

pyridine, and 20 mM HEPES buffer (pH 7.1). The reaction mixture was incubated at 37 °C for 18 h, after which time a 500- $\mu$ L aliquot was removed and divided into two 250- $\mu$ L portions. The reactions were analyzed by reverse-phase HPLC using a LiChrospher 100RP-18 Column (5  $\mu$ m) (EM Science) with a binary solvent system of (A) 20 mM  $\text{KH}_2\text{PO}_4$  (pH 4.5) and (B) methanol/water (3:2) with a linear elution gradient of 0–50% solvent B over 30 min with a flow rate of 1.0 mL/min. Analysis of a 200- $\mu$ L sample of one portion of the reaction mixture shows a peak with a retention time of 20.0 min and several peaks with longer retention times. HPLC performed on 25  $\mu$ L of a 4.0 mM stock solution (20 mM HEPES buffer, pH 7.1) of authentic 2',3'-cyclic AMP (Sigma) also produced a peak with a retention time of 20.0 min. The second portion of the reaction mixture was spiked with 10  $\mu$ L of stock 2',3'-cyclic AMP. HPLC analysis showed an increase in only the peak at retention time of 20 min. Retention times of authentic samples of adenosine 3'-monophosphate, adenosine 5'-monophosphate, adenosine 2'-mono-

phosphate, and adenosine are 18.0, 13.2, 21.9, and 23.6 min, respectively. An identical HPLC protocol was used to identify 2',3'-cyclic AMP in the hydrolysis of ApAp by polypeptides.<sup>30</sup>

**Acknowledgment.** We thank Dr. W. B. Wise for helpful suggestions regarding NMR spectroscopy, Drs. P. C. Toren and E. W. Kolodziej for mass spectral data, and Miss A. M. Huber for literature searches.

**Supplementary Material Available:** 1- and 2-D  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  NMR spectra of the novel compounds (42 pages). Ordering information is given on any current masthead page.

(30) Barbier, B.; Brack, A. *J. Am. Chem. Soc.* **1988**, *110*, 6880–6882.

## Catalytic Antibodies with Acyl-Transfer Capabilities: Mechanistic and Kinetic Investigations

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**Abstract:** Antibodies have been shown to catalyze acyl-transfer reactions. The processes by which they perform such tasks have often been postulated but largely remain unknown. An extended study is presented on three different monoclonal antibodies that catalyze the hydrolysis of an alkyl ester and an aryl amide bond. Antibodies 2H6 and 21H3 catalyze the hydrolysis of an unactivated benzyl ester and show exquisite specificity for substrates with either the *R* or *S* configuration, respectively, while 43C9 catalyzes the cleavage of a *p*-nitroanilide amide bond. New substrates were synthesized and buffer-assisted reactions were employed to determine antibody–substrate fidelity. Oxygen-18 incorporation experiments were performed providing evidence that these antibody-mediated reactions proceed through attack at the acyl carbonyl, and excluding the possibility of an  $\text{S}_{\text{N}}2$  displacement mechanism for the ester hydrolysis reaction. A pH-rate profile study in protium and deuterium oxide was performed on antibody 43C9. This revealed an apparent  $\text{pK}_{\text{a}}$  of  $\sim 9$  involved in catalysis, but both the presence and absence of a solvent isotope effect in the pH-dependent and -independent regions suggested a multistep reaction pathway may be operative.

The number of chemical transformations catalyzed by antibodies (abzymes) is rapidly increasing. Antibodies have been shown to catalyze acyl-transfer, pericyclic, elimination, and redox reactions among others.<sup>1</sup> Limits to the types of reactions that antibodies can catalyze would be more systematically explored, if our knowledge on how “abzymes” perform their catalytic processes were extended.

We have been engaged in several programs aimed at eliciting antibodies with catalytic capabilities. One such program has been targeted at the development of acyl-transfer abzymes.<sup>2</sup> To date, our main successes have relied on the utilization of transition-state theory in the design of the haptens (antigens) used in the production of these hydrolytic antibodies. Specifically we have utilized tetrahedral phosphorus moieties as haptens to mimic the putative tetrahedral intermediate in the acyl-transfer reactions. While an extensive body of knowledge has been developed as to the manner in which these transition-state analogues inhibit enzymatic reactions, little is known about the complementary molecular surfaces these entities elicit when they are used as haptens to challenge the immune system.

Recently we reported two separate studies of antibodies that catalyze the hydrolysis of either an amide or an ester bond. In our first report we demonstrated that phosphoramidate **2** could induce catalytic antibodies for the hydrolysis of amide **2a** (Figure 1).<sup>2f</sup> In the second communication we studied the propensity of racemic antigen **1** to induce catalytic antibodies with *R* or *S* selectivity for benzyl ester **1a**.<sup>2g</sup> Herein we describe extensions of these studies aimed at elucidating some of the catalytic characteristics of these hydrolytic abzymes, i.e., their substrate specificity, the nature of the reaction pathway, and the source of rate accelerations.

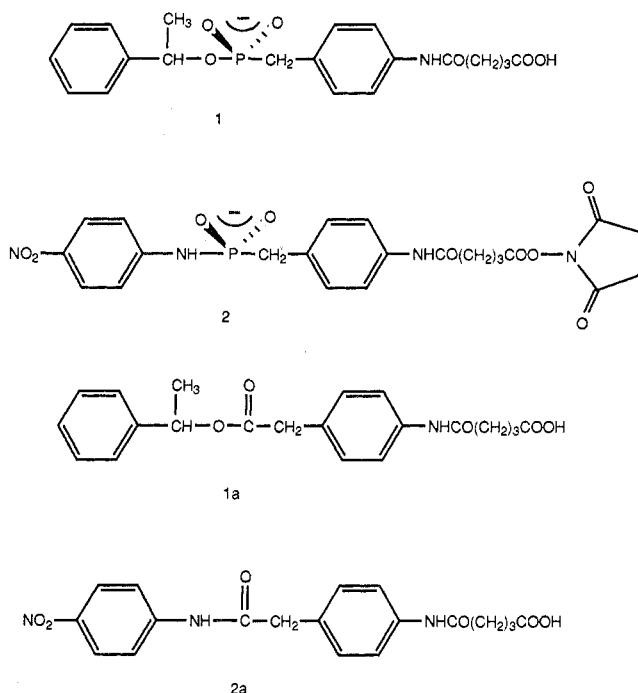
(1) Review articles on these catalytic antibody reactions: (a) Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1989**, *10*, 1283. (b) Schultz, P. G. *Acc. Chem. Res.* **1989**, *22*, 287. (c) Powell, M. J.; Hansen, D. E. *Protein Eng.* **1989**, *3*, 69.

(2) (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 (Washington, D.C.)* **1986**, *234*, 1566. (c) Napper, A. D.; Benkovic, S. J.; Tramontano, A.; Lerner, R. A. *Science (Washington, D.C.)* **1987**, *237*, 1041. (d) Janda, K. D.; Lerner, R. A.; Tramontano, A. *J. Am. Chem. Soc.* **1988**, *110*, 4835. (e) Benkovic, S. J.; Napper, A. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5355. (f) Janda, K. D.; Schloeder, D. M.; Benkovic, S. J.; Lerner, R. A. *Science (Washington, D.C.)* **1989**, *241*, 1188. (g) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science (Washington, D.C.)* **1989**, *244*, 437. (h) Janda, K. D.; Weinhouse, M. I.; Schloeder, D. M.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 1274.

<sup>†</sup> Scripps Clinic and Research Foundation.

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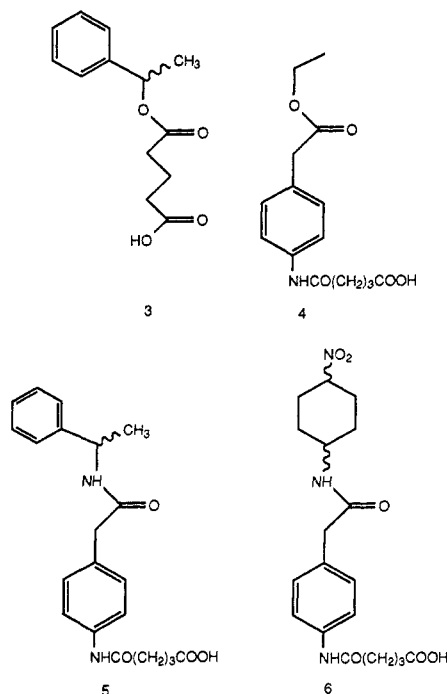
**Figure 1.** Haptens (**1** and **2**) used to induce catalytic antibodies and their respective substrates (**1a** and **2a**).

## Results and Discussion

**Antibody Substrate Specificity.** A key feature of antibody-catalyzed reactions is their ability to mimic known classes of enzymatic reactions. Antibodies 2H6 and 21H3 are no exceptions in that they catalyze reactions akin to enzymatic processes carried out by lipases.<sup>3</sup> Although their turnover numbers are less than for their enzyme counterparts, we found their stereospecificity to be exquisite.<sup>28</sup> This might imply a narrower substrate specificity for the antibodies as compared to the lipases, which, although stereoselective, are well-known to accept a large array of alkyl and aryl esters as substrates.<sup>3</sup> Consequently we investigated their substrate specificity.

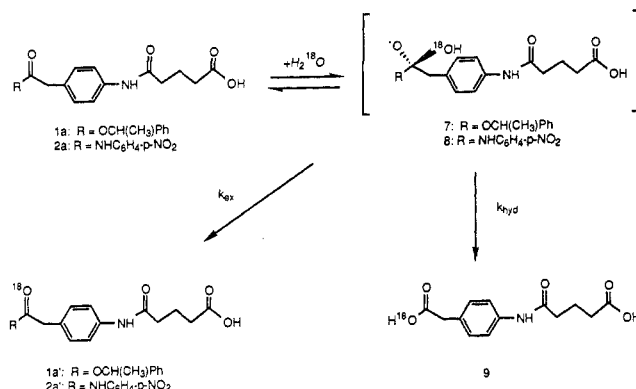
Compounds **3–5** (Figure 2) were synthesized in order to test abzymes 2H6 and 21H3 for substrate fidelity. These potential substrates were tested at 1.2 mM levels with 5  $\mu$ M antibody 2H6 and 21H3 in ATE buffer (Aces, 0.1 M; Tris, 0.052 M; ethanolamine, 0.052 M), pH 9.0,  $I = 0.1$ . All were found *not* to be substrates under conditions where less than 1% product would have been detected. While it may not be surprising that amide **5** was not a substrate because of its greater intrinsic hydrolytic stability relative to the ester, it was somewhat unexpected that esters **3** and **4**, differing in only the deletion of a single phenyl moiety, were not accepted. Antibody 2H6 was also tested for its ability to accelerate the hydrolysis of the ester analogue of **2a**, which contains an activated *p*-nitrophenyl moiety, but it failed to do so. For this compound, the rate of uncatalyzed hydrolysis is  $\sim 300$ -fold greater than **1a**. Buffer-assisted catalysis also was *not* observed with either antibody when such reagents as imidazole, peroxide, or ammonium were used at concentrations up to 6 mM with the consensus substrate **1a** *R* or *S*. Thus, it appears that our lipase mimics deviate profoundly in specificity from their enzymatic cousins. We see that fairly strict substrate homology to the inducing hapten must be maintained in order for antibody binding energy to be manifested as catalysis.

The substrate specificity requirements for catalytic antibody 43C9 were also investigated. Previously we demonstrated that this antibody catalyzes the hydrolysis of amide **2a** with a rate acceleration over background of greater than  $10^4$ .<sup>28</sup> Evaluation



**Figure 2.** Substrates found to be incompatible with antibodies 2H6, 21H3, and 43C9.

## Scheme I



of amide **2a**'s isomeric analogues (*m*- or *o*-nitroanilide) showed neither to be suitable substrates. While the ortho anilide could not inhibit the hydrolytic reaction at a concentration of 2 mM, the meta nitroanilide competitively inhibited the hydrolysis of the *p*-nitro substrate ( $K_i = 800 \mu\text{M}$ ),<sup>2f</sup> suggesting that the lack of antibody-assisted hydrolysis for this isomer may be due to unproductive binding. Similarly, compound **6**, in which the *p*-nitroaniline is reduced to the 4-nitrocyclohexylamine, bound to 43C9 but was not hydrolyzed (5  $\mu\text{M}$  43C9, 1.2 mM **6** in ATE buffer, pH 9.0,  $I = 0.1$ ). As expected, 43C9 does not hydrolyze either the *R* or *S* isomer of **1a** owing to lack of binding. Therefore, substrate and nucleophile fidelity is highly preserved by the antibody binding pocket and one may not expect to use this class of abzymes for the variety of substrates with the possible exception of single heteroatom switches.<sup>5</sup>

**Oxygen-18 Incorporation Experiments.** The structure of the haptens used for the induction of antibodies of 2H6 21H3, and 43C9 presumes that the hydrolysis of substrates proceeds via a tetrahedral transition state (**7** and **8**, in Scheme I). If this mechanism is correct, then the antibody-catalyzed hydrolysis of ester **1a** in  $[\text{H}_2^{18}\text{O}]$  would lead to the incorporation of  $^{18}\text{O}$  into

(3) (a) Desnuelle, P. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. VII, Chapter 19. (b) Brockerhoff, H. *Lipolytic Enzymes*; Academic Press: New York, 1974.

(4) Bruice, T. C.; Benkovic, S. J. In *Bioorganic Mechanisms*; Benjamin: New York, 1966; Chapter 1.

(5) Janda, K. D.; Schloeder, D. M.; Lerner, R. A. *Abstracts of Papers*, 194th National Meeting of the American Chemical Society, New Orleans, LA.; August 30–September 4, 1987; American Chemical Society: Washington, DC, 1987; ORGN 196.

**Table I.** Oxygen-18 Incorporation Experiments with Catalytic Antibodies 21H3 2H6, and 43C9

reaction	% M + 2 peak of $m/z$ 265 or 151 peak of acid <b>9</b> (mole fractn $^{18}\text{O}$ incorp into sample)	% M + 2 peak of $m/z$ 369 peak of ester <b>1a</b> or $m/z$ 271 peak of amide <b>2a</b> or <b>10</b> (mole fractn $^{18}\text{O}$ )
21H3 + ester (S)- <b>1a</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$ (90% reaction)	12.28 ( $\pm 0.07$ ) <sup>a</sup> (1.02) 10.76 ( $\pm 0.25$ ) <sup>b</sup> (0.99)	
21H3 + ester (S)- <b>1a</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$ (63% reaction)	9.80 ( $\pm 0.35$ ) <sup>b</sup> (0.90)	3.73 ( $\pm 0.01$ ) (0.01)
21H3 + acid <b>9</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$	2.24 ( $\pm 0.02$ ) <sup>a</sup> (0.02)	
2H6 + acid <b>9</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$	2.08 ( $\pm 0.12$ ) (0.01)	
ester <b>1a</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$ (57% reaction)	12.30 ( $\pm 0.05$ ) <sup>a</sup> (1.02)	4.01 ( $\pm 0.05$ ) (0.03)
amide <b>2a</b> in 20% $^{18}\text{O}$ $\text{H}_2\text{O}$ (11% reaction)	22.38 ( $\pm 0.20$ ) <sup>b</sup> (1.02)	7.39 ( $\pm 0.05$ ) (0.27)
43C9 + amide <b>2a</b> in 18% $^{18}\text{O}$ $\text{H}_2\text{O}$ (36% reaction)	22.05 ( $\pm 0.26$ ) <sup>a</sup> (1.06)	2.03 ( $\pm 0.04$ ) (0.01)
43C9 + amide <b>10</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$		1.63 (-0.02)
amide <b>10</b> in 10% $^{18}\text{O}$ $\text{H}_2\text{O}$		1.68 ( $\pm 0.04$ ) (-0.01)
acid <b>9</b> std (265 peak)	1.98 ( $\pm 0.05$ ) [cald 2.02] <sup>c</sup>	
acid <b>9</b> std (151 peak)	1.00 ( $\pm 0.04$ ) [cald 0.78] <sup>c</sup>	
ester <b>1a</b> std (369 peak)		3.45 ( $\pm 0.06$ ) [cald 3.67] <sup>c</sup>
amide <b>2a</b> std (271 peak)		1.73 ( $\pm 0.03$ ) [cald 1.79] <sup>c</sup>
amide <b>10</b> std (271 peak)		1.76 ( $\pm 0.01$ ) [cald 1.79] <sup>c</sup>

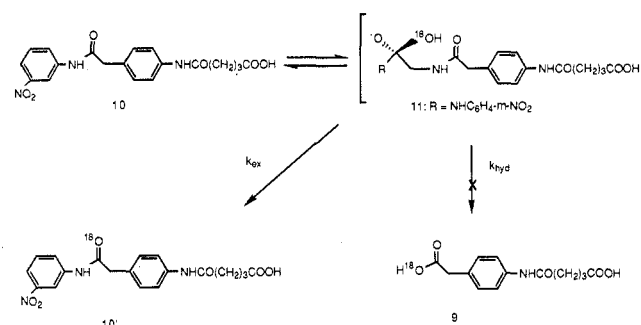
<sup>a</sup> % M + 2 peak for  $m/z$  265 peak. <sup>b</sup> % M + 2 peak for  $m/z$  151 peak.<sup>c</sup> Calculated by using the formula from Silverstein, Bassler, and Morill, p 13.17

the acid **9** (acyl oxygen bond cleavage) rather than into the alcohol (alkyl oxygen bond cleavage).

The 21H3-catalyzed hydrolysis of the S(-) ester **1a** was carried out in an aqueous solution containing 10%  $^{18}\text{O}$   $\text{H}_2\text{O}$ . Isolation of the acid **9** followed by mass spectral (MS) analysis clearly indicated that one solvent oxygen atom was incorporated into this product (Table I). Two separate reactions were performed, one to 63% completion and one to 90% completion, and **9** was isolated from both reactions. Mass spectrometric analysis of both samples indicated that they contained similar levels of  $^{18}\text{O}$  label (Table I). The starting material **1a** from the reaction run to 63% completion was also analyzed by MS and was shown to contain no  $^{18}\text{O}$  label from the solvent. Similar results were obtained for the uncatalyzed hydrolysis of **1a** in 10%  $^{18}\text{O}$   $\text{H}_2\text{O}$  in the absence of antibody 21H3: the acid **9** produced contained one oxygen from the solvent, and the recovered ester contained little, if any  $^{18}\text{O}$  label from the solvent.

Due to strong product inhibition by the diacid **9**, we did not perform similar experiments on abzyme 2H6.<sup>28</sup> However, we were able to determine with both antibodies whether they could catalyze the exchange of  $^{18}\text{O}$  into the carbonyl of the acid product **9**. No significant amount of  $^{18}\text{O}$  incorporation was seen in either experiment. These experiments also demonstrated that  $^{18}\text{O}$  is not spontaneously exchanged into **9** either during the reaction or during the quenching with perchloric acid.<sup>6</sup> It has been established that certain proteolytic enzymes (such as chymotrypsin) are competitively inhibited by some of their carboxylic acid products and furthermore that they can catalyze the exchange of  $^{18}\text{O}$  into these carboxylic acids.<sup>7</sup>

The hydrolysis of the *p*-nitro amide **2a** by antibody 43C9 in 18%  $^{18}\text{O}$   $\text{H}_2\text{O}$  was also examined. Only one solvent oxygen

**Scheme II**

(within experimental error) was incorporated into **9**. No  $^{18}\text{O}$  label was incorporated into the amide recovered from the reaction mixture. For the uncatalyzed hydrolysis of **2a**, however, a significant amount of exchange into the carbonyl of the recovered **2a** was observed (after 74 days at 37 °C).

Our observation that  $^{18}\text{O}$  is incorporated into acid **9** during the hydrolysis of substrates **1a** and **2a** by antibodies 21H3 and 43C9 provides evidence for the reaction proceeding via attack at the acyl carbonyl. It excludes the possibility of an alternative  $\text{S}_{\text{N}}2$  displacement mechanism for the hydrolysis of **1a** by 21H3.

The finding that  $^{18}\text{O}$  is incorporated into **9** is not surprising, given the large body of evidence which demonstrates that the majority of ester and amide hydrolyses proceed via a tetrahedral transition state.<sup>4,8</sup> However, the use of  $^{18}\text{O}$  labeling also gives information on an otherwise cryptic feature of the mechanism, the ratio of the rates at which the tetrahedral intermediate reacts to give either products ( $k_{\text{hyd}}$ ) or starting material ( $k_{\text{ex}}$ ). As shown in Scheme I, addition of  $^{18}\text{O}$   $\text{H}_2\text{O}$  to **1a** or **2a** leads to a tetrahedral intermediate, which can either lose RH to give products or lose  $^{18}\text{O}$   $\text{H}_2\text{O}$  to give  $^{18}\text{O}$ -labeled starting material. A significant rate of exchange should be apparent in two different ways: (a) the recovered ester or amide should possess more  $^{18}\text{O}$  than the starting material and (b) the acid produced should have higher  $^{18}\text{O}$  incorporation than that expected due to the amount of  $^{18}\text{O}$   $\text{H}_2\text{O}$  in the solvent. Significant  $^{18}\text{O}$  exchange has been observed during the hydrolysis of many carboxylic acid derivatives and is particularly prominent in the hydrolysis of many amides.<sup>8,9</sup> The data in Table I show that no significant  $^{18}\text{O}$  exchange occurs during the hydrolysis of ester **1a** by antibody 21H3 or the hydrolysis of amide **2a** by antibody 43C9 ( $k_{\text{hyd}}/k_{\text{ex}} > 10$ ).<sup>10</sup> However, a large amount of exchange occurred during the non-antibody-catalyzed hydrolysis of amide **2a** ( $k_{\text{hyd}}/k_{\text{ex}} \approx 0.48$ ).<sup>9</sup> There are several possible explanations for the lack of exchange seen during the antibody-catalyzed hydrolysis of **2a**. The antibody could have increased  $k_{\text{hyd}}/k_{\text{ex}}$  to the point where  $k_{\text{ex}}$  is insignificant. Alternatively, if the oxygens in the tetrahedral intermediate never become equivalent (due to the manner in which the transition intermediate is bound by the antibody) then **8** could return to unlabeled starting material **2a** but would not be able to fragment in such a way to give the labeled amide **2a'**. Finally, the possibility of exchange may be excluded if the reaction proceeds through an acyl-antibody intermediate. In any case it should be noted that certain proteolytic enzymes have been found to hydrolyze amides and esters without concomitant carbonyl oxygen exchange (due

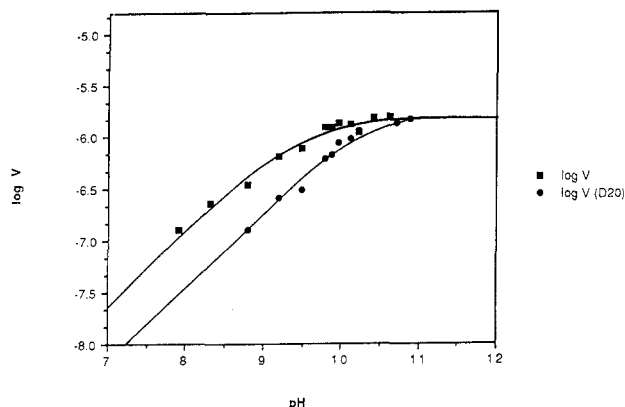
(8) Bender, M. L. *Chem. Rev.* **1959**, *59*, 53.

(9) (a) Bender, M. L.; Thomas, R. J. *J. Am. Chem. Soc.* **1961**, *83*, 4183. (b) Bender, M. L.; Matsui, H.; Thomas, R. J.; Tobey, S. W. *J. Am. Chem. Soc.* **1961**, *83*, 4193. (c) The ratio of  $k_{\text{hyd}}/k_{\text{ex}}$  calculated for *p*-nitroacetanilide in 0.239 M hydroxide from the data in ref 9a is  $\sim 0.04$ , compared to our number of 0.48 for compound **2a** in pH 9.0 ATE. This difference in  $k_{\text{hyd}}/k_{\text{ex}}$  may be due to a change in the mechanism by which acetanilides are hydrolyzed by hydroxide upon changing from *p*-toluyl, *p*-methoxy, and *p*-chloro substituents (studied in ref 11a) to the *p*-nitro substituent: Pollack, R. M.; Bender, M. L. *J. Am. Chem. Soc.* **1970**, *92*, 7190.

(10) The levels of  $k_{\text{hyd}}/k_{\text{ex}}$  that can be detected are limited by the accuracy of the MS experiment, which can be affected by rearrangements that occur in the mass spectrometer as well as other factors: Rose, M. E.; Johnstone, R. A. W. *Mass Spectrometry for Chemists and Biochemists*; Cambridge University Press: Cambridge, England, 1982; Chapter 12.

(6) It is known that strongly acidic conditions catalyze the incorporation of  $^{18}\text{O}$  into carboxylic acids: Bender, M. L. *J. Am. Chem. Soc.* **1951**, *73*, 1626.

(7) Bender, M. L.; Kemp, K. C. *J. Am. Chem. Soc.* **1957**, *79*, 116, and references cited therein.



**Figure 3.** The pH- $\log V_{\max}$  profiles for the antibody 43C9 catalyzed hydrolysis of amide **2a** in  $\text{H}_2\text{O}$  (■) and  $\text{D}_2\text{O}$  (●). Solid lines through the data represent a nonlinear least-squares fitting according to eq 1.

to the formation of an acyl-enzyme intermediate).<sup>7,11</sup>

The manner in which *m*-nitroanilide **10** acts as a competitive inhibitor in the reaction of *p*-nitroanilide **2a** with 43C9 was also investigated. Inhibition by **10** might be due to the formation of a tightly bound tetrahedral intermediate **11** in the substrate binding site that cannot be converted to product (Scheme II). The *m*-nitroanilide **10** was incubated with antibody 43C9 in the presence of 10% [ $^{18}\text{O}$ ] $\text{H}_2\text{O}$  in the same manner as was done with the *p*-nitroanilide **2a**. The anilide **10** was recovered from the reaction mixture and it was determined by MS that no  $^{18}\text{O}$  had been incorporated into **10**. This suggests either that **11** is not formed in the antibody combining site or that upon formation it is bound in an asymmetric manner such that the oxygen atoms on the tetrahedral carbon never become equivalent.

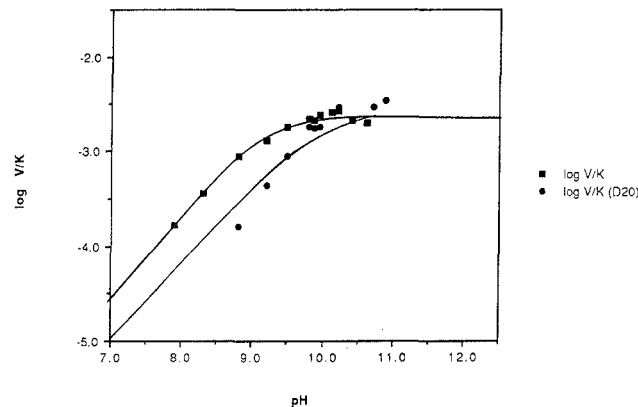
#### pH-Rate Profile for the Hydrolysis of **2a** by Antibody 43C9.

As discussed previously,<sup>2e,12</sup> the ratio of  $k_{\text{cat}}/k_{\text{uncat}}$  may be predicted from  $K_{\text{m}}/K_{\text{i}}$  for a typical abzyme reaction, provided that the hapten is an ideal transition-state mimic. Most hydrolytic abzymes show modest rate accelerations ( $k_{\text{cat}}/k_{\text{un}} = 10^2\text{--}10^3$ ).<sup>1</sup> However, we have reported that a select few of these antibodies display rate enhancements too large to be rationalized solely by transition-state binding energy alone.<sup>2f,g</sup> Abzymes 2H6 and 43C9 deviate markedly from what would be expected on the basis of a kinetic analysis derived from transition-state theory. A comparison of  $k_{\text{cat}}/k_{\text{uncat}}$  to  $K_{\text{m}}/K_{\text{i}}$  reveals that antibody 43C9 deviates from the expected norm by almost  $10^3$  and 2H6 by  $0.5 \times 10^2$ . These results led us to postulate that chemical catalysis (e.g., general acid/general base and/or nucleophilic) may be involved at the binding site, which might be detected in pH-rate profile studies.

The  $\log V_{\max}$ - and  $\log V_{\max}/K_{\text{m}}$ -pH profiles (Figures 3 and 4) for antibody 43C9 catalyzed hydrolysis of anilide substrate **2a** show the appearance of a  $\text{p}K_{\text{a}} \approx 9$  in both profiles. The correspondence further suggests that binding of the substrate is at equilibrium relative to subsequent steps. Turnover of the binary complex 43C9-**2a** then can be described by either of two kinetically indistinguishable rate laws:

$$k_{\text{cat}} = k_{\text{AM}} \frac{[\text{OH}^-]a_{\text{H}}}{K_{\text{a}} + a_{\text{H}}} = k_{\text{AM}}' \frac{K_{\text{a}}}{K_{\text{a}} + a_{\text{H}}} \quad \text{(case 1) \quad (case 2)}$$

where  $k_{\text{AM}}$  and  $k_{\text{AM}}'$  are first-order rate coefficients for decomposition of the 43C9-**2a** complex to products and  $K_{\text{a}}$  is the dissociation constant of the ionizing group. In  $\text{D}_2\text{O}$  (Figures 3 and 4), the observed  $\text{p}K_{\text{a}}$  is perturbed to 10.0 but no deuterium solvent isotope effect is observed in the plateau region. The two cases are not unequivocally distinguished by deuterium solvent isotope effects.<sup>4,13</sup> However, the occurrence of an isotope effect



**Figure 4.** The pH- $\log V_{\max}/K_{\text{m}}$  profiles for the antibody 43C9 catalyzed hydrolysis of amide **2a** in  $\text{H}_2\text{O}$  (■) and  $\text{D}_2\text{O}$  (●). Solid lines through the data represent a nonlinear least-squares fitting according to eq 1.

( $k^{\text{H}_2\text{O}}/k^{\text{D}_2\text{O}} \approx 3.8$ ,  $\log V$  plot, Figure 3) in the pH-dependent region of the profile but its absence in the plateau region suggests that two chemically different steps are being observed. Consequently Figures 3 and 4 may represent an apparent  $\text{p}K_{\text{a}}$  arising from a change in a rate-limiting step around an antibody-reactant species. The effect of  $\text{D}_2\text{O}$  on the apparent  $\text{p}K_{\text{a}}$  (ca. 1 unit) is larger than usually found for oxygen or nitrogen bases and may well be a consequence of this interpretation.<sup>14,15</sup> More detailed kinetic investigations are needed to choose unequivocally between these possibilities, but the present evidence is more in accord with a multistep hydrolytic pathway rather than simple attack by hydroxide ion on **2a** bound to antibody.

#### Summary

The catalytic antibodies that we have investigated in this study are extremely specific with respect to both substrate and nucleophile. It thus appears that catalytic antibodies will permit little latitude in choice of potential substrates. The  $^{18}\text{O}$  incorporation studies provide evidence that both the ester and amide antibody catalyzed hydrolysis reactions proceed via attack at the acyl carbonyl and exclude the possibility of an  $\text{S}_{\text{N}}2$  displacement mechanism for the ester hydrolysis reaction. An interpretation of the pH-rate profiles and solvent isotope effects favors the operation of a multistep reaction pathway for anilide hydrolysis that may include participation by functional groups at the antibody combining site.

#### 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. All reagents were purchased from Aldrich Chemical Co. All chromatography solvents were obtained commercially and used as received. Fast protein liquid chromatography (FPLC) was done on a Pharmacia system using a Pep RPC (15  $\mu\text{M}$ ) HR 16/10 column (Code No. 17-0577-01). Haptens **1** and **2** and their homologous substrates **1a** and **2a** were synthesized as described previously.<sup>2a,f</sup>

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet 510 FT-IR spectrophotometer as films between KBr plates for oils or in KBr disks for solids. All proton NMR spectra (300 and 100 MHz) were obtained in  $\text{CDCl}_3$ ,  $\text{CD}_3\text{CN}$ , or DMSO solutions at ambient temperature on either a Bruker AM-300 or Bruker 100 spectrometer. Chemical shifts ( $\delta$ ) are reported in parts per million relative to internal tetramethylsilane (0.00 ppm), elemental analyses (C, H, N) were performed by Galbraith Laboratories, Knoxville, TN.

**Mono(1-phenylethyl) Pentanedioate (3).** Glutaric anhydride (750 mg, 6.57 mmol) was added to a solution of *sec*-phenethyl alcohol (528  $\mu\text{L}$ , 4.38 mmol) and triethylamine (731  $\mu\text{L}$ , 5.24 mol) in 3 mL of dry dichloromethane. After being stirred at 40  $^\circ\text{C}$  for 20 h, ethyl acetate was added and the organic phase was washed with 0.5 M HCl (2  $\times$  15 mL) and brine (15 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The residue was

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purified on silica gel with a gradient of dichloromethane/ethyl acetate (9:1–1:1), yielding the thin clear oil product: 330 mg, 32%; IR (neat) 2982, 2938, 1732, 1709, 1453, 1207  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  7.32 (s, 5 H), 5.90 (q, 1 H), 2.41 (app dt, 4 H), 2.12–1.79 (m, 2 H), 1.53 (d, 3 H). Anal. Calcd for  $\text{C}_{13}\text{H}_{16}\text{O}_4$ : C, 66.09; H, 6.83. Found: C, 66.10; H, 6.91.

**4-[(Trifluoroacetyl)amino]benzeneacetic Acid.** 4-Aminophenylacetic acid (2.00 g, 13.23 mmol) was dissolved in 20 mL of acetonitrile and 0.6 mL of water at room temperature. The mixture was then cooled to 0 °C and sodium carbonate (2.80 g, 26.42 mmol) was added with vigorous stirring. Trifluoroacetic anhydride (5.61 mL, 39.72 mmol) was added dropwise and the reaction was stirred vigorously for an additional 10 min. The acetonitrile was then removed by rotary evaporation and the mixture was rediluted with ethyl acetate. Water was added and the pH was adjusted to ~2 with concentrated HCl. After shaking, the aqueous phase was removed and the organic phase was washed with 0.5 M HCl (3  $\times$  15 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The solid remaining was rinsed three times with hexanes and pumped dry to leave the tan-colored protected amine: 2.78 g, 85%; mp ~175 °C dec; IR (KBr) 3290, 1705, 1602, 1545, 1519, 1420  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz, DMSO)  $\delta$  7.45 (dd, 4 H), 3.59 (s, 2 H). Anal. Calcd for  $\text{C}_{10}\text{H}_8\text{NO}_3\text{F}_3$ : C, 48.59; H, 3.26; N, 5.67. Found: C, 48.41; H, 3.15; N, 5.51.

**4-[(Trifluoroacetyl)amino]benzeneacetyl Chloride (12).** A suspension of the carboxylic acid **11** (500 mg, 2.02 mmol) in 10 mL of thionyl chloride was stirred and heated at 55 °C for 5 h. The excess thionyl chloride was removed by rotary evaporation, leaving an orange-brown solid that was rinsed three times with hexanes and the pumped dry to leave the solid acid chloride product: 523 mg, 98%; mp 173–175 °C; IR (KBr) 3304, 1799, 1708, 1616, 1546, 1518, 1420  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  9.30 (br s, 1 H), 7.54 (dd, 4 H), 4.32 (s, 2 H). Anal. Calcd for  $\text{C}_{10}\text{H}_7\text{NO}_2\text{F}_3\text{Cl}$ : C, 45.22; H, 2.66; N, 5.27. Found: C, 45.34; H, 2.71; N, 5.37.

**Ethyl 4-[(Trifluoroacetyl)amino]benzeneacetate (13).** The acid chloride **12** (500 mg, 1.88 mmol) was added to a solution of ethyl alcohol (163  $\mu\text{L}$ , 2.83 mmol) and pyridine (457  $\mu\text{L}$ , 5.65 mmol) in 20 mL of dry dichloromethane. The reaction mixture was stirred at room temperature for 7 h. Ethyl acetate was added and the organic phase was washed with 0.2 M  $\text{CuSO}_4$  (3  $\times$  20 mL), 0.5 M HCl (3  $\times$  20 mL), 5–10%  $\text{NaHCO}_3$  (3  $\times$  20 mL), and brine (2  $\times$  20 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated. The solid remaining was purified by chromatography on silica gel using a gradient of dichloromethane/ethyl acetate (19:1–9:1). Solvent evaporation yielded the white solid product: 336 mg, 65%; mp 121–123 °C; IR (KBr) 3290, 2995, 1715, 1602, 1546, 1420  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  8.20 (br s, 1 H), 7.38 (dd, 4 H), 4.19 (q, 2 H), 3.60 (s, 2 H), 1.25 (t, 3 H). Anal. Calcd for  $\text{C}_{12}\text{H}_{12}\text{NO}_3\text{F}_3$ : C, 52.37; H, 4.39; N, 5.09. Found: C, 52.05; H, 4.19; N, 5.01.

**Ethyl 4-Aminobenzeneacetate (14).** Sodium borohydride (222 mg, 5.87 mmol) was added to a solution of the protected amine **13** (322 mg, 1.17 mmol) in 20 mL of dry ethyl alcohol. After being stirred at room temperature overnight (19 h), the reaction mixture was poured into 10% ammonium hydroxide (100 mL) and extracted with ethyl acetate (4  $\times$  50 mL). The combined organic washes were dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated to a crude oil. The crude product was purified by chromatography on silica gel using 9:1 dichloromethane/ethyl acetate. Solvent evaporation left the pure amine product as a yellow-green oil: 138 mg, 66%; IR (neat) 3459, 3374, 2981, 1729, 1631, 1518, 1441, 1370  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  6.83 (dd, 4 H), 4.17 (q, 2 H), 3.50 (s, 2 H), 1.25 (t, 3 H). Anal. Calcd for  $\text{C}_{10}\text{H}_{13}\text{NO}_2$ : C, 67.02; H, 7.31; N, 7.82. Found: C, 66.85; H, 7.13; N, 7.64.

**Ethyl 4-[(4-Carboxy-1-oxobutyl)amino]benzeneacetate (4).** Glutaric anhydride (101 mg, 0.89 mmol) was added to a solution of the amine **14** (132 mg, 0.74 mmol) and triethylamine (130  $\mu\text{L}$ , 0.93 mmol) in 2 mL of dry dichloromethane. The reaction was stirred at 50 °C for 4 h, ethyl acetate was added, and the organic phase was washed with 0.5 M HCl (3  $\times$  10 mL) and brine (10 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated. The residue was dissolved in a 7:1 acetonitrile/water solution. The product was then isolated by FPLC (UV detection at  $\lambda$  = 254 nm) using a 15-min gradient program (4 mL/min) of 10–90% acetonitrile in water (0.1% TFA), (product  $t_R$  = 11.6 min). Acetonitrile and TFA were removed by rotary evaporation, and the water was removed by lyophilization to leave the white powder product: 136 mg, 62%; mp 70–72 °C; IR (KBr) 3304, 2966, 1736, 1694, 1659, 1602, 1546, 1413, 1223, 1173  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.36 (dd, 4 H), 4.15 (q, 2 H), 3.60 (s, 2 H), 2.48 (app dt, 4 H), 2.08 (app pentet, 2 H), 1.25 (t, 3 H). Anal. Calcd for  $\text{C}_{15}\text{H}_{19}\text{NO}_5$ : C, 61.42; H, 6.53; N, 4.78. Found: C, 61.11; H, 6.31; N, 4.52.

**N-(1-Phenylethyl)-4-[(trifluoroacetyl)amino]benzeneacetamide (15).** Triethylamine (805  $\mu\text{L}$ , 5.78 mmol) was added to a well-stirred solution of (R)-(+)- and (S)-(–)- $\alpha$ -methylbenzylamine (249  $\mu\text{L}$ , 1.93 mmol) in 5 mL of dry dichloromethane. After 10 min, the acid chloride **12** (512

mg, 1.93 mmol, dissolved in 3 mL of dichloromethane) was added to the reaction mixture, which was then stirred overnight (~15 h) at room temperature. Ethyl acetate was added and the organic phase was washed with 0.5 M HCl (3  $\times$  10 mL) and 5–10%  $\text{NaHCO}_3$  (3  $\times$  15 mL) and brine (15 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated. The remaining solid was purified by chromatography on silica gel with 9:1 dichloromethane/ethyl acetate. Solvent removal by rotary evaporation left the white solid product: 387 mg, 57%; mp 169–171 °C; IR (KBr) 3361, 3251, 1708, 1655, 1612, 1542, 1207, 1157,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  8.36 (br s, 1 H), 7.39 (dd, 4 H), 7.30 (app s, 5 H), 5.75 (br d, 1 H), 5.14 (app pentet, 1 H), 3.80 (s, 2 H), 1.45 (d, 3 H). Anal. Calcd for  $\text{C}_{18}\text{H}_{17}\text{N}_2\text{O}_2\text{F}_3$ : C, 61.71; H, 4.89; N, 8.00. Found: C, 61.81; H, 4.95; N, 8.15.

**N-(1-Phenylethyl)-4-aminobenzeneacetamide (16).** Sodium borohydride (195 mg, 5.15 mmol) was added to a solution of the protected amine **15** (361 mg, 1.03 mmol) in 20 mL of dry ethyl alcohol. After stirring overnight (~15 h) at room temperature, more sodium borohydride (78 mg, 2.06 mmol) was added and stirring continued for another 5 h. The reaction mixture was poured into 10% ammonium hydroxide (100 mL) and extracted with ethyl acetate (4  $\times$  50 mL). The combined organic washes were dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated to leave the solid amine: 144 mg, 55%; mp 98–100 °C; IR (KBr) 3500–3200, 2971, 1737, 1712, 1648, 1546, 1518, 1365,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  7.30 (app s, 5 H), 6.85 (dd, 4 H), 5.68 (br d, 1 H), 5.11 (app pentet, 1 H), 3.50 (s, 2 H), 1.39 (d, 3 H). Anal. Calcd for  $\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}$ : C, 75.56; H, 7.13; N, 11.01. Found: C, 75.21; H, 7.03; N, 10.94.

**5-Oxo-5-[(4-[2-oxo-2-[(1-phenylethyl)amino]ethyl]phenyl)amino]pentanoic Acid (5).** Glutaric anhydride (63 mg, 0.55 mmol) was added to a solution of the amine **16** (127 mg, 0.50 mmol) and triethylamine (85  $\mu\text{L}$ , 0.61 mmol) in 2 mL of dry dichloromethane. After the resultant mixture was stirred at 50 °C for 3.5 h, ethyl acetate was added and the organic phase was washed with 0.5 M HCl (2  $\times$  15 mL) and brine (15 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The residue was dissolved in a 1:1 water/acetonitrile solution by adding a small amount of trifluoroacetic acid (TFA). The product was then isolated by FPLC ( $\lambda$  = 254 nm) using a 15-min gradient program (4 mL/min) of 10–90% acetonitrile in water (0.1% TFA) (product  $t_R$  = 11.5 min). Acetonitrile and TFA were removed by rotary evaporation and the water was removed by lyophilization to leave the white powder product: 55 mg, 30%; mp 164–165 °C; IR (KBr) 3315, 3029, 2971, 1739, 1737, 1667, 1645, 1528,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (100 MHz, DMSO)  $\delta$  10.09 (s, 1 H), 8.71 (d, 1 H), 7.56 (dd, 4 H), 7.52 (s, 5 H), 5.10 (app pentet, 1 H), 4.0 (s, 1 H), 3.21 (s, 2 H), 2.54 (dt, 4 H), 2.19–1.85 (m, 2 H), 1.59 (d, 3 H). Anal. Calcd for  $\text{C}_{21}\text{H}_{24}\text{N}_2\text{O}_4$ : C, 68.46; H, 6.57; N, 7.60. Found: 68.31; H, 6.31; N, 7.49.

**N-(4-Nitrocyclohexyl)-4-[(trifluoroacetyl)amino]benzeneacetamide (17).** Diastereomeric 4-nitrocyclohexanamine monohydrobromide<sup>16</sup> (169 mg, 0.75 mmol) was dissolved in a solution of dry dichloromethane (4 mL) and triethylamine (275  $\mu\text{L}$ , 1.97 mmol) at room temperature. After 5 min the acid chloride **12** (172 mg, 0.65 mmol, dissolved in 6 mL of dichloromethane) was added to the reaction mixture, followed by a catalytic amount (27 mg, 0.22 mmol) of 4-(dimethylamino)pyridine (DMAP), and the reaction then heated to 45 °C. Additional triethylamine (90  $\mu\text{L}$ , 0.65 mmol) and DMAP (20 mg, 0.16 mmol) were added after 1 h and again after 2.5 h. After stirring at 45 °C for a total of 3 h, ethyl acetate was added and the organic phase was washed with 0.5 M HCl (3  $\times$  20 mL) and 5–10%  $\text{NaHCO}_3$  (3  $\times$  20 mL) and brine (2  $\times$  20 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated. The crude product was purified by chromatography on silica gel using a gradient of dichloromethane/ethyl acetate (9:1–1:1). Solvent evaporation yielded the pure diastereomeric product as a tan chalky solid: 164 mg, 68%;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  8.07 (br s, 1 H), 7.42 (dd, 4 H), 5.40–5.03 (m, 1 H), 4.59–4.24 (m, 1 H), 4.00–2.70 (m, 1 H), 3.55 (s, 2 H), 2.51–0.70 (m, 8 H). Anal. Calcd for  $\text{C}_{16}\text{H}_{18}\text{N}_3\text{O}_4\text{F}_3$ : C, 51.48; H, 4.86; N, 11.26. Found: C, 51.15; H, 4.51; N, 10.95.

**N-(4-Nitrocyclohexyl)-4-aminobenzeneacetamide (18).** Sodium borohydride (95 mg, 2.51 mmol) was added to a solution of the protected amine **17** (164 mg, 0.44 mmol) in 8 mL of dry ethyl alcohol. The reaction mixture was stirred at 60 °C and an additional 5 equiv of sodium borohydride (83 mg, 2.19 mmol) was added after 4.5 h and again after 10.5 h. After the reaction stirred at 60 °C for a total of 24 h, the mixture was poured into 10% ammonium hydroxide (50 mL) and extracted with ethyl acetate (4  $\times$  24 mL). The combined organic washes were dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated to an oil product, which was not purified further: 34 mg, 27%;  $^1\text{H}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  6.86 (dd, 4 H), 5.90–4.90 (br s, 1 H), 3.70–3.52 (br m, 1 H), 3.45 (s, 2 H), 2.45–0.70 (br m, 8 H). Anal. Calcd for  $\text{C}_{14}\text{H}_{19}\text{N}_3\text{O}_3$ : C, 60.63; H, 6.91; N, 15.15.

(16) Janda, K. D.; Ashley, J. A. Syntheses of the saturated analogues of 4-nitrophenol and 4-nitroaniline. *Synth. Commun.* 1990, 20, 1073.

Found: C, 60.44; H, 6.69; N, 14.83.

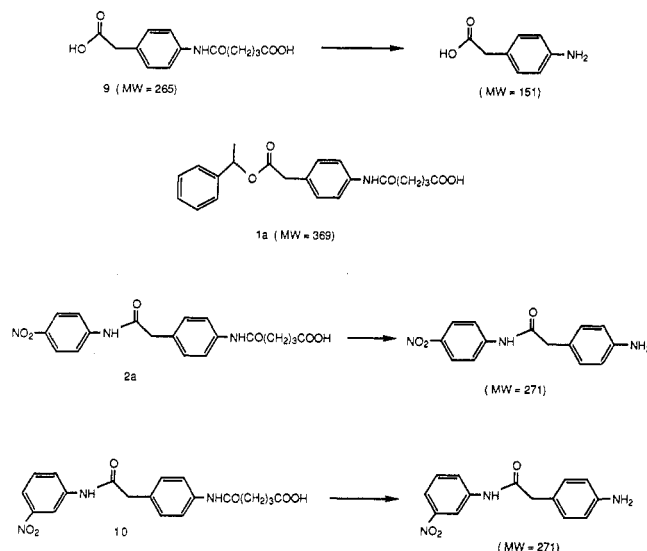
**5-[[4-[2-[(4-Nitrocyclohexyl)amino]-2-oxoethyl]phenyl]amino]-5-oxopentanoic Acid (6).** Glutaric anhydride (17 mg, 0.15 mmol) was added to a solution of the amine **18** (34 mg, 0.12 mmol) and triethylamine (21  $\mu$ L, 0.15 mmol) in 1 mL of dry acetonitrile. After the resultant mixture was stirred at 57 °C for 2 h, additional triethylamine (21  $\mu$ L, 0.15 mmol) and glutaric anhydride (17 mg, 0.15 mmol) were added to the reaction. After another 2 h, ethyl acetate and acetonitrile were added and the organic phase was washed with 0.5 M HCl (3  $\times$  5 mL) and brine (5 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The residue remaining was dissolved in 2:1 methanol/water and the product isolated by FPLC ( $\lambda = 254$  nm) using a 15-min gradient program (4 mL/min) of 10–90% acetonitrile in water (0.1% TFA) (product  $t_R \approx 10.7$  min). Acetonitrile and TFA were removed by rotary evaporation and the water was removed by lyophilization to leave the pure product: 7 mg, 17%;  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  9.81 (s, 1 H), 7.99 (d, 1 H), 7.32 (dd, 4 H), 4.75–4.50 (m, 1 H), 3.80–3.55 (m, 1 H), 3.44 (s, 2 H), 2.60–1.00 (m, 8 H + 6 H). Anal. Calcd for  $\text{C}_{19}\text{H}_{25}\text{N}_3\text{O}_6$ : C, 58.30; H, 6.44; N, 10.74. Found: C, 58.18; H, 6.37; N, 10.59.

**Preparation of Antibodies and Routine Assays.** Monoclonal antibodies 43C9, 2H6, and 21H3 were obtained from immunization, using phosphonate **1** for 2H6 and 21H3 or phosphonamide **2** for 43C9, and were purified from ascites by the procedures of Janda et al.<sup>2f,28</sup> All preparations were typically 95% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Antibody concentrations were determined from a bicinchoninic acid (BCA) assay, (absorbance at 280 nm) assuming a molecular weight of 150 000 for IgG [and are expressed as antibody concentration rather than active site concentration (two active sites per antibody)]. Kinetic determinations involving the release of *p*-nitroanilide were performed as described by Janda.<sup>2f</sup> Assays involving the formation of 4-[(4-carboxy-1-oxobutyl)amino]phenylacetic acid (**9**) from substrates **4–6** were done via an HPLC assay devised by Janda et al.<sup>28</sup> Buffer-assisted reactions (imidazole, peroxide, ammonia) were performed in a manner analogous to that described above for substrates **4–6**, except that the aminolysis reaction was followed by using the HPLC gradient assay employed for the isolation of products from the  $^{18}\text{O}$  labeling experiments (vide infra). The product 4-[(4-carboxy-1-oxobutyl)amino]benzeneacetamide had a retention time of 16.5 min. Kinetic determinations on (*R*)- and (*S*)-mono(1-phenethyl) pentanedioate **3** were performed using the HPLC assay of Janda et al.<sup>28</sup> The product *sec*-phenethyl alcohol had a retention time of 16 min.

**Oxygen-18 Incorporation Experiments.** HPLC was utilized to purify the products from  $^{18}\text{O}$  incorporation experiments. The chromatographic system consisted of a Waters 600E solvent delivery module equipped with a Waters 484 UV detector (254 nm), an HP3394A integrator, and a Vydac 5- $\mu$ m C-18 reverse-phase analytical column. A flow rate of 1 mL/min was employed with a linear gradient of 95% A to 95% B over 30 min (A, 0.1% trifluoroacetic acid in  $\text{H}_2\text{O}$ ; B, acetonitrile). Under these HPLC conditions, the retention times of the compounds of interest were as follows: acid **9**, 18.9 min; acetophenone (internal standard), 23.5 min; *m*-nitro amide **10**, 24.2 min; *p*-nitro amide **2a**, 24.6 min; ester **1a**, 27.2 min. The amount of acid **9** present in the reaction mixture was determined by calculating the ratio of the peak area for **9** to the acetophenone peak area and then comparing this ratio to a standard curve established by use of HPLC separation of samples containing known ratios of acid **9** to acetophenone.

A Kratos MS-25 mass spectrometer equipped with a DS-90 data system was used. Samples were ionized via electron impact with a source voltage of 70 eV. All analyses were performed by the direct probe method. The sample was dissolved in a minimal volume of methanol, and 1  $\mu$ L of this solution was placed in a capillary tube that was held by the probe. The probe was then inserted into the source, which was maintained at 200° at all times. Isotopic analysis was performed by raw data acquisition, with the magnet scanning only over those mass units of interest. Generally, five to seven mass spectra were obtained for each sample from an  $^{18}\text{O}$  incorporation experiment, and the average value obtained is reported in Table I.

The percentage of  $^{18}\text{O}$  incorporated into acid **9** was usually calculated from the relative area of the  $m/z$  267 ( $M + 2$ ) peak versus the area of the peak for the molecular ion at  $m/z$  265. In two cases, the ratio of the peaks at  $m/z$  153 and 151 due to loss of glutaric anhydride from labeled and unlabeled acid (see Figure 5) was used; however, we have found that this fragment give less reproducible results for isotopic incorporation than the molecular ion. The percentage of  $^{18}\text{O}$  incorporated into **1a** was calculated from the ratio of the peak at  $m/z$  371 ( $M + 2$ ) to the peak at  $m/z$  369 (molecular ion). For the amides **2a** and **10**, the molecular ion at  $m/z$  385 was much too weak to obtain an accurate measurement of its  $M + 2$  peak. Thus, the fragment at  $m/z$  271 (Figure 5) due to loss of glutaric anhydride and its  $M + 2$  peak were used to determine the  $^{18}\text{O}$  content of **2a** and **10**. Corroborating evidence for the identity of the  $m/z$



**Figure 5.** Fragmentation ions observed in oxygen-18 mass spectrometry experiments.

271 fragment of the *p*-nitro amide **2a** was obtained by high-resolution mass spectroscopy (calcd for  $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_3$  271.0958, found 271.0948). In each MS experiment, the area of the  $M + 1$  peak was also measured and was found to be in good agreement with the theoretical value.<sup>17</sup>

The mole fraction of  $^{18}\text{O}$  incorporated was calculated on the basis of the assumption that one solvent oxygen would be incorporated into the molecule of interest. The expected area for the  $M + 2$  peak for the labeled molecule (based on this assumption) was calculated by using the following formula (adapted from ref 17):  $\% M + 2 = (1.1a)^2/200 + 0.20b + (c/d \times 100\%)$  where  $a$  is the number of carbons in the molecule;  $b$  is the number of oxygens in the molecule  $- 1$ ;  $c$  is the molarity of  $^{16}\text{O}$  in the reaction mixture; and  $d$  is the molarity of  $^{18}\text{O}$  in the reaction mixture. The mole fraction of  $^{18}\text{O}$  incorporated into the molecule was subsequently calculated by using the following formula: mole fraction =  $(x - y)/(z - y)$  where  $x$  is the observed area of the  $M + 2$  peak;  $y$  is the calculated area of the  $M + 2$  peak for unlabeled sample; and  $z$  is the calculated area of the  $M + 2$  peak for labeled sample.

**Hydrolysis of Ester **1a** in  $^{18}\text{O}$   $\text{H}_2\text{O}$ .** The reaction mixture contained 10  $\mu$ M antibody 21H3, 10% (v/v)  $^{18}\text{O}$   $\text{H}_2\text{O}$  [ $^{16}\text{O}$ , 2.8%;  $^{17}\text{O}$ , 1.8%;  $^{18}\text{O}$ , 95.4% (Monsanto, St. Louis, MO); 4.8 M], 400  $\mu$ M ester *S*-(-)-**1a** and 5% DMF in ATE buffer (0.1 M Aces, 0.052 M Tris, 0.052 M ethanolamine  $I = 0.1$ , pH 9.0).<sup>18</sup> Acetophenone (50  $\mu$ M) was utilized as an internal standard. The reaction mixture was kept at room temperature. The progress of the reaction was monitored by removing 25- $\mu$ L aliquots, subsequently quenched with 1  $\mu$ L of 23%  $\text{HClO}_4$ ; the precipitated protein was removed by centrifugation, and the amount of acid **9** was determined by HPLC. Upon detection of the desired percent conversion the reaction was quenched with 23%  $\text{HClO}_4$  (to a final pH  $\sim 4$ ), centrifuged to remove precipitated protein, and separated by HPLC. The fractions containing acid **9** and ester **1a** were collected in 13  $\times$  100 mm test tubes, which had been immersed overnight in a KOH-saturated 2-propanol bath, washed with deionized water, immersed overnight in 50%  $\text{H}_2\text{SO}_4$ , washed with deionized water, and finally rinsed with HPLC-grade water. Test tubes not cleaned in this manner possessed significant amounts of impurities which interfered with the MS analyses. The recovered samples were concentrated to dryness (at ambient temperature) in vacuo in a Savant Speedvac and analyzed for  $^{18}\text{O}$  content by MS. The uncatalyzed hydrolysis was performed in 10%  $^{18}\text{O}$   $\text{H}_2\text{O}$  in a similar manner, with the exceptions that no antibody was present and the concentration of ester **1a** was 800  $\mu$ M. The results of these experiments are shown in Table I.

The ability of antibody 2H6 or antibody 21H3 to incorporate  $^{18}\text{O}$  into acid **9** was determined as follows: Solutions of 2H6 or 21H3 (10  $\mu$ M) in ATE, pH 9.0, were prepared with final concentrations of 4.8 M  $^{18}\text{O}$

(17) Silverstein, R. M.; Bassler, G. C.; Morill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; John Wiley & Sons: New York, 1981; Chapter 2.

(18) (a) The amount of  $^{18}\text{O}$  in a typical reaction mixture was verified by converting a small aliquot of  $^{18}\text{O}$   $\text{H}_2\text{O}$  to  $^{18}\text{O}$ -labeled triethyl phosphate. The  $^{18}\text{O}$  content of the triethyl phosphate was determined by MS.<sup>18b,c</sup> (b) Stempel, K. E.; Boyer, P. D. *Methods Enzymol.* **1986**, 63, 618. (c) Sharp, T. R.; Benkovic, S. J. *Biochemistry* **1979**, 18, 2910.



O]H<sub>2</sub>O, 400  $\mu$ M **9**, and 5% DMF. The mixture was maintained at room temperature for 1.5 (21H3) or 21 h (2H6) and quenched to pH  $\sim$ 4 with 23% HClO<sub>4</sub>. The resulting acid was isolated for MS analysis as described above.

**Hydrolysis of Amide 2a by NPN 43C9 in [<sup>18</sup>O]H<sub>2</sub>O and Incubation of Amide 10 with NPN 43C9 in [<sup>18</sup>O]H<sub>2</sub>O.** The reaction mixtures contained NPN 43C9 (7.8  $\mu$ M), *p*-nitro amide **2a** (2 mM), and either 10% (v/v) of 95% [<sup>18</sup>O]H<sub>2</sub>O (4.8 M) or 18% of 95% [<sup>18</sup>O]H<sub>2</sub>O (8.8 M) in ATE, pH 9.0. The mixtures were incubated at 37 °C for 7 days, at which point the 10% [<sup>18</sup>O]H<sub>2</sub>O reaction had proceeded to 38% completion and the 18% [<sup>18</sup>O]H<sub>2</sub>O reaction had proceeded to 36% completion. The reactions were quenched with 23% HClO<sub>4</sub> to pH  $\sim$ 4 and acid **9** and amide **2a** were isolated by HPLC for MS as described for the hydrolysis of ester **1a** in [<sup>18</sup>O]H<sub>2</sub>O. The uncatalyzed hydrolysis was performed in 20% [<sup>18</sup>O]H<sub>2</sub>O in a similar manner, with the exceptions that no antibody was present and the concentration of **2a** was 800  $\mu$ M.

A 2 mM solution of the *m*-nitro amide **10** in 10% (v/v) [<sup>18</sup>O]H<sub>2</sub>O in ATE (pH 9, *I* = 0.1) was also incubated with NPN 43C9 (7.8  $\mu$ M) at 37 °C as described above for the reaction of *p*-nitro amide **2a**. The reaction was quenched after 7 days with 23% HClO<sub>4</sub>. Analysis by HPLC indicated that a negligible amount (<1  $\mu$ M) of acid **9** was obtained. The *m*-nitro amide was then isolated by HPLC and analyzed for <sup>18</sup>O content as described above. The results of these experiments are shown in Table I.

**pH-Rate Studies.** pH-rate profile assays for antibody 43C9 were conducted in the following manner. Velocities were determined spectrophotometrically by measuring the initial linear absorbance change at

404 nm (release of *p*-nitroaniline). Antibody 43C9 [10  $\mu$ M; average value determined from a bicinchoninic acid (BCA) assay and absorbance at 280 nm, assuming a molecular weight of 150 000 for IgG] was preincubated at 37 °C (ATE buffer, pH 7.9–10.62, *I* = 0.1) and reactions were initiated by addition of amide **2a** in DMF to give a substrate concentration of 200–2000  $\mu$ M, (total organic phase was 5%). Matching ATE buffers in 99.7% D<sub>2</sub>O were prepared analogously as described above in water. pD was determined by adding +0.4 unit to the meter reading. Assays in D<sub>2</sub>O were conducted and monitored in a similar manner as described above for assays conducted in H<sub>2</sub>O.

**Data Analysis for pH-Rate Profile Assays.** The pH and pD variation of the rate constants for antibody 43C9 was fitted to eq 1 by the HA-

$$\log y = \log [C/(1 + H/K)] \quad (1)$$

BELL program of Cleland.<sup>19</sup> This program assumes a drop in kinetic constant only at a low pH, where *y* data input is either in the form *V*<sub>max</sub> or *V*<sub>max</sub>/*K*.

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## Differential Binding Energy: A Detailed Evaluation of the Influence of Hydrogen-Bonding and Hydrophobic Groups on the Inhibition of Thermolysin by Phosphorus-Containing Inhibitors

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**Abstract:** Two series of phosphorus-containing peptide analogues, **3** (Cbz-Gly- $\psi$ (PO<sub>2</sub><sup>-</sup>CH<sub>2</sub>)Leu-Xaa) and **4** (Cbz-Gly- $\psi$ (PO<sub>2</sub><sup>-</sup>NH)Leu $\psi$ (CO<sub>2</sub>)Xaa), have been synthesized and evaluated as inhibitors of the zinc endopeptidase thermolysin. In comparison with the previously reported phosphonamidates **1**, the phosphinates **3** lose only 0.1 kcal/mol in binding affinity, whereas the depsipeptides **4** are bound 2.7 kcal/mol more weakly; these values are contrasted to the 4.0 kcal/mol reduction in binding affinity observed for the phosphonates **2** (Cbz-Gly- $\psi$ (PO<sub>2</sub><sup>-</sup>O)Leu-Xaa) in comparison to **1** (Cbz-Gly- $\psi$ (PO<sub>2</sub><sup>-</sup>NH)Leu-Xaa). The observed effects are interpreted through consideration of the differences in active-site and solvent interactions. For the comparison between the diamides **1** and the depsipeptides **4**, a full accounting of the balance between these interactions can be approached. The arylphosphonates **5** (Aryl- $\psi$ (PO<sub>2</sub><sup>-</sup>O)Leu-Leu) were synthesized and evaluated to investigate the importance of phosphonate basicity on the overall binding affinity of these zinc-coordinating inhibitors; the inhibitor *K*<sub>i</sub> values were found to be independent of phosphonate p*K*<sub>a</sub>, indicating that the basicity of the phosphonate moiety exerts counterbalancing effects on the energies of zinc coordination and solvation. For analysis of the influence of structural variations on observed affinity, the definition of "differential binding energy" is introduced as a practical alternative to the concept of "intrinsic binding energy".

The principles of molecular recognition are important for understanding the selectivity of biological transformations and for attempts to mimic such processes through the design of artificial inhibitors and catalysts. Quantitative information on the energy of interaction between protein binding sites and the molecules that associate with them is indispensable in this regard, since this energy is the currency that governs all such transactions. The contribution that an individual functional group can make to the overall energy of interaction can be determined by comparison of related systems in which the functional group has been removed from either the ligand<sup>1</sup> or the protein,<sup>2</sup> and it is most accurately assessed when

structural information on the complex is available.

The bacterially derived endopeptidase thermolysin has proven to be an advantageous system for the detailed evaluation of protein-ligand binding effects. Along with the mammalian digestive enzyme carboxypeptidase A, thermolysin is one of the prototypical members of the class of zinc proteases, and it has played a prominent role in the design of inhibitors for this medically important class of enzymes.<sup>3</sup> The selectivity of ther-

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