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Hydrolysis of an *N*-methylcarbamate by a catalytic antibody†

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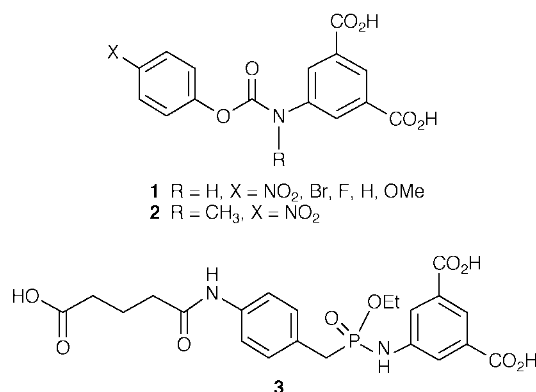
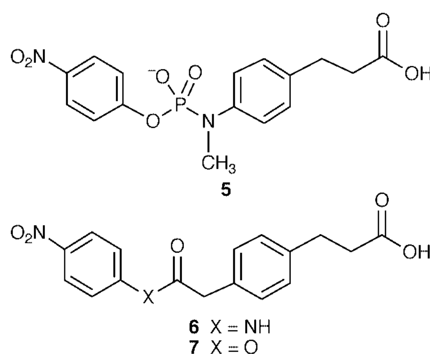
The first example of antibody-catalyzed hydrolysis of an *N*-methylcarbamate, a highly challenging reaction for antibody catalysis, was achieved by raising a monoclonal antibody to a phosphoramidate transition state analogue.

The vast majority of antibody-catalyzed hydrolyses of carbonyl derivatives, such as esters, amides and carbamates, have been performed using substrates that are amenable to hydrolysis: half-lives for the spontaneous reactions usually range from hours to a few months under the assay conditions.[1] Antibody catalysis of more energetically demanding hydrolytic reactions of carbonyl derivatives in which the half-life for the spontaneous reaction is measured in years, rather than hours to months, is rare.[2a–f] As part of a project related to antibody-catalyzed 'remote' prodrug activation,[3] we became interested in examining the possibility of generating antibodies capable of catalyzing the hydrolysis of *N*-methylcarbamates. *N*-methylcarbamates are very resistant to hydrolysis[4] and there has yet to be a report describing an antibody capable of catalyzing this reaction. Several papers have appeared describing antibodies capable of catalyzing the hydrolysis of N–H carbamates.[2e,5] Of particular note is the work of Wentworth *et al.*[2e] These researchers obtained an antibody, DF8-D5, that catalyzes the hydrolysis of N–H carbamates of type **1** by raising antibodies to



the phosphonamidate transition state analogue (TSA) **3**. Although this antibody did not hydrolyze *N*-alkylcarbamates such as **2**, the significantly smaller Hammett ρ value ($\rho = +0.53$) obtained for the antibody-catalyzed reaction compared to that obtained for the uncatalyzed reaction ($\rho = +2.68$) suggested that the antibody reaction proceeded *via* the highly disfavoured $B_{Ac}2$ mechanism rather than the more favoured $E1_cB$ process found for the uncatalyzed reaction.[2e] These results suggest that antibodies raised to an appropriately designed TSA of the $B_{Ac}2$ process for *N*-methylcarbamate hydrolysis might be capable of catalyzing the hydrolysis of these stable compounds. Here we report the first example of antibody-catalyzed hydrolysis of an *N*-methylcarbamate, a highly challenging reaction for antibody catalysis.

Carbamate **4** was chosen as a model substrate for the antibody-catalyzed reaction (Scheme 1). Although this carbamate contains a *p*-nitrophenol leaving group, it is fairly resistant to hydrolysis, exhibiting a half life of approximately 5.7 years at pH 9.0.[4a,6] In the TSA used by Wentworth *et al.*,[2e] the phenolic oxygen of the substrate was replaced with a methylene unit. This was done to promote the $B_{Ac}2$ mechanism over the $E1_cB$ mechanism by minimizing recognition of the phenolate anion, an important feature of the $E1_cB$ transition state.[2e] However, during *N*-methylcarbamate hydrolysis, there is no competing $E1_cB$ mechanism. Consequently, we chose to employ a phosphoramidate TSA (hapten **5**), reasoning that a phosphoramidate should be a better mimic of the $B_{Ac}2$ transition state than a phosphonamidate.



Hapten **5** was constructed† and conjugated to Bovine Serum Albumin (BSA) and Keyhole Limpet Hemocyanin (KLH) *via* its *N*-hydroxysulfosuccinimide ester, which was prepared *in situ via* reaction of **5** with *N*-hydroxysulfosuccinimide in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). The degree of conjugation to the carrier protein was determined using the trinitrobenzene sulfonic acid (TNBS) assay of Habeeb.[7] Balb/c mice were hyperimmunized with the KLH conjugate and monoclonal antibodies were obtained using hybridoma technology.[8]†

Thirty-two monoclonal antibodies were screened for catalytic activity by removing aliquots from a solution containing antibody (~4 μM) and substrate **4** (1 mM) in 50 mM bicine, 5% DMSO, pH 9.0, at various time intervals, and then examining the aliquots for *p*-nitrophenol using HPLC. Under these conditions, we were unable to detect any hydrolysis of **4** in the absence of antibody after 40 h. Several of the antibodies

† The synthesis and characterization of **4**, **5**, **6** and **7**, preparation of monoclonal antibodies and ST51 Fab, experimental details for kinetic studies and Lineweaver–Burk plots are provided as electronic supplementary information, see http://www.rsc.org/suppdata/cc/b0/b000468p/

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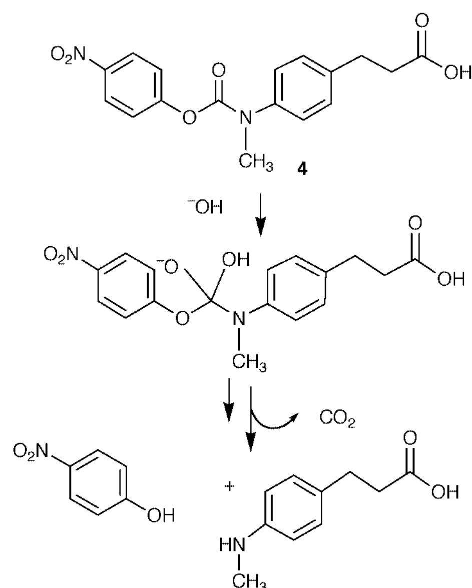

Scheme 1

exhibited catalytic activity. The most active antibody, ST51, was selected for a more detailed kinetic analysis. The ST51-catalyzed reaction obeys saturation kinetics and at pH 9.0 exhibited a $k_{cat} = 9.1 \times 10^{-2}$ h^{-1}, a $K_m = 2.6 \times 10^2$ μM, and $k_{cat}/K_m = 3.5 \times 10^{-4}$ μM^{-1} h^{-1}. The rate enhancement obtained for the reaction (k_{cat}/k_{uncat}) is 6.5×10^3, which is in the range of the average rate enhancement obtained for other antibody-catalyzed hydrolytic reactions of carbonyl derivatives such as esters, amides and carbamates.[1] The rate enhancement obtained with **4** and ST51 (6.5×10^3) is approximately 20 times greater than that obtained by Wentworth *et al.*[2e] for the hydrolysis of **1** when X = NO_2 with DF8-D5 (k_{cat}/k_{uncat} = 300). However, when DF8-D5 is assayed with other substrates (**1**, X = Br, F and OMe), it exhibits greater rate enhancements than that obtained with ST51 and substrate **4**.[2e]

That catalysis is indeed a result of the abzyme and not a contaminating protease or esterase is supported by the following results. First, the reaction is completely inhibited by stoichiometric quantities of the TSA. Second, pure ST51 Fab[10] exhibited the same catalytic activity as pure ST51 mAb. Finally, ST51 did not catalyze the hydrolysis of amide **6** *or even ester* **7**, two compounds that should be readily hydrolyzed by proteases or esterases and both of which undergo spontaneous hydrolysis under the assay conditions more readily than **4**.[11,12] The fact that ST51 does not hydrolyze **6** and **7** also indicates that ST51 is a remarkably selective abzyme compared to other abzymes that catalyze the hydrolysis of amides[2a] or carbamates.[5a]

Although ST51 is also active at pH 10.0 and exhibits a higher k_{cat} (2.5×10^{-1} h^{-1}) at pH 10.0 than at pH 9.0, it exhibits a considerably higher K_m (1.3 mM), and lower rate enhancement (1.8×10^3) at pH 10. ST51 is a robust abzyme, as evidenced by the fact that it can be stored at room temperature at pH 10.0 over several days without any loss of catalytic activity. In addition, ST51 is capable of multiple turnover, indicating that it is not subject to strong product inhibition as is often the case for hydrolase abzymes.[13] This may be due to the fact that carbamate hydrolysis yields CO_2, as opposed to a negatively charged carboxylic acid found with ester or amide hydrolysis, which should experience minimal charge interactions with positively-charged residues in the active site generated in response to the negatively-charged TSA.[13]

In summary, this work represents the first example of antibody-catalyzed hydrolysis of an *N*-methylcarbamate, a highly challenging reaction for antibody catalysis. This was achieved by raising antibodies to a phosphoramidate TSA. Studies concerning the application of this antibody to 'remote' prodrug activation[3] are in progress. In addition, X-ray crystallographic analyses to determine the structure of ST51 Fab are also in progress.[14] The results of the X-ray analysis and further kinetic studies will be used to elucidate the mechanism of this interesting abzyme.

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6 The second order rate constant for the hydrolysis of **4** was determined by measuring its rate of hydrolysis in NaOH solutions (0.1–1.0 M) at 25 °C using spectrophotometry. This yielded a second order rate constant of 3.8×10^{-4} M^{-1} s^{-1}. This value is very close to that obtained (3.7×10^{-4} M^{-1} s^{-1}) by other workers [see ref. (4*a*)] for the hydrolysis of phenyl *N*-methyl-*N*-(*p*-nitrophenyl)carbamate in aq. NaOH solutions at 25 °C. Using a base concentration of 1×10^{-5} M , we obtained a half-life for **4** at pH 9.0 of 5.7 years.

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