Hapten **1**[a]



[a] Key: (a) $P(OMe)_3$, 79%; (b) PCl_5, 110% (crude); (c) 4-hydroxypiperidine, 99%; (d) LDA, 19%; (e) NaOH, 50%.

Hapten Synthesis and Hybridoma Production. Hapten **1** was synthesized according to Scheme 1. Thus, 4-nitrobenzyl bromide was converted to dimethyl (4-nitrophenyl)phosphonate via the Arbusov reaction in 79% yield. The phosphonate was then transformed to phosphonochloridate **4** with PCl_5, and without further purification, **4** was allowed to reaction with alcohol **5** to provide the penultimate phosphonate hapten **6**. Subsequent hydrolysis of the phosphonate and carboxylate funtionalities granted hapten **1** with an overall yield of 7.4%.

Small compounds such as hapten **1** are not immunogenic unless they are coupled to a carrier protein molecule. Thus, **1** was coupled to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) via amide bond formation with the surface lysine residues. The ratio of hapten/carrier monomer, the so-called epitope density, was found to be 14 and 12 for the KLH and BSA conjugates, respectively, as determined by measuring the nitrophenyl absorbance at 300 nm.

Monoclonal antibodies were elicited against KLH−**1** conjugate using hybridoma methodology.[11] In brief, 129GIX$^+$ mice were injected intraperitoneally with KLH−**1** emulsified in RIBI (MPL/TDM) adjuvant. After three rounds of immunization using KLH−**1** and RIBI (MPL/TDM) adjuvant, mice were hyperimmunized with KLH−**1** in the absence of adjuvant and fused after 3 d. Spleen cells were fused with a SP2/0 myeloma cell line, and the BSA−**1** conjugate was used in the screening of hybridoma cells in an enzyme-linked immunosorbant assay (ELISA). A total of 21 antibody-secreting hybridomas were isolated specifically for hapten **1**. All monoclonal antibodies were of the IgG class and were purified by salt precipitation, anion-exchange chromatography (DEAE), and affinity chromatography (protein G). Antibodies were judged to be >95% homogeneous by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−Page).

Kinetics and Inhibition Analysis. Antibodies at a concentration of 5 μM were initially screened under biphasic conditions (15% Bicine, 50 mM, pH = 9.0, 83% hexane/2% chloroform)

(5) Wermuth, C. G.; Koga, N.; Konig, H.; Metcalf, B. W. *Medicinal Chemistry for the 21st Century*; Blackwell Scientific Publications: London, 1992; pp 3−12.

(6) (a) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science* **1989**, *244*, 437. (b) Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961. (c) Ikeda, S.; Weinhouse, M. I.; Janda, K. D.; Lerner, R. A. *J. Am. Chem. Soc.* **1991**, *113*, 7763.

(7) (a) Janda, K. D.; Ashley, J. A.; Jones, T. M.; Mcleod, D. A.; Schloeder, D. M.; Weinhouse, M. I.; Lerner, R. A.; Gibbs, R. A.; Benkovic, P. A.; Hilhorst, R.; Benkovic, S. J. *J. Am. Chem. Soc.* **1992**, *113*, 291. (b) Janda, K. D.; Weinhouse, M. I.; Danon, T.; Pacelli, K. A.; Schloeder, D. M. *J. Am. Chem. Soc.* **1991**, *113*, 5427.

(8) For examples of using phosphonate as a transition state mimic for ester hydrolysis, see: (a) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566. (b) Jacobs, J. W.; Schultz, P. G.; Sugasawara, R.; Powell, M. *J. Am. Chem. Soc.* **1987**, *109*, 2174. (c) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Ibid.* **1989**, *244*, 437.

(9) (a) Landsteiner, K. *The Specificity of Serological Reactions*; Charles C. Thomas: Springfield, IL, 1936. (b) Nakayama, G. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 780. (c) Hsieh, L. C.; Yonkovich, S.; Kochersperger, L.; Schultz, P. G. *Science* **1993**, *260*, 337.

(10) A binding site for variable amino acids was achieved recently for an antibody-catalyzed peptide synthesis: 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.

(11) Kohler, G.; Milstein, C. *Nature* **1975**, *256*, 495.

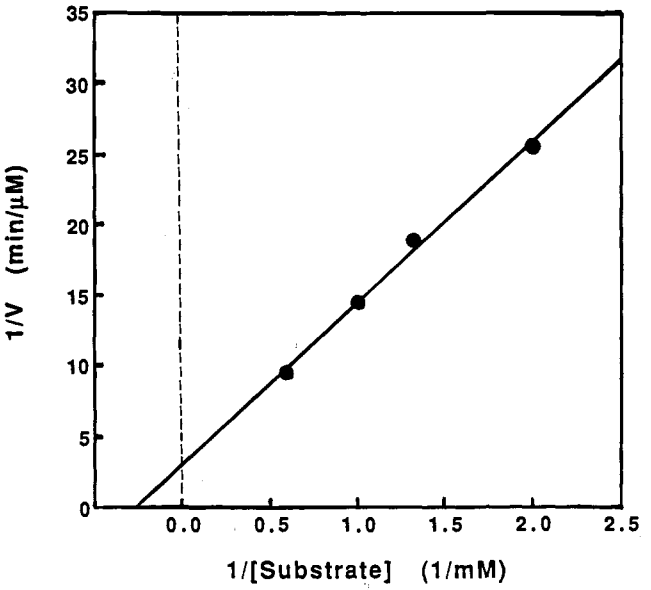


Figure 2. Lineweaver–Burk plot of IgG 27H9-catalyzed hydrolysis of ester **2c** (5 μM IgG 27H9, 15% Bicine, 50 mM, pH = 9.0, 83% pentane/2% chloroform).

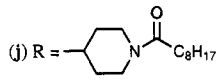
to accommodate the water insoluble substrate **2c**. These conditions, while unusual to typical antibody work, have proven quite reliable in our catalytic antibody assays.[12] From the panel of 21 antibodies specific to BSA–**1**, three were found to cleave **2c** as determined by an HPLC assay following the release of *p*-nitrophenylacetic acid. Of the three catalytic IgG's, 27H9 showed significantly greater activity and was selected for further study. The 27H9-catalyzed hydrolysis of **2c** was shown to obey Michaelis–Menten kinetics with a k_{cat} of 0.062 min^{-1} and a K_m of 3.4 mM (Figure 2). The rate of the uncatalyzed reaction was found to be 2.1×10^{-6} min^{-1}. The k_{cat}/k_{uncat} value of the antibody-catalyzed reaction was determined to be 2.9×10^4. This antibody-catalyzed reaction was competitively inhibited by **1** (K_i = 12 μM), demonstrating that the catalytic activity is associated with binding in the antibody-combining site.

Substrate Synthesis, Protecting Group Specificity, and Substrate Tolerance. To investigate the antibody's protecting group specificity and substrate tolerance, a number of nitrophenylacetates were synthesized (Figure 1 and Table 1). The syntheses of **2a**–**l** and **3a**–**l** were accomplished by carbodiimide coupling between 4-nitrophenylacetic acid or 3-nitrophenylacetic acid and the corresponding commercially available alcohols. The mixed bis(nitrophenylacetate) **8** was synthesized in two steps (Scheme 2). Thus, treatment of 1 equiv of 1,3-propanediol and 1 equiv of 3-nitrophenylacetic acid with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide method-*p*-toluenesulfonate (CMC metho-*p*-toluenesulfonate) gave propanediol monoester **7** in 53% yield after chromatographic purification. The bis(nitrophenylacetate) **8** was obtained by coupling of monoester **7** with 4-nitrophenylacetic acid.

A key feature typically associated with antibody catalysis is the exquisite antibody–substrate binding fidelity. To relax specificity, antigenicity was directed toward the protecting group while the scaffolding portion of the haptenic molecule was left to be hydrophobic and thus rather nonspecific. The success of this approach can be seen in Table 1. A wide variety of primary alcohols protected as 4-nitrophenylacetates were utilized as substrates by IgG 27H9. The best substrate from this group of 12 esters was **2f**; the ratio of k_{cat}/k_{uncat} here was almost 1.5 million.[13] When we investigated the selectivity of 27H9 with

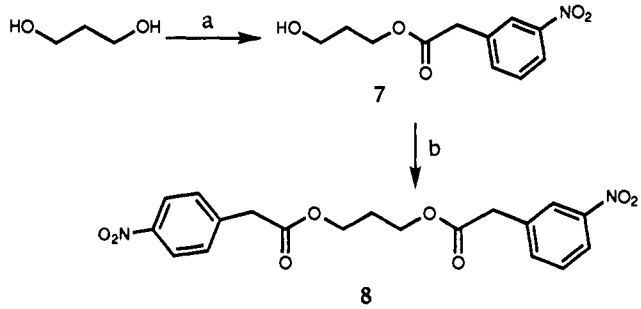
(12) Ashley, J. A.; Janda, K. D. *J. Org. Chem.* **1992**, *57*, 6691.

Table 1. Summary of the Kinetic Parameters for Antibody 27H9-Catalyzed Reactions[a]

	2			
substrates	K_m	k_{cat}	k_{cat}/k_{uncat}	3
(**a**) R = ethyl	2.8	0.011	8.8×10^3	no catalysis
(**b**) R = benzyl	1.5	0.029	8.5×10^4	no catalysis
(**c**) R = allyl	3.4	0.062	2.9×10^4	no catalysis
(**d**) R = 2-chloroethyl	0.78	0.29	1.8×10^4	no catalysis
(**e**) R = isobutyl	3.7	0.0038	1.9×10^4	no catalysis
(**f**) R = *m*-nitrobenzyl	1.5	0.47	1.4×10^6	no catalysis
(**g**) R = $(CH_2)_2$-*t*-Bu		no catalysis		no catalysis
(**h**) R = neopentyl	0.62	0.00055	6.5×10^3	no catalysis
(**i**) R = $CH(CF_3)_2$	1.2	0.16	1.9×10^4	no catalysis
(**j**) R = 		no catalysis		no catalysis
(**k**) R = isopropyl		no catalysis		no catalysis
(**l**) R = cyclohexyl		no catalysis		no catalysis

[a] Kinetic constants K_m and k_{cat} were obtained from double-reciprocal plots of initial rate data (described in the Experimental Section). The units for K_m are mM, and k_{cat} and k_{uncat} are in min^{-1}. 27H9-catalyzed reactions were conducted in 83% hexane, 2% chloroform, and 15% Bicine buffer (50 mM, pH = 9.0). The uncatalyzed reactions were measured in this same buffer system without the antibody. The antibody-catalyzed velocities are corrected in that background velocity is subtracted.

Scheme 2. Synthesis of Mixed Bis(nitrophenylacetate) **8**[a]



[a] Key: (a) 3-nitrophenylacetic acid, CMC metho-*p*-toluenesulfonate, DMAP, CH_2Cl_2, 53%; (b) 4-nitrophenylacetic acid, CMC metho-*p*-toluenesulfonate, DMAP, CH_2Cl_2, 70%.

esters of secondary alcohols such as **2j**–**l**, we were surprised to find these compounds were excluded as substrates. The lack of reactivity is unlikely due to recognition as these compounds are more akin in structure to hapten **1** than esters **2a**–**h**. In an attempt to resolve this interesting observation, we synthesized ester **2i**. This 4-nitrophenylacetate of an activated secondary alcohol (1,1,1,3,3,3-hexafluoro-2-propanol) did serve as a substrate (Table 1). This result indicates that the lack of reactivity is unlikely due to recognition problems but rather the fact that esters of secondary alcohols are more resistant than esters of primary alcohols toward hydrolysis.[14] The fact that ester **2j** competitively inhibits (K_i = 730 μM) the 27H9-catalyzed hydrolysis of **2d** provides additional evidence for this argument. Because catalysis of esters of secondary alcohols is well within the purview of catalytic antibodies,[6] we would suggest that the lack of catalysis seen in the cases at hand is

(13) As one reviewer pointed out, k_{uncat} values can be directly related to the hydrophobicity of the substrate employed in the reaction. Therefore, the k_{uncat} term will depend not only on the rate of hydrolysis of the particular ester but also on the partitioning of the substrate between the organic and the aqueous phases (i.e. a more hydrophilic substrate will increase the background reaction).

(14) A combination of the steric effect and the basicity of the leaving group contributes to the relative resistance of esters of secondary alcohols toward hydrolysis: (a) Bender, M. L. *Chem. Rev.* **1960**, *60*, 53. (b) Taft, R. W. In *Steric Effects in Organic Chemistry*; Newman, M. S., Ed.; Wiley: New York, 1956; Chapter 13.

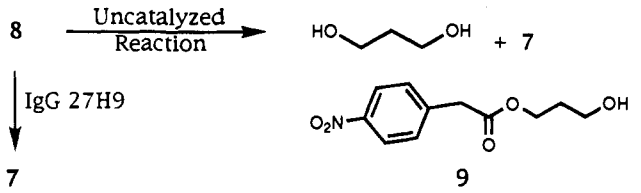


Figure 3. Reaction of bis(nitrophenylacetate) **8** with IgG 27H9 and without antibody catalyst.

due to the distribution of antibody binding energy to just a small portion of the substrate's structural makeup, rather than the entire molecule.[15]

A major goal of the work conducted here was to demonstrate that antibody catalysis could discriminate between closely related chemical protecting groups. Clearly, the failure to observe catalysis with any of the 3-nitrophenylacetates (Table 1) demonstrates antibody protecting group selectivity. This protecting group selectivity challenge was extended to the regioselective hydrolysis of the bis-ester **8**. The similar chemical reactivities of these two esters render this selective cleavage event difficult to achieve by current chemical methods. Thus, the hydrolysis of **8** (15% Bicine, 50 mM, pH = 9.0, 83% hexane/2% chloroform) without antibody present provides a variety of products (Figure 3), among which a 1/1 ratio of 4-nitrophenylacetic acid and 3-nitrophenylacetic acid was produced. However, inclusion of IgG 27H9 routes the reaction to that of a complete regioselective hydrolysis of the 4-nitrophenylacetyl moiety (k_{cat} = 0.013 min^{-1}, K_m = 590 μM).

Conclusion

The proper introduction and removal of protecting groups is one of the most important and widely carried out synthetic transformations in organic chemistry. We have presented a model system for the investigation of catalytic antibodies as deprotecting reagents. Our strategy was to apply a two-prong haptenic approach which would allow discrete protecting group selectivity while also providing a broad base substrate acceptance.

While this method was successful, it is limited to chemical scenarios where immunogenic imbalance can be achieved within an antigenic molecule. We would suggest that a more general approach to achieve greater substrate tolerance would be through a dual haptenic-immunization and screening approach. Here, immunization and antibody screening (specificity) would be accomplished using different haptenic molecules. For example, if mice are immunized with hapten **1** and subsequently monoclonal antibodies were selected according to their binding with hapten, say **10**, then the epitope of recognition for antibodies isolated should reside within the 4-nitrophenylacetyl portion of the molecule. Future efforts to apply this latter methodology would extend the scope of protecting group removal with catalytic antibodies.

Experimental Section

General Procedures. HPLC analysis was carried out on a Hitachi 655A chromatograph with an analytical reversed-phase C-18 column (VYDAC 201TP54). The ^{1}H NMR spectra were recorded on a Bruker AM 300 MHz spectrometer. Flash column chromatography purifications were performed using E. Merck Kieselgel 60 (230−400 mesh) silica gel.

Methyl (4-Nitrobenzyl)phosphonochloridate (4). A mixture of 4-nitrobenzyl bromide (5.0 g, 23 mmol) and $P(OMe)_3$ (10 mL, 92 mmol) was refluxed for 6 h. Excess $P(OMe)_3$ was removed under vacuum, and the residue was purified by column chromatography on silica gel (CH_3CN/CH_2Cl_2, 1/1) to give dimethyl (4-nitrobenzyl)phosphonate (4.5 g, 79%): ^{1}H NMR ($CDCl_3$) δ 3.26 (d, 2H, J = 22 Hz), 3.72 (d, 6H, J = 13 Hz), 7.48 (d, 2H, J = 9.0 Hz), 8.20 (d, 2H, J = 9.0 Hz); HRMS (FAB) m/z calcd for ($C_9H_{12}NO_5P$ + H) 246.0531, found 246.0528.

Dimethyl (4-nitrobenzyl)phosphonate (600 mg, 2.45 mmol) and phosphorus pentachloride (663 mg, 3.19 mmol) in $CHCl_3$ (5 mL) were stirred at 45 °C. After 3 h, sulfur dioxide (generated from heating sodium bisulfite) was bubbled through the mixture for 5 min. The solvent was removed under vacuum, and the crude product, methyl (4-nitrobenzyl)phosphonochloridate (**4**) (680 mg, 110%), was used in the subsequent reaction without further purification: ^{1}H NMR ($CDCl_3$) δ 3.65 (d, 2H, J = 22 Hz), 3.88 (d, 3H, J = 13 Hz), 7.51 (d, 2H, J = 2.9, 9.0 Hz), 8.22 (d, 2H, J = 8.6 Hz).

***N*-(Benzylglutaryl)-4-hydroxypiperidine (5).** A solution of glutaric anhydride (6.0 g, 53 mmol), benzyl alcohol (6.0 mL, 58 mmol), triethylamine (9.5 mL, 58 mmol), and a catalytic amount of DMAP (100 mg) in CH_2Cl_2 (50 mL) was stirred at room temperature for 12 h. The solvent was removed in vacuo, and the residue was dissolved in ether (150 mL) and washed with saturated $NaHCO_3$. The aqueous layer was acidified with 1 N HCl to pH = 2, and this was extracted with ethyl ether. The ether layer was dried with Na_2SO_4 and removed in vacuo to provide benzylglutaric acid (6.1 g, 52%). The acid (5.0 g, 22.5 mmol) was dissolved in CH_2Cl_2 (50 mL). To it was added oxalyl chloride (2.5 mL, 29 mmol), followed by a drop of DMF. After the mixture was stirred at room temperature for 1 h, the solvent was removed to give the desired benzylglutaryl chloride (5.4 g, 100%).

Benzylglutaryl chloride (1.6 g, 7.0 mmol) was added to 4-hydroxypiperidine (1.6 g, 16 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred for 5 min and washed with 1 N HCL, followed by water. The organic layer was dried over sodium sulfate, and the solvent was removed in vacuo to give the desired product **5** (2.1 g, 100%): ^{1}H NMR ($CDCl_3$) δ 1.50 (m, 2H), 1.85 (m, 2H), 1.97 (quintet, 2H, J = 7.2 Hz), 2.37 (t, 2H, J = 7.6 Hz), 2.46 (t, 2H, J = 7.1 Hz), 3.16 (m, 2H), 3.66 (m, 1H), 3.91 (m, 1H), 4.07 (m, 1H), 5.10 (s, 2H), 7.32 (m, 5H); HRMS (FAB) m/z calcd for ($C_{17}H_{23}NO_4$ + H) 306.1705, found 306.1708.

***N*-(Benzylglutaryl)-4-((methoxy(4-nitrobenzyl)phosphinyl)oxy)-piperidine (6).** LDA (1.5 M, 0.40 mmol) was combined with alcohol **5** (122 mg, 0.40 mmol) in THF (5 mL) at −40 °C. After the mixture was stirred for 10 min, methyl (4-nitrobenzyl)phosphonochloridate **4** (200 mg, 0.801 mmol) was added. This mixture was stirred at −40 °C for 2 h, and the reaction was terminated by quenching with 1 N HCl. The aqueous solution was extracted with ethyl acetate and washed with aqueous 1 N HCl, followed by saturated NaCl. The organic layer was dried with sodium sulfate and filtered, and the solvent was removed under reduced pressure. The residue obtained was purified by preparative TLC (eluent 5% EtOH in CH_2Cl_2) to give the desired phosphonate **6** (40 mg, 19%): ^{1}H NMR ($CDCl_3$) δ 1.47−2.00 (m, 6H), 2.34 (t, 2H, J = 7.1 Hz), 2.44 (t, 2H, J = 7.0 Hz), 3.26 (d, 2H, J = 22 Hz), 3.14−3.85 (m, 4H), 3.69 (d, 3H, J = 11 Hz), 4.59 (m, 1H), 5.10 (s, 2H), 7.32 (m, 5H), 7.46 (d, 2H, J = 8.6 Hz), 8.18 (d, 2H, J = 8.5 Hz); HRMS (FAB) m/z calcd for ($C_{25}H_{31}N_2O_8P$ + H) 519.1896, found 519.1905.

Hapten 1. To a mixture of phosphonate **6** (15 mg, 0.029 mmol) in EtOH (2 mL) was added aqueous NaOH (2 N, 2 mL). The mixture was stirred for 30 min, and the ethanol was removed under vacuum. The remaining solution was stirred for an additional 2 h, where upon it was quenched with 1 N HCl (4.3 mL). The solvent was removed in vacuo, and the crude product was extracted from the solid residue with ethanol. Final purification by reverse phase high-performance chromatography (HPLC) (eluent 40% acetonitrile in 0.1% aqueous trifluroacetic acid) led to the desired product (8.0 mg, 50%): ^{1}H NMR (CD_3OD/D_2O, 1/1) δ 1.5−1.9 (m, 6H), 2.39 (t, 2H, J = 7.1 Hz), 2.44 (t, 2H, J = 7.4 Hz), 3.24 (d, 2H, J = 22 Hz), 3.35−3.70 (m, 4H), 4.44 (m, 1H), 7.53 (d, 2H, J = 8.3 Hz), 8.19 (d, 2H, J = 8.4 Hz); HRMS (FAB) m/z calcd for ($C_{17}H_{23}N_2O_8P$ + Na) 437.1090, found 437.1103.

General Procedure for the Synthesis of Esters 2a−l and 3a−l. To a mixture of either 3-nitrophenylacetic acid or 4-nitrophenylacetic acid (0.3 mmol) and commercially available alcohols (0.3 mmol) in CH_2Cl_2 (2 mL) were added CMC metho-*p*-toluenesulfonate (0.33 mmol) and a catalytic amount of DMAP (2 mg). The reaction mixture was

(15) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover Publications, Inc.: New York, 1987; Chapter 5.

stirred at room temperature for 4 h, and the solvent was removed under reduced pressure. The residues were purified by preparative TLC (EtOAc/hexanes) to give the desired products with the expected spectroscopic data.

Synthesis of the Bis(nitrophenylacetate) 8. To 3-nitrophenylacetic acid (50 mg, 0.28 mmol) and 1,3-propanediol (20 μL, 0.28 mmol) in CH_2Cl_2 (1.5 mL) were added CMC metho-*p*-toluenesulfonate (130 mg, 0.30 mmol) and a catalytic amount of DMAP (2 mg). The mixture was stirred at room temperature for 4 h, and the residue was purified by preparative TLC (EtOAc/hexanes 50/50) to give the monoester **7** (35 mg, 53%). To this monoester (30 mg, 0.13 mmol) in CH_2Cl_2 (1 mL) were added 4-nitrophenylacetic acid (23 mg, 0.13 mmol), CMC metho-*p*-toluenesulfonate (59 mg, 0.14 mmol), and a catalytic amount of DMAP (1 mg). This mixture was stirred at room temperature for 4 h, and the solvent was removed under reduced pressure. The resulting residue was purified by preparation TLC (CH_2Cl_2) to yield the desired product **8** (36 mg, 70%): ^{1}H NMR ($CDCl_3$) δ 1.98 (m, 2H), 3.73 (s, 4H), 4.17 (t, 2H, J = 6.3 Hz), 4.18 (t, 2H, J = 6.3 Hz), 7.42–7.68 (m, 4H), 8.12–8.25 (m, 4H); HRMS (FAB) *m/z* calcd for ($C_{19}H_{18}N_2O_8$ + Na) 425.0961, found 425.0975.

Antibody Production and Assays. 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) (1.5 equiv) and *N*-hydroxysulfosuccinimide (1.5 equiv) were added to 2.0 mg of **1** in 100 μL of aqueous DMF (DMF/H_2O, 9/1). After 24 h at room temperature, the solution was added to 5 mg of KLH or BSA in 900 μL of 0.05 M sodium phosphate buffer, pH = 7.5. The conjugate was used without further purification. Four 8-week-old 129G1X$^+$ mice each received two intraperitoneal injections of 100 μg of **1** conjugated to KLH and RIBI adjuvant (MPL and TDM emulsion) 2 weeks apart. One month after the second injection, the mouse with the highest titer (12 800 to 25 600) was injected intravenously with 50 μg of **1**–KLH conjugate; 3 d later, the spleen was taken from the preparation of hybridomas. Spleen cells (1.0×10^8) were fused with SP2/0 myeloma cells (2.0×10^7). The cells were plated into 34 96-well plates; each well contained 150 μL of hypoxanthine, aminopterin, thymidine–Dulbecco's minimal essential medium (HAT–DMEM) containing 1% nutridoma, and 2% bovine serum albumin.

After 2 weeks, the antibodies produced by wells containing macroscopic colonies were assayed by ELISA for binding to **1**–BSA. Colonies that initially produced antibodies that bound **1**–BSA were subcloned twice, after which 21 remained active. All monoclonal antibodies were injected into pristine-primed 129G1X$^+$ × BALB/c mice to generate ascitic fluid. For studies, IgG was purified from the ascitic fluid by salt precipitation, anion exchange chromatography (DEAE), and affinity chromatography (protein G). All antibodies were then concentrated and dialyzed into 50 mM Bicine, pH = 9.0. The antibodies were judged to be homogeneous (>95%) by sodium dodecyl sulfate–polyacylamide gel electrophoresis.

Catalytic Reactions and Kinetic Measurements. The biphasic reactions were carried out in eppendorf tubes at 23 °C on an IKA-VIBRAX-VXR vortexer shaking at 1400 rpm. Typical reactions were 1 mL in total volume, and all contained final concentrations (v/v) of 83% hexane, 2% chloroform, and 15% Bicine buffer (50 mM, pH = 9.0). Catalyzed reactions were performed in the presence of 5 μM monoclonal antibody and were initiated by the addition of various amounts of (0.2–4 mM) substrate from a chloroform stock solution. Initial velocities were obtained by HPLC analysis on an analytical reversed-phase C-18 column (VYDAC 201TP54), eluting with an isocratic mobile phase of 25% acetonitrile and 75% water (containing 0.1% trifluoroacetic acid). Formation of the 4-nitrophenylacetic acid was followed at 275 nm by first diluting 50 μL of the aqueous solution into equal volume of 4% trifluoroacetic acid in 25% aqueous acetonitrile and then injecting 50 μL of the mixture into HPLC. No 4-nitrophenylacetic acid could be detected in the organic phase.

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