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# SUBSTRATE ATTENUATION: AN APPROACH TO IMPROVE ANTIBODY CATALYSIS

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**ABSTRACT.** Antibodies raised to quinaldine phosphonamide **1a** showed no ability to hydrolyze its most homologous substrates amide and ester **2** and **3**, respectively However, within this same set of antibodies some thirteen showed a great propensity to hydrolyse a structurally similar naphthyl ester. In addition to heteroatom discrimination one of the antibodies examined in detail displayed an increase in catalytic efficiency presumably via weak apparent binding ($K_m$) when phenylesters were employed as substrates. These findings suggest abzyme catalysis may be improved via substrate attenuation.

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 [1] 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 [2] To date, the 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 phosphorous 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 complimentary molecular surfaces these entities elicit when they are used as haptens to challenge the immune system.

**Figure 1**

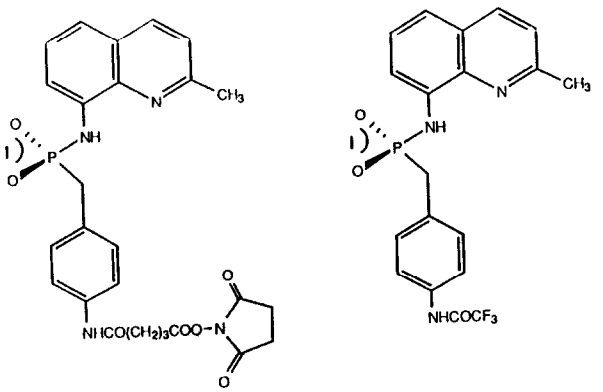
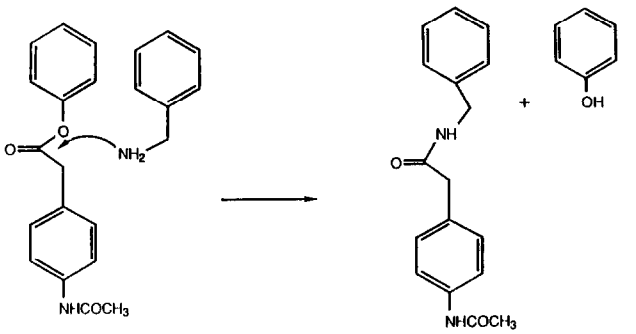


Figure 2




Recently we reported how antibodies obtained to phosphonamidate **1a** (Figure 1) could catalyze a bimolecular amide forming reaction (Figure 2).[2d] Interestingly this same set of antibodies showed no propensity to catalyze the hydrolysis of substrates (**2** and **3**, Figure 3) with greatest homology to hapten **1a**. Herein we report that within this same group of antibodies, a number of them catalyze the hydrolysis of substrates that do not retain all the heteroatom/prominent structural features of the inducing transition state analogue. From these findings an approach is suggested to improve antibody catalytic efficiency.

Immunization with KLH conjugate **1a** produced approximately 1500 hybridoma cells, 200 of which were specific for BSA conjugate **1a** by ELISA Of these 200 antibodies only 55 survived the subcloning process.[3] Initial screening for hydrolytic activity was performed via one of two assays. Amide substrate **2** (Figure 3) was assayed for release of arylamine using a microtiter well colormetric assay[4] and esters **3**, **4-6** (Figure 3) were assayed by HPLC.[2a] Surprisingly, substrates with the greatest homology to hapten **1**, **2** and **3** were not hydrolyzed appreciably over the background rate with any of the 55 antibodies tested. The 1-naphthol ester **6** proved to be a substrate for thirteen of the monoclonals Thus, a simple heteroatom change led to this striking result. Intrigued by these findings we subsequently looked at one of these abzymes in detail including its behavior with other less homologous substrates.

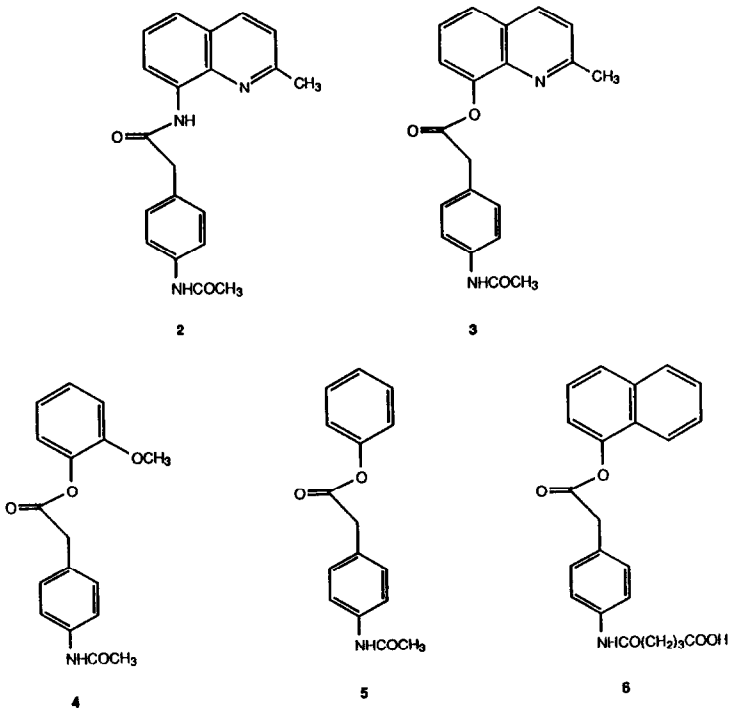
Antibody 37G2 was determined to be one of the more proficient of the thirteen catalytic antibodies. It hydrolyzed naptholester **6** ($t_{1/2}$ = 21 min) and saturation kinetics were observed (Table 1), along with competitive inhibition by transition state analogue **1b**, $K_i$ = 0 5 μm (Table 1) This antibody also demonstrated an ability to hydrolyze ester substrates **4** and **5**. Most interesting was the fact that these esters were even better substrates than naptholester **6**

Monoclonal abzyme 37G2 initial rate of hydrolysis measured as a function of either substrate **4** or **5** concentration followed Michaelis-Menten kinetics (Table 1) Quite apparent from this table is the weaker binding ($K_m$) which is displayed as the substrates (**4-6**) become less similar in structure to the original hapten Also evident is that as $K_m$ increases in this series of substrates so does $v_{max}$. The structural variations in the esters may preferentially reduce the binding of its more stable conformations This would be tantamount to weaker overall binding but increased productive binding of substrate, as the distorted conformer is more likely to lead to hydrolysis due to reduced conjugation or other effects.[5]

In previous reports, catalytic antibodies exhibited high turnover numbers with substrates most congruent to their transition state hapten. In our case, the 55 antibodies specific to hapten **1** displayed no tendencies to accelerate the rate of hydrolysis of quinaldine amide/ester **2** or **3**, respectively. Despite this lack of reactivity observed with these most homologous substrates, we found a large number of antibodies which catalyzed the hydrolysis of an ester which was structurally congruent to the hapten less one heteroatom In addition to heteroatom discrimination, one of the abzymes studied exhibited an amazing increase in catalytic efficiency presumably via weaker apparent binding manifested by an increase in $K_m$ Since substrate destabilization can be a mechanism for enzyme catalysis,[6] it seems reasonable to suggest

that abzyme catalytic efficiency may be improved by simple substrate attenuation. Further structural and theoretical studies will help assess the importance of this mechanism for catalysis by antibodies.

**Figure 3**




## EXPERIMENTAL

Proton nuclear magnetic resonance ($^1$H NMR) spectra were obtained on a Bruker WP100 100 MHz spectrophotometer in $CDCl_3$ or $Me_2SO$-$d_6$. Chemical shifts of samples dissolved in $CDCl_3$ and $Me_2SO$-$d_6$ are reported in ppm downfield from tetramethylsilane. All reagents that are not referenced were obtained from Aldrich Chemical Company, Inc. Hapten **1a** and inhibitor **1b** were synthesized as reported previously.[2d]

**2**, **3**, **5**, and **6**: To a 20 mL methylene chloride solution of the arylamine or arylalcohol (0.5 mmol) was added 4-acetamidophenylacetic acid (0 5 mmol) followed by triethylamine (1 5 mmol) and the solution was stirred 5 min. Bis(2-oxo-3-oxazolidinyl)phosphinic chloride (0.6 mmol) was then added, the reaction was stirred 1 h, followed by dilution with 20 mL methylene chloride The solution was washed with 10% aq. $NaHCO_3$, brine, dried ($Na_2SO_4$), and the solvent was removed in vacuo.

**2**: Chromatography, silica gel ($CH_2Cl_2$-EtOAc 1:1) gave a white solid: 0.14 g (83%); mp 188-189 °C; $^1$H NMR ($CDCl_3$) 9.9 (br s, 1), 8.0 (d, 1, $J$ = 8.6), 7.6 (d, 2, $J$ = 7.1), 7.45-7.3 (m, 4), 7.2 (d, 2, $J$ = 7.1), 3 92 (2), 2.6 (s, 3), 2 2 (s, 3); mass spectrum, exact *m/e* calcd. for $C_{20}H_{19}N_3O_2$ 333 390, found 333 1477

**3**: Chromatography, silica gel ($CH_2Cl_2$-EtOH 20:1) gave a white solid: 0.122 g (73%); mp 99-102 °C, $^1$H NMR ($CDCl_3$) 8.0 (d, 1, $J$ = 8.6), 7 6-7.4 (m, 5), 7.25 (app ABq, 4, $J$ = 12.9, 2 9), 4.05 (s, 2), 2.7 (s, 3), 2.1 (s, 3); mass spectrum, exact *m/e* calcd. for $C_{20}H_{18}N_2O_3$ 334.360, found 334.133.

**5**: Chromatography, silica gel ($CH_2Cl_2$-EtOAc 1:1) gave a white solid. 0.0808 g (58%); mp 132-134 °C, $^1$H NMR ($CDCl_3$) 7 4-7.2 (m, 8), 7.05 (d, 2), 3.8 (s, 2), 2.1 (s, 3); mass spectrum, exact *m/e* calcd. for 269.285, found 269.152

**6**: Chromatography, silica gel (gradient $CH_2Cl_2$ then $CH_2Cl_2$-EtOAc 1:1) gave a white solid: 0.0306 g (20%); mp 148-159 °C, $^1$H NMR ($CDCl_3$) 7.65 (br s, 7), 7.55-7 05 (m, 6), 6.9 (m, 2), 3 85 (s, 2), 3.7 (s, 3), 2.05 (s, 3); mass spectrum, exact *m/e* calcd. for 300.335, found 300.274.

**4**: To a solution of the phenyl 4-(amino)napthylacetate (0.08 g, 0 28 mmol) in 3 mL methylene chloride was added 0.044 g (0.28 mmol) glutaric anhydride. The reaction mixture was stirred and heated at 40 °C for 1 h, diluted with 10 mL ethyl acetate, and washed with 0.5 M HCl (2 x 10 mL). The organic layer was dried ($Na_2SO_4$) and the solvent was removed in vacuo. Chromatography, silica gel ($CH_2Cl_2$-MeOH 5:1) gave a white solid: 0 085 g (77%): mp 148-149 °C, $^1$H NMR (DMSO-$d_6$) 9.8 (br s, 1), 7.9-7.3 (m, 7), 7.25 (d, 2, $J$ = 8.4), 7.1 (d, 2, $J$ = 8.4), 3.9 (s, 2), 2.1 (t, 4, $J$ = 7.1), 1 6 (m, 2); mass spectrum, exact *m/e* calcd. for $C_{23}H_{18}N_2O_3$ 392.210, found 392.149.

Table 1. Kinetic parameters for antibody 37G2 with various substrates [a]

| Ab | Substrate | $v_{max}$ (nM sec$^{-1}$) | $K_m$ (μM) | $K_i$ (μM) | $k_{cat}$ (sec$^{-1}$) | $k_{cat}/k_{uncat}$ |
|---|---|---|---|---|---|---|
| 37G2 | **4** | 3 3 | 33 | 0 5 | 00054 | 71 |
| 37G2 | **5** | 5.5 | 143 | - | 00092 | 153 |
| 37G2 | **6** | 20 | 768 | - | .0033 | 1976 |

[a]Esterolysis rates were measured on a HP 8452a Dioad array UV-vis spectrophotometer at 308 nm for naptholester substrates **4**, 274 nm for **5**, and 282 nm for **6**. Reactions were preformed in a 1 mL quartz cuvette maintained at 25 °C ± 0.10 °C by a water-jacketed cell holder equilibrated to a Lauda RM6 circulation water bath. A stock solution of ester **4**, **5** or **6** was prepared in DMF and diluted to the desired concentration in phosphate buffer [50 mM, pH 8.0] giving a final volume of 5% DMF. Reaction mixtures were equilibrated for 5 min and initiated by the addition of an aliquot of antibody solution. The initial linear rates were measured at < 10% hydrolysis of total substrate. The observed rate was corrected for the uncatalyzed rate of hydrolysis in the absence of antibody. Kinetic parameters $v_{max}$, $K_m$, and $K_i$ were determined by nonlinear least-squares fitting of the initial rate vs substrate concentration to a hyperbolic curve described by the Michaelis-Menten equation.

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