Antibody Bait and Switch Catalysis: A Survey of Antigens Capable of Inducing Abzymes with Acyl-Transfer Properties

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Abstract: Antibodies have been shown to catalyze acyl-transfer reactions. Various antigens have been applied to these hydrolytic reactions, but typically all encompass the same theme of incorporating a monoanionic phosphonate/phosphonamidate. To expand the scope and capabilities of these abzymes, we have directed our attention toward new strategies in antigen design. One method, which we have termed "bait and switch" catalysis, uses haptens to elicit amino acid(s) within the binding pocket of an antibody that can accelerate hydrolysis. We reported initial success of this methodology utilizing the cationic hapten **1** for obtaining abzymes that hydrolyzed benzoate ester **6**. In addition, we showed how the structurally identical but neutral hapten **2** was unable to induce catalytic antibodies. To further identify those factors critical in the generation of hydrolytic abzymes via our bait and switch methodology, we have (1) designed and synthesized three new homologues of **1** in which we have varied the type of charge/no charge and its location, (2) screened and identified catalytic antibodies from these antigens, (3) determined affinity constants of a number of these monoclonal antibodies (catalytic and noncatalytic) for their respective haptens and possible substrates (ester/amide), (4) performed steady-state kinetics, including a pH-rate profile on one of these abzymes, and (5) used chemical modifying reagents to identify which amino acid residue(s) are involved in these catalytic processes.

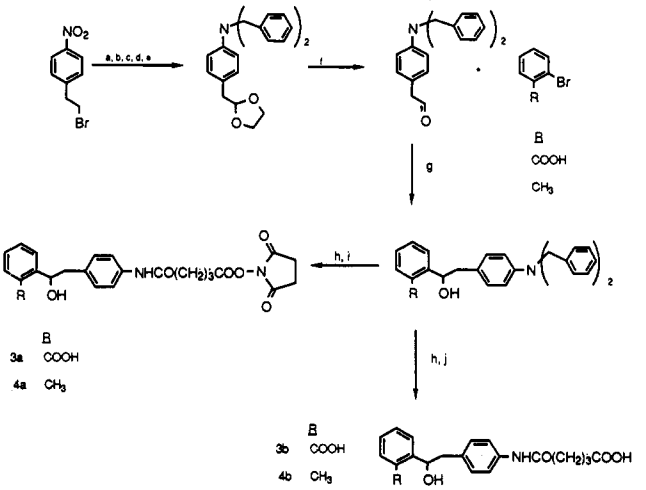
Introduction

The immune system of vertebrates generates proteins called immunoglobulins that can bind both macromolecules and lower molecular weight compounds. An important aspect of the immune system is its ability to produce an array of antibodies of the highest possible binding energy (greatest affinity) for a given antigen.[1] The primary repertoire of antibodies consists of about 10^8 different binding specificities, which, when coupled with the additional diversity arising from the process of somatic mutation, generates the most diverse set of binding proteins in biology.[2]

Nearly three decades ago, Linus Pauling postulated that the binding forces utilized by both enzymes and antibodies are fundamentally the same.[3] Yet nature has dictated that an enzyme, not an antibody, can use this binding energy to stabilize a transition state and promote a chemical reaction. If one had an antibody that specifically binds the transition state of a given chemical reaction, the antibody should catalyze that reaction. It is possible to model the transition states of some reactions with stable chemical species. Indeed, when suitable transition-state analogue haptens were used to induce monoclonal antibodies, catalysis was observed.[4] To date, antibodies have been shown to catalyze a variety of chemical transformations including hydrolytic, concerted, bimolecular, carbon–carbon bond forming, and redox reactions.[5] Many of these reactions proceed with high rates and enantioselectivity.

An area of antibody catalysis that we have been quite interested in has been acyl-transfer reactions.[6] Haptens (antigens) that have been applied in these hydrolytic reactions typically encompass the same theme, the utilization of a tetrahedral phosphorus moiety. To expand the scope and capabilities of these hydrolytic abzymes, we have directed our attention to new strategies in the design of haptens. One such method, which we have termed "bait and switch" catalysis, involves the use of haptens for explicit elicitation of amino acid (acids) within the antibody binding pocket to assist in the acyl-transfer reaction of interest.[7,8] We reported initial

Scheme I.[a] Synthesis of Haptens **3a** and **4a** and Inhibitors **3b** and **4b**



[a] Key: (a) Triethanolamine/H_2O, reflux 12 h, 55%; (b) $Pb(OAc)_4$, TFA/CH_2Cl_2, 3 h, room temperature, 50%; (c) Rexyn, $CaSO_4$, CH_2Cl_2, 24 h, 60%; (d) catalytic Pd/C, H_2, MeOH, room temperature, 95%; (e) 5 equiv of benzyl bromide, Et_3N, CH_2Cl_2, room temperature, 90 min, 80%; (f) acetone, 4 M HCl, 12 h, 40%; (g) 2-bromobenzoic acid and then *n*-BuLi, −78 °C, THF, 4 h, 62%; (h) catalytic Pd/C, H_2, MeOH, 2 h, room temperature, 95%; (i) $COCl(CH_2)_3COON(COCH_2)_2$, Et_3N, CH_2Cl_2, room temperature, 20 min, 90%; (j) glutaric anhydride, Et_3N, CH_2Cl_2, room temperature, overnight, 80%.

(1) Pressman, D.; Grossberg, A. L. *The Structural Basis of Antibody Specificity*; Benjamin: New York, 1968.
(2) Lerner, R. A. Proceedings of the XVIIIth Solvany Conference on Chemistry, Design and Synthesis of Organic Molecules Based on Molecular Recognition, Nov 20–Dec 1, 1983; pp 43–49.
(3) Pauling, L. *Am. Sci.* **1948**, *36*, 51.
(4) (a) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6736. (b) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566.
(5) For review articles on catalytic antibody reactions see: (a) Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1989**, *10*, 1283. (b) Schultz, P. G. *Acc. Chem. Res.* **1989**, *22*, 287. (c) Powell, M. J.; Hansen, D. G. *Protein Eng.* **1989**, *3*, 69.
(6) (a) Janda, K. D.; Lerner, R. A.; Tramontano, A. *J. Am. Chem. Soc.* **1988**, *110*, 4835. (b) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science (Washington, D.C.)* **1988**, *241*, 1199. (c) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science (Washington, D.C.)* **1989**, *244*, 437.
(7) (a) Janda, K. D. *Abstracts of Papers, 198th National Meeting of the American Chemical Society*, New Orleans, LA, Aug 1987; American Chemical Society: Washington, DC, 1987; ORGN 196. (b) Stinson, S. C. *Chem. Eng. News* **1987**, *65*, 30. (c) Janda, K. D.; Weinhouse, M. I.; Schloeder, D. M.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 1274. (d) Janda, K. D. *Biotechnol. Prog.* **1990**, *6*, 178.

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Table I. Antibodies Induced to **1a** and Their Binding to **1b**, **6** and **7**[a]

antibody	isotype	K_D(1),[b] M	K_D(6),[c] M	K_D(7),[d] M
30C6*[e]	$K\gamma_1$	3.9×10^{-5}	0.8×10^{-3}	1.0×10^{-3}
51B5*	$K\gamma_1$	2.0×10^{-5}	1.5×10^{-3}	0.5×10^{-3}
57H5*	$K\gamma_1$	1.6×10^{-4}	1.0×10^{-3}	1.1×10^{-3}
77D2*	$K\gamma_{2a}$	2.0×10^{-5}	2.0×10^{-3}	1.3×10^{-3}
57A5	$K\gamma_1$	1.2×10^{-6}	$>3 \times 10^{-3}$	$>3 \times 10^{-3}$
66D6	$K\gamma_1$	0.5×10^{-7}	1.2×10^{-3}	1.0×10^{-3}
62H8	$K\gamma_{2a}$	0.6×10^{-6}	0.4×10^{-3}	2.5×10^{-4}

[a] Binding determined at 37 °C in 10 mM pH 7.2 phosphate containing 150 mM NaCl and 5% Blotto.[24] [b] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **1a** by added **1b**.[10] [c] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **1a** by added **6**. [d] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **1a** by added **7**. [e] Asterisk denotes catalytic antibody.

success of this methodology utilizing the positively charged pyridinium salt **1** (Figure 1). This hapten induced a significant number of antibodies that catalyzed the hydrolysis of benzoate ester **6** (Figure 2). In addition, we showed that the structurally identical but neutral hapten **2** was unable to induce catalytic antibodies. We now report on other haptens in which we have varied the type of charge (or lack of one) as well as its placement in an effort to identify those factors critical in the generation of hydrolytic abzymes via our bait and switch methodology.

Results

Hapten Synthesis and Antibody Preparation. Haptens **3a** and **4a** and inhibitors **3b** and **4b** were synthesized in nine steps starting with 4-nitrophenethyl bromide (Scheme I). The dimethylaniline antigen **5a** was prepared in three steps starting with 4-aminobenzyl alcohol (Scheme II). Inhibitor **5b** was prepared in two steps starting from *p*-acetamidobenzyl alcohol. All three haptens were coupled (via the *N*-hydroxysuccinimide ester) to the carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). 129G1X mice were immunized with the KLH conjugate of **3a**, **4a**, and **5a** and produced 18, 22, and 26 hybridomas, respectively. All monoclonals were of the IgG class and were purified as previously described.[7c] Antibodies were judged to be homogeneous by SDS (sodium dodecyl sulfate)–polyacrylamide gel electrophoresis.

Esterolytic Assay. Antibodies at a concentration of 20 μM were initially screened (phosphate buffer, 50 mM, pH 7.5, 100 mM NaCl, 37 °C) via an HPLC assay against benzoate ester **6** (1 mM) for the production of 5-[(4-hydroxyphenyl)amino]-5-oxopentanoic acid. From the 18 monoclonals obtained to **3**, three were found to be catalytic. The 22 and 26 antibodies obtained from immunizations with haptens **4** and **5**, respectively, showed a *negligible* or an inhibitory effect on the spontaneous rate of hydrolysis of **6**. The three antibodies found to be catalytic were completely inhibited by the presence of 50 μM of carboxylate **3b**.

Kinetics and Inhibition Data. The kinetics of the most efficient catalytic antibody (27A6) obtained from immunizations with hapten **3** was characterized in detail.[9] The initial rate of hydrolysis of **6** (50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), 100 mM NaCl, pH 8.5, 37 °C) catalyzed by 27A6 followed Michaelis–Menten kinetics with values of k_{cat}^{app} and K_m of 0.01 ± 0.002 min^{-1} and (243 ± 15) × 10^{-6} M, respectively. The antibody-catalyzed hydrolysis of benzoate **6** was competitively inhibited (K_i = 6 ± 2 μM) by the addition of carboxylate **3b**.

pH–Rate Profile. The pH dependence of the hydrolysis of **6** was examined in the presence of 27A6 (20 μM) between pH 7.2–8.4 (EPPS) and pH 8.4–10.0 (2-(cyclohexylamino)ethanesulfonic acid, (CHES) (50 mM buffer and 100 mM NaCl) at 37 °C (Figure 2). The pH dependence of k_{cat}^{app} was linear in this region, as was the background rate of hydrolysis (log k_{obsd}). The rate of hydrolysis for ester **6** extrapolated to zero buffer concentration (k_{OH^-} = 2.3 × 10^{-2} min^{-1}) (Figure 2). The variation of the buffer concentration (12.5–50 mM) in the presence of 27A6 indicated no dependence of k_{cat}^{app} on the concentration of buffer

Table II. Antibodies Induced to **2a** and Their Binding to **2b**, and **6**[a]

antibody	isotype	K_D(2b),[b] M	K_D(6),[c] M
6F6	$K\gamma_1$	1.2×10^{-5}	1.7×10^{-5}
20G4	$K\gamma_1$	3.8×10^{-8}	2.5×10^{-4}
25B6	$K\gamma_{2a}$	6.0×10^{-8}	$>3 \times 10^{-3}$
34A4	$K\gamma_1$	1.2×10^{-5}	0.8×10^{-5}
34C9	$K\gamma_{2a}$	4.5×10^{-9}	1.2×10^{-5}
35G2	KY_{2a}	1.9×10^{-8}	2.2×10^{-5}
40D3	KY_{2a}	2.5×10^{-7}	$>3 \times 10^{-3}$

[a] Binding determined at 37 °C in 10 mM pH 7.2 phosphate containing 150 mM NaCl and 5% Blotto.[24] [b] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **2a** by added **2b**.[10] [c] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **2a** by added **6**.

Table III. Antibodies Induced to **3a** and Their Binding to **3b**, **6**, and **7**[a]

antibody	isotype	K_D(6),[b] M	K_D(6),[c] M	K_D(7),[d] M
27A6*[e]	$K\gamma_{2a}$	4.0×10^{-6}	2.0×10^{-3}	2.2×10^{-3}
57G11*	$K\gamma_1$	4.0×10^{-5}	1.5×10^{-3}	1.0×10^{-3}
52D11	$K\gamma_1$	1.6×10^{-5}	1.8×10^{-3}	0.9×10^{-3}
60A4	$K\gamma_1$	3.0×10^{-5}	0.6×10^{-3}	1.5×10^{-3}
18G10	$K\gamma_1$	2.0×10^{-5}	1.3×10^{-3}	1.4×10^{-3}
53F8	$K\gamma_1$	2.2×10^{-5}	$>3 \times 10^{-3}$	$>3 \times 10^{-3}$
20G10	$K\gamma_1$	3.2×10^{-5}	$>3 \times 10^{-3}$	1.1×10^{-3}

[a] Binding determined at 37 °C in 10 mM pH 7.2 phosphate containing 150 mM NaCl and 5% Blotto.[24] [b] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **3a** by added **3b**.[10] [c] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **3a** by added **6**. [d] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **3a** by added **7**. [e] Asterisk denotes catalytic antibody.

Table IV. Antibodies Induced to **4a** and Their Binding to **4b** and **6**[a]

antibody	isotype	K_D(4b),[b] M	K_D(6),[c] M
2G4	$K\gamma_1$	6.0×10^{-8}	1.3×10^{-4}
4E3	$K\gamma_1$	6.8×10^{-8}	6.5×10^{-5}
6A11	$K\gamma_1$	3.9×10^{-8}	2.5×10^{-4}
1C8	$K\gamma_1$	3.8×10^{-8}	6.3×10^{-5}
2H4	$K\gamma_1$	6.1×10^{-7}	1.5×10^{-3}
11H2	$K\gamma_1$	7.6×10^{-8}	7.5×10^{-4}
1B12	$K\gamma_1$	1.5×10^{-7}	5.0×10^{-4}

[a] Binding determined at 37 °C in 10 mM pH 7.2 phosphate containing 150 mM NaCl and 5% Blotto.[24] [b] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **4a** by added **4b**.[10] [c] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **4a** by added **6**.

Table V. Antibodies Induced to **5a** and Their Binding to **5b** and **6**[a]

antibody	isotype	K_D(5a),[b] M	K_D(6),[c] M
20C10	$K\gamma_1$	7.5×10^{-8}	0.2×10^{-3}
35D7	$K\gamma_1$	1.0×10^{-7}	2.0×10^{-3}
45C7	$K\gamma_1$	4.4×10^{-9}	1.5×10^{-3}
48C4	$K\gamma_{2a}$	7.0×10^{-9}	1.8×10^{-3}
51F1	$K\gamma_{2a}$	6.5×10^{-7}	1.8×10^{-4}
25BB	$K\gamma_1$	7.8×10^{-8}	$>3 \times 10^{-4}$
42B2	$K\gamma_1$	3.0×10^{-8}	3.5×10^{-4}

[a] Binding determined at 37 °C in 10 mM pH 7.2 phosphate containing 150 mM NaCl and 5% Blotto.[24] [b] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **5a** by added **5b**.[10] [c] Binding determined by measuring inhibition of antibody binding to surface-bound protein-conjugated **5a** by added **6**.

(8) For effective methods for the specific elicitation of amino acid(s) in an antibody combining site to be used in catalysis see: (a) Cochran, A. G.; Sugasawara, R.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 7888. (b) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Nature (London)* **1989**, *338*, 269.

(9) This antibody and 57G11 have been deposited at the American-type culture collection.

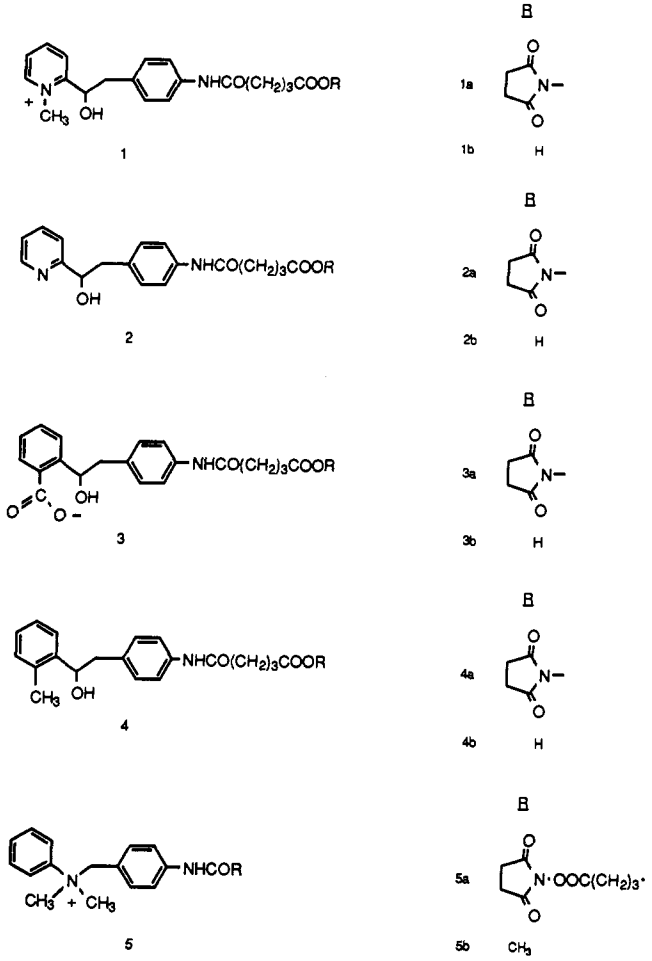


Figure 1. Structure of antigens (**1a**, **2a**, **3a**, **4a**, **5a**) and inhibitors (**1b**, **2b**, **3b**, **4b**, **5b**).

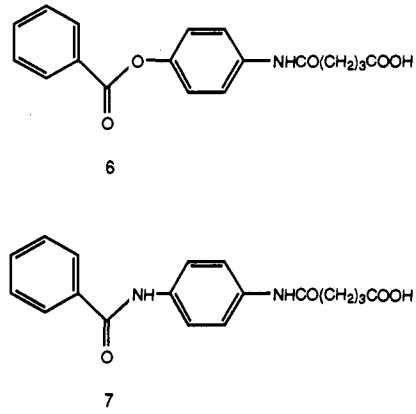


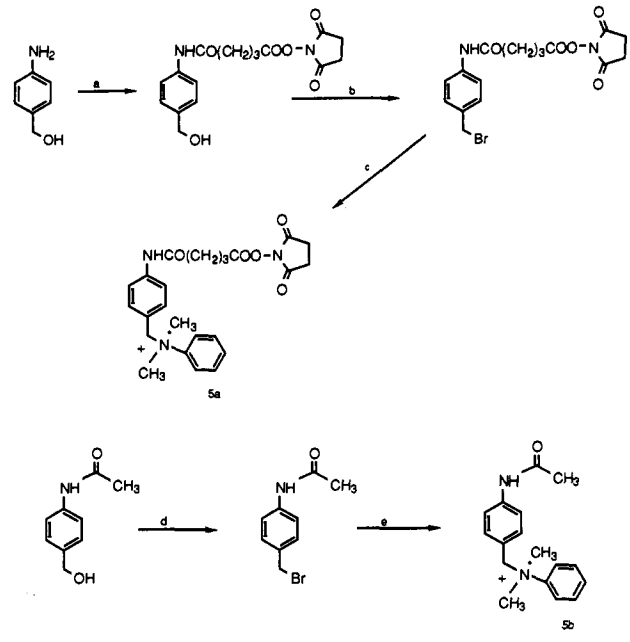
Figure 2. Structures of substrates.

species. There was no difference in the observed rates with antibody 27A6 when tested at pH 8.6 in EPPS and CHES (50 mM buffer, 100 mM NaCl).

Antibody Binding Constants. Competition ELISA[10] was used to determine dissociation constants for the antibodies and their respective haptens and substrates. The raw data obtained from ELISA experiments were fit to a nonlinear least-squares program developed by Dr. Alan Schwabacher.[11] Each dissociation constant was determined in triplicate, and the average of the three determinations was used as the final value reported in Tables I–V.

(10) Friguet, B.; Chaffotte, A. F.; Djauadi-ohaniance, C.; Goldberg, M. E. *Immunol. Methods* **1985**, *77*, 305.
(11) Schwabacher, A. W.; Weinhouse, M. I.; Auditor, M. T. M.; Lerner, R. A. *J. Am. Chem. Soc.* **1989**, *111*, 2344.

Scheme II.[a] Synthesis of Antigen **5a** and Inhibitor **5b**



[a] Key: (a) $COCl(CH_2)_3COON(COCH_2)_2$, Et_3N, CH_2Cl_2, room temperature, 1 h, 90%; (b) dibromotriphenylphosphorane, DMF, 50 °C, 12 h, 50%; (c) *N,N*-dimethylaniline, CH_2Cl_2, room temperature, 1 h, 90%; (d) dibromotriphenylphosphorane, DMF, 60 °C, 2.5 h, 75%; (e) *N,N*-dimethylaniline, ethyl acetate, room temperature, 30 min, 85%.

Dissociation constants listed as greater than 3 mM suggest no binding. Affinity constants of greater than 3 mM were unattainable due to solubility limits of ester **6** or amide **7**. Under the assay conditions in which the competition ELISA's were performed, depletion of ester **6** was minimal and therefore no correction was needed.

Chemical Modification of Antibodies. The esterolytic activity of monoclonal antibody 27A6 was unaffected by treatment with diethyl pyrocarbonate or maleic anhydride in 50-fold molar excess to protein. Lysine residues can be in excess of 50 residues per antibody molecule. As such, sequestered critical residues may be missed by the level of modifying reagent used. We therefore treated antibody 27A6 with 200 molar excess of maleic anhydride. Under these conditions a 100% loss of catalytic activity and a 5-fold decrease in titer (ELISA) was observed. Identical treatment of antibody 27A6 in the presence of inhibitor **3** (5-fold excess to protein) resulted in no protection (i.e., 100% loss in catalytic activity and 5-fold drop in titer). Treatment of antibody 27A6 with phenylglyoxal (50-fold molar excess) resulted in a 75% loss of catalytic activity and a 4-fold drop in titer (binding to hapten **3**) as observed by ELISA. Identical treatment of the protein in the presence of inhibitor **3b** (5-fold molar excess to protein) resulted in only 35% loss of catalytic activity and no appreciable change in titer. Because of these findings, other catalytic and noncatalytic antibodies to haptens **3**, **4**, and **5** were chemically modified with phenylglyoxal in exactly the same manner as described above. Antibodies to **4** (2G4, 4E3, 2H4, 6A11, 1CB) and **5** (35D7, 45C7, 48C4, 42B2, 25B8) were unaffected (ELISA). In contrast, five antibodies induced with **3** (57G12, 60A4, 52D11, 70F3, 5G3) all showed some loss of binding (ELISA). The catalytic antibodies 57G12 and 70F3 showed a 4-fold decrease in titer, and antibodies 60A4, 52D11, and 5G3 displayed a 3-, 2-, and 1-fold drop in titer, respectively.

Discussion

Background. Two general methodologies in hapten design have emerged in the catalytic antibody field. The initial approach was based on transition-state theory. More recently, a tactic requiring antibody–hapten charge complementarity, what we term bait and switch catalysis, has been developed. Currently, a main interest in antibody catalysis lies in peptide hydrolysis.[12] We felt a good

starting point using the bait and switch strategy would be investigations into the hydrolysis of simple aryl esters. Our belief was that this simplified system should provide insight as to what role (if any) bait and switch catalysis could play in antibody-mediated hydrolysis.

Our strategy was based on the utilization of antibodies that were induced from a homologous series of haptens (Figure 1), each of which possessed a point charge in close proximity to, or in direct substitution for, the chemical group (ester) we wished to transform in the respective substrate (Figure 1). It was envisioned that antibodies raised to these haptens should possess amino acid residue(s) at the binding site having a charge complementary to the haptenic charge. The substrate ester **6** (Figure 2) will lack this charge, but will retain a similar structure (steric dimensions). Hence, the amino acid at the binding site will be free from its original charge stabilization role and can now serve as a potential general acid/base or a transition-state stabilizing element. Thus, a central tenet in this strategy lies in our ability to generate a chemical catalyst at the binding site.

While antibody–hapten charge complementarity was deemed essential for the overall success of the project, we believed two other issues of hapten design to be important: The first was to develop an adequate model of the acyl functionality to be hydrolyzed. The second necessitated the use of uncharged haptens. The first point was addressed by using a suitable acyl moiety isostere. We employed a hydroxylic group having a tetrahedral geometry that could serve as an adequate representation of the developing transition state. We intentionally left this position uncharged so that there would be no additional electrostatic effects. However, it is foreseeable that "second-generation haptens" might include a charged phosphorus group at this position. The uncharged haptens (i.e., **2** and **4** (Figure 1)) were needed as controls to ensure the validity of our hypothesis. They are virtually structurally identical to **1–3** but lack a charge.

Our previous communication showed the bait and switch strategy catalyzing acyl-transfer reactions to be useful when we employed the *N*-methylpyridinium salt **1a** for antibody induction.[7] With this hapten we found that 30% of the antibodies obtained were catalytic. While this number was impressive, more interesting was our finding that one of these abzymes employed the basic form of an ionizable group ($pK_a = 6.26 \pm 0.05$) in the catalytic process. In addition, the optimum pH of the antibody-catalyzed reaction was near neutrality, and the utilization of neutral hapten **2** showed no propensity to induce catalytic antibodies. To further expand the scope of bait and switch methodology in abzyme acyl-transfer reactions, we have synthesized haptens **3**, **4**, and **5** (Figure 1). Our hope was that each hapten would increase our understanding of antibody–hapten charge complementarity catalysis.

General Antibody Binding and Specificity. Antibody–antigen recognition (binding) is a complex array of intermolecular interactions and forces, which include hydrogen-bonding, apolar/hydrophobic/ionic interactions, van der Waals, and steric forces. Such interactions and forces will obviously play an important role in not only antigen but also prospective substrate binding. Because the nature of our study required a delicate interplay between charged/uncharged interactions and steric forces, we felt it necessary to determine the immunogenicity of our haptens along with antibody binding constants to their respective haptens and possible substrates. We believed these findings would serve three purposes. First, they would provide clear evidence that the lack of antibody catalysts (from a particular hapten) results from inappropriate active-site charge placement or lack of one rather than poor immune response. Second, they would demonstrate the detection of catalytic events was not missed because of insufficient substrate binding. Last, the information obtained would assist us in designing future catalysts, especially in the area of antibodies that could accelerate amide hydrolysis.

Because of the large set of antibodies examined in these study (110, five haptens), we felt it impractical to determine affinity constants for each antibody. As such, we chose a representative sample of catalytic as well as noncatalytic antibodies and measured binding constants for their respective antigens as well as ester substrate **6**. In addition, we determined affinity constants to benzamide **7** (Figure 2) with antibodies from haptens that yielded catalysts.

Haptens **1–5** (Figure 1) produced mice with antibody mouse titers well over 25 600 (before fusion); therefore, *all* haptens provided an extremely strong immunological response. These results substantiate that our hapten design is the driving force for obtaining antibody catalysts and the lack of catalytic antibodies produced to a particular hapten is not due to a weak immune response. The dissociation constants of haptens **1–5** and their respective antibodies were found to lie in the range 10^{-9}–10^{-4} M (Tables I–V). This range can be considered typical. However, it is interesting to note the binding constants obtained for antibodies induced to cationic haptens **1** and **5** (Tables I and V) displayed a large disparity among each set as a whole. Hapten **5**, which yielded no abzymes (see below), induced antibodies with tight binding constants (nanomolar), while **1**, which induced a large number of catalysts, presented us with antibodies displaying relatively weak affinity constants (mainly micromolar). Of the four antibody catalysts induced to **1** that we tested, all had dissociation constants above 20 μM!

Antibody dissociation constants to benzoate ester **6** were in the micro- to millimolar range, with a few antibodies showing no binding at all (maximum substrate concentration 3 mM). While these results are not surprising, they do provide good evidence of overall substrate binding to the majority of the antibodies and thus a low probability of our missing catalysis due to insufficient binding. Of note is that the best dissociation constants to **6** occurred among hapten **2** antibodies (Table II). Two of these antibodies displayed binding constants very similar to that seen with the antigen. It would be reasonable to assume that this lack of antibody discrimination is due to similar ionic interactions and steric forces that can operate equally well between either compound and these antibodies.

Finally, we probed for simple binding with benzamide **7**. Because of the structural differences between amide and ester molecules, it would be extremely informative to know if antibodies made to haptenic structures within our bait and switch regime could bind to a possible amide substrate. Such information would assist in our future design of compounds for the creation of antibody peptidases. From Tables I and III we see simple binding is observed with benzamide **7** and antibodies obtained to haptens **1** and **3**. In fact, dissociation constants are quite comparable to the ones obtained for ester **6**. While antibody–antigen interactions can show a high degree of specificity, it would appear that sufficient cross-reactivity is allowed among these chemically different structures.

Hapten 5. Hapten **5** was prepared as shown in Scheme II. The most notable feature of this molecule is the tetrahedral cationic charge that directly replaces the acyl carbon of the substrate. While this hapten might be considered even more of a radical departure from the typical phosphonate hapten surrogates, it should address a number of previously unanswered questions concerning our bait and switch strategy. Two of concern are the importance of the cationic charge, including its placement relative to the scissile bond of the substrate and the relevance of our acyl carbon replacement with the hydroxy isostere. From the 26 antibodies raised to hapten **5**, none were found to accelerate the rate of hydrolysis to any appreciable extent over the background rate. This result was quite intriguing in lieu of the fact that a similar antigen designed by Schultz showed a high propensity (66%) to induce catalytic antibodies for an elimination reaction.[8b] Although the reactions here are quite different, Schultz found compelling evidence that a carboxylate was involved in the catalytic process as we found using the methylpyridinium hapten **1** to induce antibodies for the esterolytic reaction. These results, in conjunction with our previous and current findings (catalytic and binding studies) for antibodies obtained to haptens **1** and **2**, suggest the following: (1) The rate enhancements seen with abzymes induced

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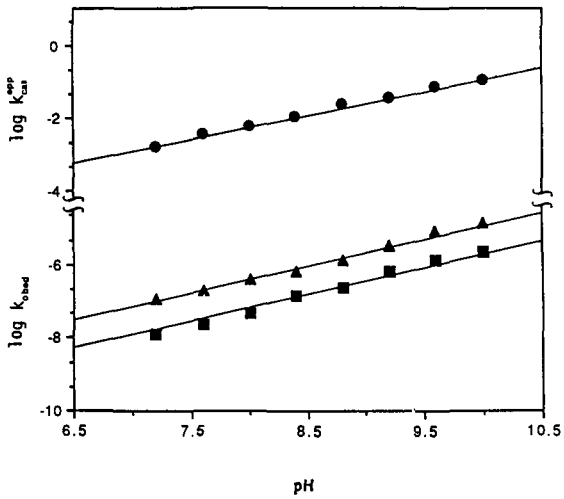

Figure 3. log k_{cat}^{app} vs pH plot (●) of antibody (27A6) catalyzed reaction of **6**. log k_{obsd} vs pH (▲) of **6**. log k_{obsd} vs pH (■) of **6** extrapolated to zero buffer concentration. The calculated line was obtained by using $k_{obsd} = k_{OH^-}[OH^-]$.

from hapten **1** are not solely due to the presence of a carboxylate acting as a catalytic base. (2) The functionality in the hapten that is used to represent the transition state is critical. (3) The combination of a cationic charge and at least a neutral representation of the transition state is required to induce hydrolytic abzymes.[13]

Haptens 3 and 4. Encouraged by results obtained using the cationic hapten **1**, we believed an analogous process could be realized with a structurally similar (steric dimensions) anionic hapten. The benzoic acid hapten **3a** filled the necessary requirements. Its backbone was homologous to antigens **1** and **2**, while possessing an anionic point charge in close proximity to the acyl moiety we planned to hydrolyze. Our choice of a carboxylate group was based on findings by Pressman indicating that this type of functionality within a haptenic molecule has a strong propensity to induce a positively charged amino acid (i.e., lysine or arginine) within the antibody binding pocket.[14] We believed either amino acid could assist in the catalytic process via general acid or electrostatic stabilization of a transition state. The latter process involving arginine residues has been implicated in enzyme catalysis.[15]

The (hydroxyethyl)benzoic acid **3a** was synthesized in nine steps (Scheme I) with an *N*-hydroxysuccinimide ester appended for ease of coupling to the protein carrier. Immunizations to the **3a**-KLH conjugate produced 18 monoclonal antibodies, three of which were catalytic and inhibited by free hapten **3b**. Although these results are not as good as those obtained with hapten **1a**, we were pleased to find that none of the 22 antibodies obtained to the neutral homologue of **3a** (**4**) were catalytic. Once again, we see the importance of the charged functionality contained within our antigen design.

Earlier observations on the pH–rate profile of mAb 30C6 (induced from **1a**) indicated the basic form of a dissociable group was involved in catalysis. Also noted was the independence of k_{cat}^{app} on the concentration of the buffer species as found with abzyme 27A6, which was induced from **3** (Figure 1). In contrast to the behavior of mAb 30C6, we observed a pH dependence of k_{cat}^{app} with abzyme 27A6 (Figure 3). While this appears to contradict the essence of our bait and switch theory, there exists the possibility that the pK_a of the residue(s) may lie outside the pH range investigated or that protein–substrate electrostatic interactions (electrostatic catalysis) are the essential feature of this abzyme's ability to accelerate the reaction.[16]

Chemical Modification of Antibodies. While we were unable to detect any amino acid involvement in 27A6 hydrolytic reaction via pH effects, we did see specific inactivation[17] of all three (27A6, 57G12, 70F3) catalytic antibodies through the use of the arginine-modifying reagent phenylglyoxal.[18] The loss of activity (catalytic/binding) can be interpreted as due to reaction of the reagent with an amino acid in the binding site; it can be reduced significantly by the presence of inhibitor **3b**. However, a conformation change following reaction of the reagent at a different site would lead to a similar conclusion. Thus, it is possible that an arginine residue somewhere other than in the binding site is chemically altered leading to stabilization of conformations of the protein in which the binding site is altered so that it no longer binds substrate **6** or hapten **3**. This complication does not seem to apply here. As we demonstrated through catalytic and ELISA assays (see Results) and previous binding studies noted by Freedman et al.[19] and Mayers et al.,[20] the glyoxalation of guanidinium groups destroys catalytic and/or binding activity *only* of antibodies against negatively charged haptens, and not of antibodies against neutral hapten **4** or positively charged hapten **5**. If glyoxalation exerted an effect by altering a guanidinium distant from the binding site by the above mechanism, it is difficult to see why antibodies to the **4** or **5** haptens would not be similarly affected.

Conclusion

A bait and switch methodology can be a powerful tool for obtaining catalytic antibodies with acyl-transfer capabilities. Five haptens of similar structure, each of which featured a point charge or lack of one, were used to induce antibodies. Our results strongly implicate amino acid functional group participation within the binding pocket of the antibodies. Furthermore, these findings begin to define what properties the immune system can make available to assist in our ultimate goal of antibody-catalyzed peptide hydrolytic reactions. The production of abzymes as hydrolytic catalysts that utilize participation of amino acid side chains (as general acids/bases/nucleophiles or for electrostatic stabilization) is developing a sound foundation.

Experimental Section

General Methods (Synthesis). Unless otherwise noted, reactions were carried out in flame-dried glassware under an atmosphere of nitrogen. Reagent and solvent transfers were made with oven-dried syringes and needles. Dichloromethane and acetonitrile were continuously distilled from calcium hydride. Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone ketyl. All reagents were purchased from Aldrich Chemical Co. All chromatography solvents were obtained commercially and used as received. Reactions were monitored by analytical thin-layer chromatographic methods (TLC) with the use of E. Merck silica gel 60F glass plates (0.25 mm). Flash chromatography was carried out with the use of E. Merck silica gel 60 (230–400 mesh) as described by Still.[21] Benzoate substrate **6** and haptens **1** and **2** were synthesized as described previously.[7d]

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. All proton NMR spectra (300 MHz) were obtained in $CDCl_3$, CD_3CN, or DMSO solutions at ambient temperature on a Bruker AM-300 spectrometer, chemical shifts (δ) are reported in

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(17) Complete inactivation was also observed with antibody 27A6 and maleic anhydride (200 molar excess); see Results. However, we ascribe this loss in catalysis and binding *not* to modification of the binding pocket but rather to denaturation of the protein. This is based on the finding that **3b** had no effect on blocking the loss of catalytic activity or binding (ELISA).

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parts per million relative to internal tetramethylsilane (0.00 ppm), elemental analyses (C, H, N) were performed by Galbraith Laboratories, Knoxville, TN.

2-[(4-Nitrophenyl)methyl]-1,3-dioxolane (8). Ethylene glycol (5.4 mL, 97 mmol) was added to a stirred solution of *p*-nitrophenylacetaldehyde[22] (3.0 g, 18.2 mmol) in 10.0 mL of methylene chloride. To this were added 1.0 g of Rexyn 101 (H) (Fisher Scientific) cation-exchange resin and 3.0 g of powdered calcium sulfate, which had been oven dried (120 °C) overnight. The mixture was stirred for 24 h at room temperature, and subsequently, the reaction mixture was poured into 100 mL of H_2O and extracted three times with 50-mL portions of methylene chloride. Combined organic layers were dried with sodium sulfate and purified by flash chromatography (2:1 hexanes–ethyl acetate), yielding 2.28 g (60%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 8.18 (d, J = 8.6 Hz, 2 H), 7.42 (d, J = 8.6 Hz, 2 H), 5.08 (t, J = 4.3 Hz, 1 H), 4.0–3.8 (m, 4 H), 3.06 (d, J = 4.3 Hz, 2 H). Anal. Calcd for $C_{10}H_{11}NO_4$: C, 57.42; H, 5.26; N, 6.70. Found: C, 57.51; H, 5.19; N, 6.65.

4-(1,3-Dioxolan-2-ylmethyl)benzenamine (9). Compound **8** (2.0 g, 9.6 mmol) was added to 20 mL of methanol. To this suspension was added 10% palladium on activated carbon (200 mg), and the flask was fitted with a balloon of hydrogen and stirred rapidly at room temperature for 6 h. The reaction mixture was filtered through Celite and concentrated, yielding 1.63 g (95%) of the theoretical amount. This material was then employed in the subsequent step: TLC, R_f = 0.3 (1:1 ethyl acetate–hexanes).

***N*-[4-(1,3-Dioxolan-2-ylmethyl)phenyl]-*N*-(phenylmethyl)benzenemethanamine (10).** To a stirred suspension of **9** (2 g, 11.2 mmol) in 10 mL of methylene chloride was added triethylamine (4.5 mL, 32 mmol). Addition of benzyl bromide (6.4 mL, 107 mmol) was done dropwise over 30 min with rapid stirring; this was additionally allowed to stir for an extra 60 min. The reaction mixture was diluted with methylene chloride (50 mL) and extracted with (2 × 25 mL) 0.5 M HCl. The combined organic extracts were dried with sodium sulfate and purified by flash chromatography in 4:1 methylene chloride–hexanes, yielding 3.2 g (80%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 7.35–7.23 (m, 10 H), 7.06 (d, J = 8.6 Hz, 2 H), 6.66 (d, J = 8.6 Hz, 2 H), 5.50 (t, J = 4.3 Hz, 1 H), 4.62 (s, 4 H), 4.0–3.8 (m, 4 H), 2.82 (d, J = 4.3 Hz, 2 H). Anal. Calcd for $C_{24}H_{25}NO_2$: C, 80.22; N, 6.96; N, 3.90. Found: C, 80.35; H, 7.11; N, 3.86.

4-[Bis(phenylmethyl)amino]benzeneacetaldehyde (11). To a solution of 2.0 g (5.6 mmol) of **10** in 15 mL of acetone was added 2 mL of 4 M HCl. This solution was allowed to stir 24 h at room temperature, whereupon 10 g of silica was added to the reaction mixture which was concentrated dryness. Flash chromatography was run in 4:1 methylene chloride–hexanes with the preadsorbed crude yielding 0.7 g (40%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 9.7 (t, J = 2.9 Hz, 1 H), 7.40–7.20 (m, 10 H), 7.00 (d, J = 8.6 Hz, 2 H), 6.7 (d, J = 8.6 Hz, 2 H), 4.66 (s, 4 H), 3.54 (d, J = 2.9 Hz, 2 H). Anal. Calcd for $C_{22}H_{21}NO$: C, 83.81; H, 6.67; N, 4.44. Found: C, 84.06; H, 6.58; N, 4.50.

2-[2-(4-Aminophenyl)-1-hydroxyethyl]benzoic Acid (12). 2-Bromobenzoic acid (957 mg, 4.8 mmol) was dissolved in 20 mL of tetrahydrofuran and the resultant solution cooled to −78 °C (CO_2/acetone). *n*-Buli (5.8 mM, 1.6 M in hexanes, 9.2 mmol) was added and the mixture stirred for 1 h. Aldehyde **11** (1.0 g, 3.2 mmol) dissolved in 10 mL of tetrahydrofuran cooled to −78 °C was added via cannula and the resultant solution stirred for 4 h at −78 °C. The reaction mixture was poured into saturated ammonium chloride followed by extraction with 2 × 50 mL of ethyl acetate. The combined organic extracts were dried with sodium sulfate and purified by flash chromatography using neat methylene chloride, yielding 860 mg (62%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 7.90–7.80 (m, 1 H), 7.68–7.40 (m, 2 H), 7.40–7.05 (m, 11 H), 7.0 (d, J = 8.6 Hz, 2 H), 6.64 (d, J = 8.6 Hz, 2 H), 5.62 (t, J = 7.1 Hz, 2 H), 3.64 (br s, 2 H), 3.4–2.8 (m, 2 H). Anal. Calcd for $C_{29}H_{27}NO_3$: C, 80.37; H, 6.24; N, 3.23. Found: C, 80.44; H, 6.29; N, 3.19.

2-[2-[4-[Bis(phenylmethyl)amino]phenyl]-1-hydroxyethyl]benzoic Acid (13). Carboxylate **12** (320 mg, 7.3 × 10^{-4} mol) was dissolved in 20 mL of methanol. This was followed by the addition of 10% palladium on activated carbon (32 mg), charging of the flask with hydrogen, and rapid stirring for 90 min. Filtration through Celite followed by concentration yielded 179 mg (95%) of the theoretical amount. This material was employed without further purification in the subsequent step: TLC, R_f = 0.6 (1:1 methylene chloride–ethyl acetate).

2-[2-[4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]-phenyl]-1-hydroxyethyl]benzoic Acid (3a). Carboxylate **13** (100 mg, 3.9 × 10^{-4} mol) was dissolved in 800 μl of methylene chloride. Triethylamine (109 μl, 7.8 × 10^{-4} mol) was added followed by 5-[(2,5-dioxo-1-pyrrolidinyl)oxy]-5-oxopentanoyl chloride (118 mg, 5.1 × 10^{-4} mol) and stirring for 20 min. Purification was performed by loading the crude reaction mixture onto a flash chromatography column and eluting with 1:1 methylene chloride–ethyl acetate, yielding 159 mg (90%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 9.24 (s, 1 H), 7.36–6.8 (m, 6 H), 6.42 (d, J = 8.6 Hz, 2 H), 5.20 (t, J = 7.1 Hz, 1 H), 2.66–2.36 (m, 2 H), 2.15 (5, 4 H), 2.1–1.96 (m, 2 H), 1.96–1.7 (m, 2 H), 1.5–1.16 (m, 2 H). Anal. Calcd for $C_{24}H_{24}N_2O_8$: C, 61.54; H, 5.13; N, 5.98. Found: C, 61.62; H, 5.10; N, 5.89.

2-[2-[4-[(4-Carboxy-1-oxobutyl)amino]phenyl]-1-hydroxyethyl]benzoic Acid (3b). To a stirred suspension of **13** (41 mg, 0.16 mmol) in 5 ml of methylene chloride was added triethylamine (48 μl, 0.34 mmol). This was followed by the addition of glutaric anhydride (27 mg, 23 mmol), stirring overnight at room temperature, and evaporation of the reaction mixture to dryness. The reaction mixture was dissolved in 1:1 acetonitrile–water, and purification was performed by FPLC (Pharmacia pro RPC 15/10 column): CH_3CN–H_2O (0.1% TFA), 10:90 to 90:10 gradient, 4 mL/min, 15 min, retention time 10 min; yield 47.5 mg (80%) of the theoretical amount; ^{1}H NMR (CD_3CN) δ 8.28 (s, 1 H), 7.8–7.5 (m, 4 H), 7.45 (d, J = 9 Hz, 2 H), 7.10 (d, J = 9 Hz, 2 H), 5.78 (t, J = 4.5 Hz, 1 H), 3.4–3.1 (m, 2 H), 2.38 (t, J = 3 Hz, 4 H), 1.9 (M, 2 H). Anal. Calcd for $C_{20}H_{21}NO_6$: C, 64.69; H, 5.66; N, 3.77. Found: C, 64.75; H, 5.71; N, 3.80.

2-[2-(4-Aminophenyl)-1-hydroxyethyl]-1-methylbenzene (14). *n*-BuLi (2.8 mL, 1.6 M in hexanes, 4.4 mmol) was added to 30 mL of tetrahydrofuran and the resultant mixture cooled to −78 °C (CO_2/acetone). To this solution was added 2-bromotoluene (0.573 mL, 4.8 mmol), and the resultant mixture allowed to stir 30 min. Aldehyde **11** (1.0 g, 3.2 mmol), dissolved in 15 mL of tetrahydrofuran and cooled to −78 °C, was next added and stirred for 2 h. The reaction mixture was poured into a saturated ammonium chloride solution and extracted with 2 × 25 mL of methylene chloride. The combined organic layers were dried with sodium sulfate and purified by flash chromatography in 6:1:1.5 hexanes–methylene chloride–ethyl acetate, yielding 900 mg (69%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 7.9–7.8 (m, 1 H), 7.68–7.40 (m, 2 H), 7.40–7.15 (m, 12 H), 7.05 (d, J = 8.6 Hz, 2 H), 6.70 (d, J = 8.6 Hz, 2 H), 5.3 (t, J = 7.1 Hz, 1 H), 4.62 (s, 4 H), 3.0–2.7 (m, 2 H), 2.3 (s, 3 H). Anal. Calcd for $C_{29}H_{29}NO$: C, 85.50; H, 7.13; N, 3.44. Found: C, 85.58; H, 7.08; N, 3.41.

2-[2-[4-[Bis(phenylmethyl)amino]phenyl]-1-hydroxyethyl]-1-methylbenzene (15). To a solution of **14** (900 mg, 2.2 mmol) in 50 mL of ethyl acetate was added 10% palladium on activated carboh (100 mg). The reaction vessel was pressurized to 50 psi with hydrogen on a Parr hydrogenation apparatus. The reaction was complete after 4 h, filtered through Celite, and concentrated in vacuo, yielding 476 mg (95%) of the theoretical amount. This material was used without further purification in the next step: TLC, R_f = 0.05 (4:1 methylene chloride–hexanes).

2-[2-[4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]-phenyl]-1-hydroxyethyl]-1-methylbenzene (4a). To a solution of **15** (476 mg, 2.1 mmol) in 10 mL of methylene chloride containing triethylamine (351 μl, 2.5 mmol) was added 5-[(2,5-dioxo-1-pyrrolidinyl)oxy]-5-oxopentanoyl chloride (572 mg, 2.3 mmol). The solution stirred 30 min after which time it was diluted with ethyl acetate (25 mL), washed with 1 M HCl (2 × 15 mL), and dried with sodium sulfate. The crude material was purified by flash chromatography (1:1 methylene chloride–ethyl acetate), yielding 780 mg (85%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 9.24 (s, 1 H), 7.4–6.85 (m, 6 H), 6.45 (d, J = 8.6 Hz, 2 H), 5.20 (t, J = 7.1 Hz, 1 H), 2.65–2.4 (m, 2 H), 2.3 (s, 3 H), 2.15 (s, 4 H), 2.1–1.96 (m, 2 H), 1.96–1.72 (m, 2 H), 1.5–1.20 (m, 2 H). Anal. Calcd for $C_{24}H_{26}N_2O_6$: C, 65.75; H, 5.94; N, 6.39. Found: C, 65.79; H, 5.91; N, 6.41.

5-[[4-[2-Hydroxy-2-(2-methylphenyl)ethyl]phenyl]amino]-5-oxopentanoic Acid (4b). To a stirred solution of **15** (51 mg, 0.23 mmol) in 0.5 mL of methylene chloride was added glutaric anhydride (26 mg, 0.23 mmol). The reaction was complete in 4 h (room temperature), and the crude reaction mixture was evaporated to dryness and redissolved in 1:1:2 methanol–acetonitrile–water for FPLC purification (Pharmacia pro RPC 15/10 column): CH_3CN–H_2O (0.1% TFA), 10:90 to 90:10 gradient, 4 mL/min, 15 min, retention time 13 min. After purification, **4b** was obtained as a clear oil, 73 mg (93%) of the theoretical amount: ^{1}H NMR (CD_3CN) δ 8.28 (s, 1 H), 7.55–7.4 (m, 5 H), 7.28–7.15 (m, 5 H), 5.05 (m, 1 H), 2.95–2.8 (m, 2 H), 2.35 (t, J = 7.5 Hz, 4 H), 2.20 (s, 3 H), 1.9–1.8 (m, 2 H). Anal. Calcd for $C_{20}H_{23}NO_4$: C, 70.38; H, 6.74; N, 4.22. Found: C, 70.46; H, 6.77; N, 4.04.

5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-*N*-[4-(hydroxymethyl)phenyl]-5-oxopentanamide (16). To a solution of triethylamine (1.13 mL, 8.2 mmol) in 10 mL of methylene chloride was dissolved *p*-aminobenzyl alcohol (1.0 g, 8.1 mmol). To this was added 5-[(2,5-dioxo-1-

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pyrrolidinyl)oxy]-5-pentanoyl chloride (2.21 g, 8.9 mmol) and the resultant mixture stirred for 1 h. The reaction mixture was diluted with methylene chloride (25 mL) and extracted with 2 × 15 mL of a 1 M HCl solution. The resultant organic layer was dried with sodium sulfate and purified by flash chromatography in 9:1 methylene chloride–methanol, yielding 2.43 g (90%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 8.0 (br s, 2 H), 7.52 (d, J = 8.6 Hz, 2 H), 7.30 (d, J = 8.6 Hz, 2 H), 4.62 (s, 2 H), 3.45 (s, 1 H), 2.9 (s, 4 H), 2.75 (t, J = 10 Hz, 2 H), 2.5 (t, J = 10 Hz, 2 H), 2.38–2.10 (m, 2 H). Anal. Calcd for $C_{16}H_{18}N_2O_6$: C, 57.49; H, 5.39; N, 8.38. Found: C, 57.42; H, 5.44; N, 8.29.

***N*-[4-(Bromomethyl)phenyl]-5-[(2,5-dioxo-1-pyrrolidinyl)oxy]-5-oxopentanamide (17).** To a solution of **16** (2.9 g, 6 mmol) in 20 mL of dimethylformamide was added dibromotriphenylphosphorane (3.04 g, 7.2 mmol); this was subsequently warmed to 50 °C and left to stir for 4 h. This reaction was diluted with 1 L of ethyl acetate, extracted with brine (4 × 200 mL), and dried over sodium sulfate. Purification via flash chromatography (1:1 ethyl acetate–methylene chloride) yielded 1.20 g (50%) of the theoretical amount: ^{1}H NMR ($CDCl_3$) δ 8.0 (br s, 1 H), 7.50 (d, J = 8.6 Hz, 2 H), 7.30 (d, J = 8.6 Hz, 2 H), 4.5 (s, 2 H), 2.9 (s, 4 H), 2.75 (t, J = 10 Hz, 2 H), 2.5 (t, J = 20 Hz, 2 H), 2.38–2.10 (m, 2 H). Anal. Calcd for $C_{16}H_{17}N_2O_5$: C, 58.72; H, 5.20; N, 8.56. Found: C, 58.66; H, 5.24; N, 8.50.

***N*-[4-(Bromomethyl)phenyl]acetamide (18).** To a solution of *p*-acetamidobenzyl alcohol (1 g, 6 mmol) in 5 mL of dimethylformamide under a nitrogen atmosphere was added dibromotriphenylphosphorane (5 g, 12 mmol). The reaction was warmed to 60 °C and stirred for 2.5 h. The crude reaction was diluted with ethyl acetate (30 mL) and washed with water (3 × 25 mL). The organic layer was separated, dried with sodium sulfate, and purified by flash chromatography (4:1 methylene chloride–hexanes) yielded 1.03 g (75%) of the theoretical amount: ^{1}H NMR (CD_3CN) δ 8.45 (s, 1 H), 7.58 (d, J = 8.6 Hz, 2 H), 7.38 (d, J = 8.6 Hz, 2 H), 4.60 (s, 2 H), 2.10 (s, 3 H). Anal. Calcd for $C_9H_{10}NOBr$: C, 47.37; H, 4.39; N, 6.14. Found: C, 47.45; H, 4.37; N, 6.21.

4-[[5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-1,5-dioxopentyl]amino]-*N*,*N*-dimethyl-*N*-phenylbenzenemethanaminium Bromide (5a). Bromide **17** (1.0 g, 2.5 mmol) was dissolved in 60 mL of methylene chloride, followed by the addition of dimethylaniline (1.6 mL, 12.5 mmol). The solution stirred for 30 min upon which time a precipitate formed; stirring continued for 4 h. The suspension was transferred to a separating funnel and extracted with 3 × 30 mL of distilled water. The combined aqueous washes were lyophilized to obtain 1.17 g of product (90% of the theoretical amount): ^{1}H NMR (D_2O) δ 7.60 (s, 5 H), 7.38 (d, J = 8.6 Hz, 2 H), 7.0 (d, J = 8.6 Hz, 2 H), 4.95 (s, 2 H), 3.62 (s, 6 H), 2.9 (s, 4 H), 2.75 (t, J = 10 Hz, 2 H), 2.5 (t, J = 10 Hz, 2 H), 2.38–2.10 (m, 2 H). Anal. Calcd for $C_{24}H_{28}N_3O_5Br$: C, 55.60; H, 5.41; N, 8.11. Found: C, 55.67; H, 5.39; N, 8.07.

4-(Acetylamino)-*N*,*N*-dimethyl-*N*-phenylbenzenemethanaminium Bromide (5b). To a solution of **18** (386 mg, 1.6 mmol) in 5 mL of ethyl acetate was added *N*,*N*-dimethylaniline (2 ml, 16 mmol). The reaction mixture was stirred 30 min at room temperature, after which the reaction was diluted with ethyl acetate (10 mL) and extracted with water (2 × 10 mL). The water was lyophilized, and the crude product was diluted in 1:1 acetonitrile–water and purified by FPLC (Pharmacia pro RPC 15/10 column): CH_3CN–H_2O (0.19% TFA), 10:90 to 90:10 gradient, 4 mL/min, 15 min, retention time 9.5 min. The final yield was 475 mg of a yellow oil (85% of the theoretical amount): ^{1}H NMR (CD_3CN) δ 9.6 (s, 1 H), 7.7–7.5 (m, 7 H), 6.95 (d, J = 8.2 Hz, 2 H), 4.85 (s, 2 H), 3.55 (s, 6 H), 2.02 (s, 3 H). Anal. Calcd for $C_{17}H_{21}N_2OBr$: C, 58.45; H, 6.02; N, 8.02. Found: C, 58.56; H, 6.10; N, 7.98.

5-[[4-(Benzoylamino)phenyl]amino]-5-oxopentanoic Acid (7). To a stirred solution of *p*-aminobenzanilide (K&K Laboratories) (1 g, 4.7 mmol) in 20 mL of tetrahydrofuran was added glutaric anhydride (.645 g, 5.7 mmol). After 20 min, a precipitate formed. This suspension was stirred 2 h for complete reaction. The precipitate formed was filtered, rinsed with methylene chloride, and recrystallized from methanol–water, yielding 1.38 g (90%) of the theoretical amount: ^{1}H NMR (DMSO-d_6) δ 10.28 (s, 1 H), 10.05 (s, 1 H), 7.95 (J = 3.3 Hz, d, 2 H), 7.70 (J = 9 Hz, d, 2 H), 7.60–7.45 (m, 5 H), 2.45 (t, J = 6 Hz, 2 H), 2.38 (t, J = 6 Hz, 2 H), 1.9 (m, 2 H). Anal. Calcd for $C_{18}H_{18}N_2O_4$: C, 66.26; H, 5.52; N, 8.59. Found: C, 66.44; H, 5.65; N, 8.51.

Antibody Production and Assays. The conjugate was prepared by slowly adding 2.5 mg of **3a**, **4a**, or **5a** in 250 μL of dimethylformamide (DMF) to 2 mg of KLH in 750 μL of 0.01 M sodium phosphate buffer, pH 7.2, while the solution was stirred at 4 °C for 1 h. Four 8-week-old mice each received an intraperitoneal (ip) injection of 100 μg of **3a**, **4a**, or **5a** conjugated to KLH and emulsified in a complete Freund's adjuvant, followed by an ip injection of 50 μg of the **3a**–, **4a**–, or **5a**–KLH conjugate in alum 2 weeks later. One month after the second injection, the mouse with the highest antibody titer to **3**, **4**, or **5** (25 600) was injected intravenously with 50 μg of the **3a**–, **4a**–, and **5a**–KLH conjugate; 3 days later the spleen was taken for the preparation of hybridomas. Spleen cells (1.0×10^8) were fused with 2.0×10^7 SP2/0 myeloma cells. Cells were plated into forty-five 96-well plates; each well contained 150 μL of (hypoxanthine–aminopterin–thymidine)–Dulbecco's minimal essential medium (HAT–DMEM) containing 1% nutridoma and 2% BSA.

After 2 or 3 weeks, the antibodies produced by wells containing macroscopic colonies were assayed by ELISA for binding to **3**, **4**, or **5**. Colonies that initially produced antibodies that bound to **3**, **4**, or **5** were subcloned twice after which 18 for **3**, 22 for **4**, and 26 for **5** remained active. All monoclonal antibodies were injected into pristane-primed BALB/c × 129GIX+ mice to generate ascitic fluid. For studies, IgG was purified from the ascitic fluid by salt precipitate and anion-exchange chromatography (DEAE) and affinity chromatography (protein G); each purified monoclonal antibody was dialyzed into 50 mM phosphate (100 mM NaCl, pH 7.5). Antibodies were judged to be homogeneous (>95%) by sodium dodecyl sulfate–polyacylamide gel electrophoresis.

Kinetic Measurements. Purified monoclonal antibody was dialyzed against EPPS buffer (1 mM, pH 8.00, 100 mM NaCl) or CHES buffer (1 mM, pH 8.0, 100 mM NaCl). Its protein concentration was determined by the BCA method (Pierce). Assays were performed by HPLC (reversed-phase column, C_{18}, VYDAC 201TP54) with CH_3CN–H_2O (0.1% TFA) on an isocratic program of 10:90. An internal standard of *o*-acetamidophenol was used to calculate the amount of 5-[(4-hydroxyphenyl)amino]-5-oxopentanoic acid formed.

Antibody stock solutions were diluted into 1 mL of the appropriate buffer (50 mM, EPPS (pH 7.2–8.6), CHES (pH 8.6–10.0), 100 mM NaCl) to give a final protein concentration of 20 μM. All reactions contained 5% cosolvent dioxane, and the temperature was maintained at 37 ± 0.1 °C. Initial linear rates were measured at <5% hydrolysis of the total substrate. All antibodies tested were found to be stable for at least 48 h under reaction conditions as determined by ELISA binding assays. The observed rate was corrected for the uncatalyzed rate of hydrolysis in the absence of antibody. Kinetic parameters V_{max} and K_M were determined by nonlinear least-squares fitting of the initial rate vs substrate concentration to a hyperbolic curve described by the Michaelis–Menten equation.

The variation of initial rates as a function of pH was measured in CHES (50 mM) (100 mM NaCl) at pH above 8.6 and in EPPS (50 mM) (100 mM NaCl) otherwise. There was no difference in the observed rates with antibody 27A6, when tested at pH 8.6 in EPPS and CHES (50 mM) (100 mM NaCl). Variation of the buffer ion concentration (12.5–50 mM) showed no dependency of k_{cat} (antibody 27A6) on the presence of the buffer species.

Equation 1 describes the pH–rate profile obtained for the rate of hydrolysis (k_{obsd}) of **6** extrapolated to zero buffer concentration.[23] The

$$k_{obsd} = k_{OH^-}[OH^-] \quad (1)$$

line (Figure 2) was generated by varying the concentration of buffer (12.5–50 mM) at a fixed concentration of **6** (400 μM) over the pH range 7.2–10.0. The buffers employed and their pH range tested were exactly the same as described above. The value of k_{OH^-} may be calculated from the slope of a plot of k_{obs} vs K_W/a_H.

Antibody Binding Constants. Competition ELISA[10] was used to determine dissociation constants for the antibodies and the respective haptens and substrates. Before the competitive ELISA procedure can be run on each antibody, simple ELISA must be used to determine the smallest amount of antigen (BSA–hapten) and antibody that gives a good signal to noise ratio (sigmoidal curves of absorbance plotted vs log of concentration) and linear response in the "linear" region when absorbance is plotted verses concentration. Typically the concentration of antibody used is an amount below which linear response is obtained and all subsequent competition ELISA's are performed with use of these initial antigen/antibody amounts.

In a typical simple ELISA a BSA–hapten conjugate (2.5 mg/mL) is diluted 1:1000 in phosphate buffer salt (PBS, 10 mM pH 7.2 phosphate, 150 mM NaCl). A 50-μL portion of the diluted BSA–hapten conjugate is placed in wells A1–H1 of an ELISA plate (Costar 3696, half-area plates). These are then serially diluted across the plate, which already contains 25 μL of PBS/well. A methanol fix is performed (50 μL of CH_3OH/well) for 5 min. The methanol is removed by simple shaking and the well allowed to air-dry for 10 min. The plate is blocked by Blotto[24] (5% nonfat dry milk–PBS, 50 μL/well) at 37 °C for 30 min. The antibody can now be titered, first by removing the Blotto (simple shaking) and then adding 25 μL/well of Blotto–5% DMF, followed by

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25 μL of the hybridoma supernatent into rows A1-A12, and is serially diluted 2-fold down the plate. The plate was allowed to incubate for 2 h at 37 °C, which was then followed by a deionized water wash/shaking (10 times). All wells were next treated with 25 μL of a goat anti-mouse glucose oxidase (Cappel) solution, which was diluted previously 1:500 in Blotto. The plate was allowed to incubate for 1 h at 37 °C. This was followed by (10×) deionized water wash/shaking. The plate is finally developed with 50 μL/well of a developing solution (25 mL, 0.2 M sodium phosphate, pH 6.0, 3 mL, 20% glucose/deionized water, 200 μL, 0.1% horseradish peroxidase/0.1 M phosphate buffer, pH 6.0, 200 μL, ABTS (BMB)) (45 mg/mL of deionized water). After 30 min the plate (each well) absorbance was determined at 405 nm on a Molecular Devices (V_{max}) kinetic plate reader.

A typical competition ELISA was performed in the following manner. A "limiting" antibody and antigen concentration as determined above is used in the following manner. The BSA-hapten conjugate (concentration determined above) in PBS is added at 25 μL/well to an ELISA plate. Methanol fix was added at 50 μL/well for 5 min. The methanol is removed, and the plate is air-dried for 10 min. The plate is blocked as described above with Blotto (50 μL/well) at 37 °C for 30 min, and the excess Blotto is removed. Next, the antigen (hapten) or substrate (diluted 1:20 in PBS-5% DMF) is serially diluted 2-fold (12 times). These solutions (12.5 μL) (each dilution) are added to the ELISA plate in the appropriate well, A1-A12, followed by the antibody (concentration determined above) (12.5 μL). This is allowed to incubate for 2 h at 37 °C. The plates are washed with deionized water (10×), and goat anti-mouse glucose oxidase is added (described above) followed by incubation for 1 h at 37 °C. The plate is again washed with deionized water (10×) followed by the addition of developing solution (described above). Absorbances are read at 405 nm.

The data (absorbances) obtained were fitted to eq 2 where CP is the absorbance determined and t is the antigen or substrate concentration. $P(1)$, $P(2)$, $P(3)$, and c are adjustable parameters in the four-parameter logistic fit.[25]

$$CP = \frac{P(1)}{1 + P(2)t^c} + P(3) \quad (2)$$

Chemical Modification of Antibodies. (a) Phenylglyoxal. A 50-μL aliquot of a phenylglyoxal solution (6 mM, 125 mM $NaHCO_3$, pH 7.5) was added to buffer (195 μL; 125 mM, pH 7.5, $NaHCO_3$) containing antibody (20 μM). The mixture was vortexed and left to stand for 1 h at room temperature. This was then transferred to a microdializer (Pierce) and dialized with 125 mM pH 7.5 $NaHCO_3$ with a flow-through of approximately 150 mL/h for 2 h. This was then flushed with 4 × 60 mL portions of pH 8.4 50 mM CHES-100 mM NaCl and left to stand in this buffer overnight. This was again flushed 3 × 50 mL portions of pH 8.4 50 mM CHES-100 mM NaCl the next morning. Samples were removed, protein concentrations recalculated (BCA), and assays run for catalytic activity (HPLC) or binding (ELISA). A similar procedure was used with hapten present (200 μM).

(b) Maleic Anhydride. A 5-μL aliquot of a maleic anhydride solution (0.06 M, dioxane) was added to 229 μL of 20 mM pH 8.0 borate-100 mM NaCl containing 20 μM of antibody. The solution was vortexed and left to stand at room temperature for 1 h. This was then transferred to a microdializer and dialyzed as described above with 50 mM phosphate, pH 7-100 mM NaCl overnight, followed by 50 mM CHES, pH 8.4-100 mM NaCl for 2 h. Samples were removed, protein concentrations calculated (BCA), and assays run for catalytic activity or binding.

(c) Diethyl Pyrocarbonate. A 10-μL aliquot of a 0.6 M diethyl pyrocarbonate solution in ethanol is diluted in 1 mL of NaOAc (150 mM, pH 6-100 mM NaCl). Of this 5 μL is added to 229 μL of NaOAc (150 mM, pH 6.0-100 mM NaCl) containing 20 μM of antibody. The mixture is vortexed and left to stand at 4 °C overnight. This was then transferred to a microdializer and dialyzed as described above with CHES (50 mM CHES, pH 8.4-100 mM NaCl). Samples were removed, protein concentrations determined (BCA), and assays performed for catalytic activity and binding.

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Coupled Kinetic Analysis of Cleavage of DNA by Esperamicin and Calicheamicin

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Abstract: A coupled kinetic analysis of esperamicin, calicheamicin, and DNase I cleavage of covalently closed circular PM2 DNA has been carried out. Analysis of the optical density data derived from agarose gel electrophoresis experiments shows that esperamicin A_1, like the hydrolytic enzyme DNase I, produces mainly single-strand breaks in DNA. These agents cause covalently closed circular form I DNA to be initially converted to nicked circular form II DNA. However, the ratio of the rate constant for this process (k_1') to that associated with conversion of form II to linear form III DNA (k_2') is not consistent with completely random nicking, and some double-strand cleavage may occur. The values of k_1'/k_2' found for DNase I and esperamicin A_1 were 5.4 nd 3.9, respectively. The behavior of these agents sharply contrasts with that of esperamicin C and calicheamicin, for which double-strand cleavage of DNA is deduced from the analysis. Although the rate constant for introducing the first break in DNA for calicheamicin is lower than the corresponding rate constant for esperamicin C, the second break (in the opposing strand) is fast for calicheamicin, making it the better double-strand cleaving agent. These drugs are unique among antitumor agents in that a single activation event on the warhead portion produces a double-strand break in DNA without the need to posttreat the DNA with other agents in order to induce a cleavage. The cleavage kinetics are discussed in terms of the structural differences in these unusual anticancer drugs.

The newly discovered anticancer drugs esperamicin[1,2] and calicheamicin[3,4] (Figure 1) exhibit high potency against murine tumor lines. These compounds possess sugar groups appended to an unusual 1,5-diyn-3-ene core structure referred to as the "warhead". In the presence of reducing agents and DNA the warhead undergoes a rearrangement to create a phenylene diradical that causes strand breaks by attacking the sugar groups

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