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Squaric monoamide monoester as a new class of reactive immunization hapten for catalytic antibodies

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Abstract—A squaric monoester monoamide motif was employed as an effective reactive immunogen for the discovery of monoclonal antibodies with reactive residue(s) in their combining sites. Two antibodies, 2D4 and 3C8, were uncovered that enhance paraoxon hydrolysis over background. Kinetic analysis of these antibodies was performed and interestingly both undergo a single turnover event due to covalent modification within the antibody combining site. Because antibodies 2D4 and 3C8 result in covalent attachment and thus inactivation of paraoxon, they could be useful probes for investigating paraoxon intoxication.
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Reactive immunization, the most recently developed hapten manifold, has led to the production of new antibody catalysts.[1–3] In contrast to traditional hapten strategies, where 'non-reactive' transition state analogs are employed, reactive immunization relies on an antigen so highly reactive that a chemical reaction occurs in the antibody combining site during immunization. The importance of this approach is that it allows for the isolation of antibodies that have highly reactive residues in their binding cleft, which, if engaged with an appropriate substrate homologue, can provide proficient turnover.[3–5] Reactive immunization has been viewed as a pivotal tool for the recruitment of more sophisticated catalytic antibodies. However, despite its undeniable merits, exploration and success of new reactive immunization haptens have been limited to only two major manifolds, phosphonate diesters[3] and β-diketones.[6–8] Clearly, new reactive immunization haptens could provide additional opportunities for harnessing promising antibody catalysts.

Paraoxon is a representative phosphate insecticide that is used as the dominant form of insect control worldwide.[9] Unfortunately, these insecticides are often found responsible for the poisoning of agricultural workers.[10] The toxic effect of this class of insecticide is known to be associated with their inhibition of mammalian acetylcholinesterase.[11–13] Antibodies have a long history of being used as therapeutic tools for the treatment of poisoning via neutralization and elimination of toxins from susceptible tissues.[14–16] Catalytic antibodies would be especially advantageous for this purpose, as a catalytic antibody would not only sequester the foreign antigen, but also decompose and subsequently release innocuous products, thus regenerating the antibody for further substrate degradation.

We have mapped out the design and preparation of a new class of reactive immunization hapten based on the well-developed chemistry of squaric acid and its derivatives. The synthesis of squaric acid was first achieved in 1959 and this initial report spurred the syntheses of a vast array of squaric acid derivatives.[17–19] Exploration of this class of molecule has been constant due to its unique chemical properties, wherein the general reactive motif of squaric acid is due to its two acidic hydroxyl groups ($pK_{a1} = 0.54$, $pK_{a2} = 3.48$).[20,21] These functionalities act not only as proton acceptor sites, but also as nucleophilic binding loci for divalent metal ions.[20,22–25] The reactivity of squaric acid is exemplified further by its diester derivatives that can undergo stepwise nucleophilic substitution when treated with nucleophiles, i.e., amines, giving rise to the corresponding squaramides.[26] This unique reactivity enables the employment of squarate molecules in multiple research areas such as enzyme inhibition and the

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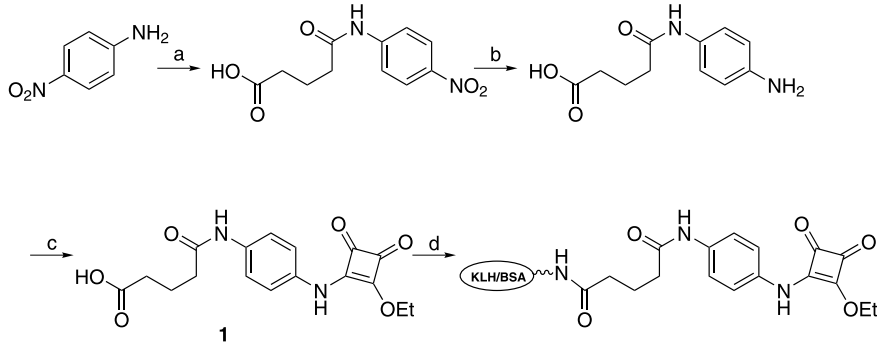
conjugation of oligosaccharides to proteins and polyazamacrocycles.[27–29] Herein, we report the design, preparation, and immunization results of a reactive immunization hapten based on a squaric monoester monoamide motif; two of the monoclonal antibodies (mAbs) elicited during immunization showed the ability to recognize and cleave paraoxon, a common phosphotriester pesticide.[9]

Hapten **1** was designed and synthesized as described in Scheme 1. Ethyl squarate **1** was programmed to trap a nucelophilic residue at the antibody combining site via a nucleophilic substitution reaction (Scheme 2). Hapten **1** was coupled to the carrier proteins, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), by prior activation with sulfo-*N*-hydroxysuccinimide and 1-[3-(dimethylamino)propyl]-3-ethylcarbidiimide hydrochloride (EDCI) while the KLH-**1** conjugate was utilized to inoculate Balb-c mice. Following antibody production by standard hybridoma technology,[30,31] 17 (mAbs) were generated that bound to the BSA-**1** conjugate. All (mAbs) were found to belong to the IgG class and were purified as described previously.[2]

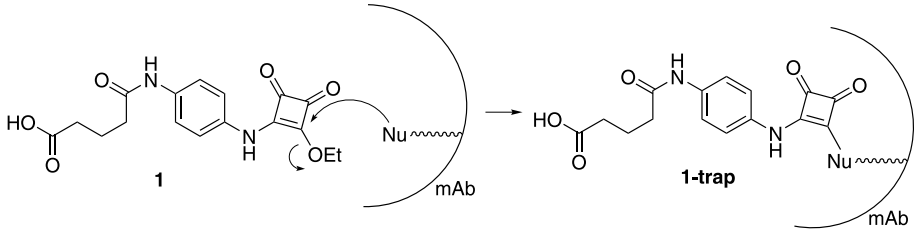
The 17 mAbs were screened for phosphotriesterase activity against the substrate paraoxon (Scheme 3), a commonly used pesticide often found poisonous among agricultural workers. We chose this substrate based on the following criteria: (A) phosphotriesters are more susceptible to nucleophilic hydrolysis relative to phosphodiester and monoester; therefore, paraoxon as substrate might provide sufficient reactivity toward the antibody catalyst during initial screening; (B) hydrolysis of paraoxon leads to the release of 4-nitrophenol, a compound that can be easily detected quantitatively by well-established UV assay methods[32]; (C) antibodies that catalyze paraoxon hydrolysis could have potential therapeutic value in the treatment of paraoxon intoxication.

The reactions were carried out in 50 mM bicine buffer (pH 8.0) and monitored at 405 nm on a Cary50 UV spectrometer to detect the release of 4-nitrophenol. In each reaction, 20 μM antibody was reacted with 1 mM substrate. From the 17 mAbs that were elicited against hapten **1**, two mAbs, 2D4 and 3C8, were found to accelerate the hydrolysis of paraoxon with moderate rate enhancement over the background reaction. However, only single turnover was observed for both antibodies. The pre-steady-state kinetic data are summarized in Table 1.

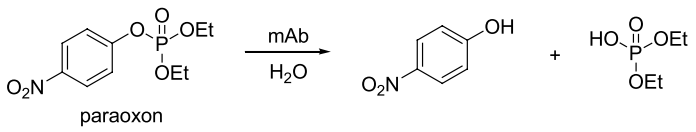
The observation of a single turnover led to the speculation that the antibodies might be susceptible to product



Scheme 1. Synthesis of the squaric monoester monoamide hapten. Reagents: (a) glutaric anhydride, $CHCl_3$, 84%; (b) Pd/C, H_2, MeOH, 84%; (c) 3,4-diethoxycyclobut-3-ene-1,2-dione, DIPEA, EtOH, 83%; (d) EDCI, sulfo-NHS, BSA/KLH.



Scheme 2. Hypothetical reactive immunization mechanism using hapten **1** to trap a nucleophilic residue within antibody combining site.



Scheme 3. Hydrolysis of paraoxon.

Table 1. Kinetic data for mAb, 2D4, and 3C8 that accelerate the paraoxon hydrolysis[a]

mAb	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/k_{uncat}	k_{cat}/K_m ($M^{-1}min^{-1}$)
2D4	2.28×10^{-3}	1.18	2.31×10^{3}	1.93
3C8	2.03×10^{-3}	1.29	2.06×10^{3}	1.57

[a] Determined in 50 mM bicine with 50 mM NaCl, pH 8.0, 5% DMSO cosolvent at 23 °C. The k_{uncat} (paraoxon) = 9.86×10^{-7} min^{-1}.

inhibition. Thus, the possibility of active site product inhibition was further investigated as follows. Both antibodies 2D4 and 3C8 were incubated with a stoichiometric amount of 4-nitrophenol, one of the products of paraoxon hydrolysis. Neither antibody was affected by 4-nitrophenol, as both maintained hydrolytic activity against paraoxon, suggesting the other product of hydrolysis might be an inhibitor. Accordingly, both antibodies were incubated with readily available diethyl phosphorochloridate, which immediately hydrolyzes to the diethyl hydrogen phosphate under the assay conditions; again, neither antibody's hydrolytic activity was impaired.

The observation of a single turnover and a lack of product inhibition strongly suggested that paraoxon could participate in a covalent modification of the antibody, inactivating it toward further hydrolysis. To investigate this hypothesis, both antibodies were incubated with paraoxon in large excess at 37 °C for 60 h. After incubation, both antibodies were subjected to exhaustive dialysis against bicine and assayed for hydrolytic activity. Neither antibody was capable of further hydrolysis of paraoxon. The inactivated antibodies were subsequently treated with hydroxylamine in 50-fold excess at 37 °C for 4 h. After dialysis, about 40% of the hydrolytic activity was recovered for both antibodies. This evidence strongly suggested that paraoxon was a covalent active site inhibitor of the antibodies 2D4 and 3C8.

To probe further possible 'reactive immunization' and its mechanism, we investigated hapten analog **2** (Scheme 4) as an inhibitor. A stoichiometric amount of this molecule was incubated with both antibodies, and to the mixture was added 1 mM paraoxon. After 2 h incubation, the reactivity of both 2D4 and 3C8 was fully quenched. This same observation was made for incubation times as short as 10 min, indicating that the binding of **2** is much more favored over that of paraoxon. For this reason, a time-dependent paraoxon hydrolysis profile for this inhibitor could not be obtained. Exhaustive dialysis of analog **2**-inhibited antibody did not regenerate antibody hydrolytic reactivity. However, treatment of the inactivated antibody with 50-fold excess hydroxylamine at 37 °C for 4 h followed by dialysis fully recovered the catalytic activities of both antibodies. Again, this evidence strongly suggests covalent modification at a reactive residue in the combining site of these antibodies.

Putting this current research effort in perspective, a catalytic antibody was previously disclosed by our laboratory to decompose paraoxon.[32] Here, the *N*-oxide **3** was utilized as the hapten, following a 'bait-and-switch' strategy[33,34] (Fig. 1). The hapten retains the nitrophenyl group and general geometry of paraoxon. The partial positive charge on the *N*-oxide nitrogen was placed to induce a general base in the antibody combining site to assist in the formation of hydroxide in proximity to the phosphorus atom, while the partial negative charge on the *N*-oxide oxygen atom could induce a general acid capable of either protonating the leaving oxide group or stabilizing the developing negative charge on the P=O bond during the transition state. Out of 25 mAbs, antibody 3H5 was uncovered as a catalyst against paraoxon hydrolysis. However, a linear dependence of the rate of catalysis upon hydroxide ion concentration implied that antibody 3H5 catalyzed the reaction primarily through transition-state stabilization rather than a 'bait-and-switch' general acid/base catalysis mechanism.

In our current study, we rely on a reactive immunogen to elicit antibody catalysts that could decompose paraoxon via covalent catalysis. In our approach, we took advantage of the susceptibility of the squaric acid monoester monoamide motif toward nucleophilic substitution to trap a nucleophile residue in the antibody combining site. A total of 17 mAbs were raised against hapten **1** and two mAbs, 2D4 and 3C8, accelerated the hydrolysis of paraoxon. Kinetic studies demonstrated that this event is mediated by a reactive residue in the antibody cleft. However, it is likely based on our kinetic investigation that both antibodies are subjected to irreversible inhibition forming an unreactive phosphoryl-antibody intermediate. This phosphoryl-antibody intermediate is stable in buffer; however, in the presence of hydroxylamine the

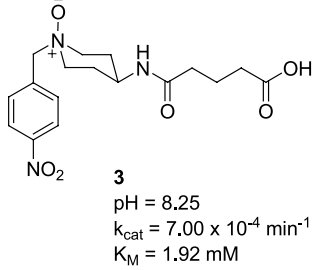
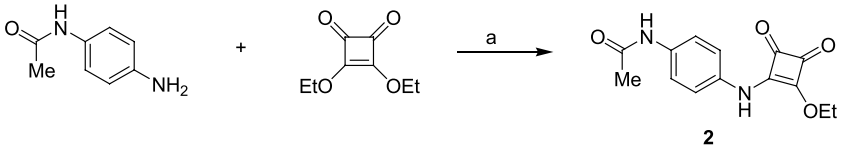


Figure 1. *N*-Oxide hapten designed and synthesized by Lavey and Janda.



Scheme 4. Synthesis of analog inhibitor **2**: (a) 3,4-diethoxycyclobut-3-ene-1,2-dione, DIPEA, EtOH, 93%.

paraoxon-inactivated antibodies were able to regenerate activity, thus restoring the reactive residue in the combining site. Inhibition studies using hapten analog **2** as inhibitor have further confirmed these findings.

We have shown that the squaric monoester monoamide motif is an effective reactive immunogen for the recovery of antibodies with reactive residue(s) in their binding pockets. It is important to note that the initial substrate we used in our study to assay antibody activity was in fact covalent in nature and thus limited our analysis to a single turnover. Future studies will continue probing the kinetic properties of these two antibodies with additional substrates in an effort to uncover true catalysis. However, we note in their current state, antibodies 2D4 and 3C8 could be useful tools for investigating immunotherapy for paraoxon poisoning.

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