Use of antibodies to dissect the components of a catalytic event. The cyclopropenone hapten

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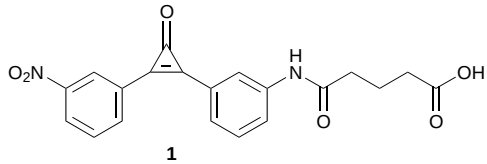
Antibodies elicited against the planar cyclopropenone hapten 1 efficiently catalyze ester hydrolysis, highlighting the importance of charge rather than shape complementarity as a design element of hydrolytic antibodies.

In the most common approach to the design of antibody catalysts the experimenter simultaneously incorporates a series of parameters that will operate in concert to achieve a catalytic event. A less frequently discussed but equally important approach is to apply special hapten design to induce only selected parameters and thereby assess their role in catalysis. Here we take the less common approach in order to examine the relative importance of two key parameters—charge *vs.* shape complementarity in the antibody active site. The relative importance of these parameters is of central significance not only in hapten design but also in immunochemistry in general.

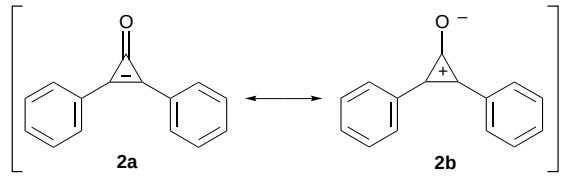
Mechanistic and X-ray crystallographic studies of both esterolytic enzymes[1] and catalytic antibodies highlight the importance of three design elements when planning inhibitors and haptens. I. *Tetrahedral geometry*, needed to mimic the rehybridization of the carbonyl carbon in the transition state. II. *Negative charge*, needed to create a stabilizing environment for the developing oxyanion. III. *Positive charge*, needed to generate a general base residue in the active site. Haptens with design elements I and II, *e.g.* phosphonates, amidophosphonates, phosphates, have been extensively used to elicit antibodies that catalyze various interconversion reactions of carboxylic acid derivatives.[2] Design elements I and III were also used to generate hydrolytic antibodies.[3] Few attempts to simultaneously employ all design elements, I, II and III, in order to achieve polyfunctional catalytic antibodies have been reported.[4,5]

A common assumption that lies at the basis of all of the above mentioned studies is that the shape complementarity element I is an indispensable feature to be considered in the design of hydrolytic antibodies. Here we report on esterolytic antibodies that were raised against the cyclopropenone functionality. Hapten **1** reflects the electrostatic design elements II and III but lacks the geometrical design element I, thereby allowing us to examine the importance of charge complementarity alone.



1

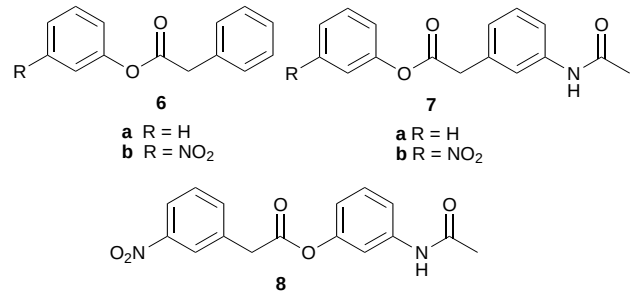
Hapten **1** presents a cyclopropenone ring as well as two aromatic recognition elements. Diphenylcyclopropenone, **2**,[6] may be described by two canonical resonance forms **2a** and **2b**. The solid state structure of **2** indicates that its carbonyl bond is highly polarized, suggesting a significant contribution of the aromatic (2π electrons) zwitterionic structure **2b**. This polarization may be enhanced by the formation of hydrogen bonds.[7] The physical properties of **2** (^{17}O NMR, IR and dipole moment) provide supporting evidence for the existence of a negative charge on the oxygen atom.[5] The remarkable chemical stability of **2** under acidic conditions is also attributed to the aromatic nature of the cyclopropenium moiety.



2a **2b**

The synthesis of **1** was achieved in four steps starting with mono-nitration of **2** (Aldrich) to give 2-(3-nitrophenyl)-3-phenylcyclopropenone **3**.[8] The nitro group was reduced with Ti^{III} to the corresponding amine, **4**. Reaction with glutaric anhydride to produce the monoamide **5** (**3**–**5** not shown),[9] followed by nitration of the unsubstituted phenyl ring, afforded hapten **1**.[10]

The five substrate esters **6**–**8** were prepared by acid catalyzed esterification of the substituted phenylacetic acid with the appropriate phenol.



6 **7** **8**

a R = H
b R = NO_2

a R = H
b R = NO_2

Following immunization of 129^{IX+} mice with the Keyhole Limpet Hemocyanin (KLH) conjugate of **1**, 15 hybridoma cells producing anti-**1** antibodies were selected for further studies. Antibodies from each cell-line were purified by ammonium sulfate precipitation, anion exchange, and protein-G affinity chromatography. The antibodies were screened for catalytic activity by monitoring the release of *m*-nitrophenol (λ_{max} = 330 nm) in the hydrolysis of **6b** and **7b** using a microplate reader. Antibodies 10E8, 11G4, 12G2, 13B6 and 15D3 were found to be catalytic and antibody 12G2 was selected for this study.

The antibody catalyzed reactions were carried out by mixing a phosphate-buffered saline (PBS) solution (50 mM phosphate, 100 mM NaCl, at different pHs) of antibody 12G2 (12 μM, 0.045 ml) with MeCN solutions (0.005 ml) of the substrate **6b** at different concentrations (0.04–0.6 mM) and monitoring them

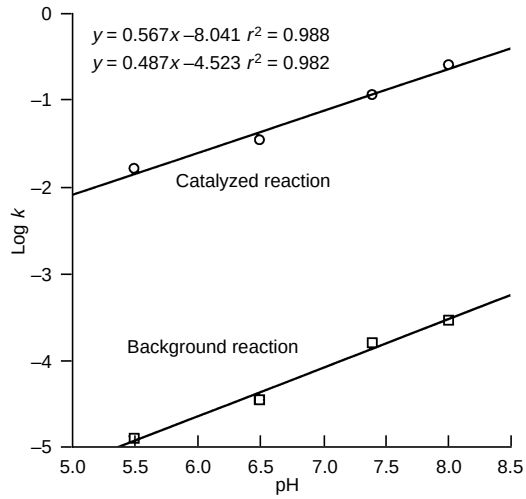


Fig. 1 pH–rate profile of 12G2-catalyzed hydrolysis of **6b**

Table 1 Kinetic parameters 10^{-5} for the 12G2 catalyzed hydrolysis of the studied substrates

Substrate	$k_{un}/10^{-5}$ min^{-1}	K_m/μM	$k_{cat}/10^{-2}$ min^{-1}	k_{cat}/k_{un}
6b	3.6	100	3.5	970
7b	7.0	480	1.4	200
8	2.7	90	0.3	110

by HPLC. The background (uncatalyzed) reaction rate constants (k_{un}) were determined in the presence of 12G2 and **1** (0.001 ml, EtOH solution, equimolar to the substrate).[11] The catalyzed reaction rate constants (k_{cat}) were obtained from Lineweaver–Burk plots of the kinetic data.

The pH rate profiles (log *k vs.* pH) of the catalyzed and uncatalyzed hydrolysis of **6b** between pH 5.5 and 8 (Fig. 1) show that both reactions have the same linear dependence on pH. The fact that both lines in Fig. 1 exhibit the same slope suggests that catalysis does not involve any strain/rehybridization effect and both reactions proceed along the same mechanism, namely, general base catalysis.

A comparative study of the substrate range was carried out at pH 6.5. The kinetic parameters for three different substrates, derived from the corresponding Lineweaver–Burk plots are given in Table 1. While rate enhancement of 2–3 orders of magnitude is achieved with substrates **6b**, **7b** and **8**, no catalysis is observed with **6a** and **7a**, a fact that highlights the importance of the nitro group as a substrate recognition element. Comparison of the kinetic data of **6b** and **7b** indicates that the presence of an acetamido group in the substrate decreases both substrate binding and catalytic efficiency. This observation supports the above-mentioned conclusion that the antibody cannot distort the substrate to fit the binding pocket of 12G2. Ester **8** binds to 12G2 as well as **6b** does but its catalyzed hydrolysis is one order of magnitude slower.

Interestingly, very little is known about natural products containing a cyclopropenone fragment.[12] A papain inhibitor possessing a cyclopropenone moiety exhibits a K_i in the submicromolar level. The mechanism for the action of this unique protease inhibitor still remains unclear.[13]

Catalytic antibodies offer unique opportunities to examine mechanistic hypotheses and the relative importance of individual design elements in catalysis. We have shown here that antibodies elicited against hapten **1** catalyze ester hydrolysis with a 1000-fold rate enhancement. As hapten **1** does not mimic the shape of the transition state, yet elicits efficient catalysts, it is likely to generate the necessary charge complementarity in the active site. Thus, haptenic tetrahedral geometry is a desirable feature but not a prerequisite to generating hydrolytic antibodies. This study, together with the information reported about a cyclopropenone-containing protease inhibitor, highlights the potential applications of the rarely used cyclopropenone functionality in the future design of both haptens and enzyme inhibitors.

We are grateful to Dr S. C. Sinha for early contributions to this work and to D. M. Kubitz for antibody preparation. We thank Pharmore Therapeutics and The Skaggs Institute for Chemical Biology for financial support. F. G. thanks the Lady Davis foundation for a postdoctoral fellowship.

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9 Preparation of **5**: **4** (0.55 g, 2.5 mmol) was dissolved in dry THF (25 ml). Glutaric anhydride (1.42 g, 12.5 mmol) was added and the mixture was stirred at room temp. for 12 h. Work-up and purification by column chromatography (silica gel, EtOAc–hexane 4 : 1) afforded **5** (0.21 g, 25%) in the form of a colorless solid. δ_H($CDCl_3$ with 2 drops CD_3OD) 8.05 (s 1 H), 7.90 (s br, 2 H), 7.84 (s br, 1 H), 7.59 (d, *J* 9.72, 1 H), 7.50 (s, 3 H), 7.41 (t, *J* 7.48, 1 H), 2.35 (t, *J* 7.68, 2 H), 2.31 (t, *J* 6.76, 2 H), 1.91 (t, *J* 7.80, 2 H); *m/z* (ESI) 334 ($M - H^+$).
10 Preparation of **1**: sodium nitrate (0.025 g, 0.298 mmol) was added to a stirred solution of 2-[3-(4-carboxybutanamido)phenyl]-3-phenylcyclopropenone (0.1 g, 0.298 mmol) in conc. sulfuric acid (1 ml). The mixture was stirred for 1 h at room temp., then heated to 100 °C for 1 h and poured into ice–water. The precipitate was washed with water, dissolved in EtOAc and passed through a silica gel bed using EtOAc to give **1** (0.053 g, 31%) in the form of a colorless solid. δ_H(CD_3OD) 8.87 (s, 1 H), 8.52 (d br, 1 H, *J* 8.28), 8.50 (s, 1 H), 8.44 (d br, 1 H, *J* 7.04), 7.95 (t, 1 H, *J* 7.72), 7.82 (d, 1 H, *J* 8.04), 7.76 (d, 1 H, *J* 7.44), 7.66 (s, 1 H), 7.61 (t, 1 H, *J* 7.80); *m/z* (LSI) 381 (MH^+).
11 No change in the rate of the 12G2-catalyzed hydrolysis of **6b** could be detected in the presence of ethanol (0.001 ml). Partial inhibition of the reaction was observed in the presence of compounds **2**, **3** and **5** (1 equiv. with respect to the substrate).
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Received in Cambridge, UK, 9th January 1998; 8/00274F