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AN ANTIBODY WITH DUAL CATALYTIC ACTIVITY

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Abstract: An antibody raised to a product analogue for a Diels Alder reaction has been found to catalyse sequentially the planned electrocyclic reaction between acetoxybutadiene and an N-alkyl maleimide followed surprisingly by hydrolysis of the acetate of the product.

The potential of catalytic antibodies to provide novel protein catalysts to a given specification has been widely recognised[1]. With the general acceptance of the value of enzymes to provide homochiral building blocks for organic synthesis[2], we decided to attempt to obtain a catalytic antibody that would provide a polyfunctional molecule containing several chiral centres of potential use in organic synthesis. The fullest expression of the value of catalytic antibodies would be in those reactions for which naturally occurring enzymes are not known. We therefore selected the Diels Alder cycloaddition for investigation[3]. The target molecule is shown in figure 1. Others have also investigated antibody catalysis of the Diels Alder reaction[4,5] also using maleimides as substrates.

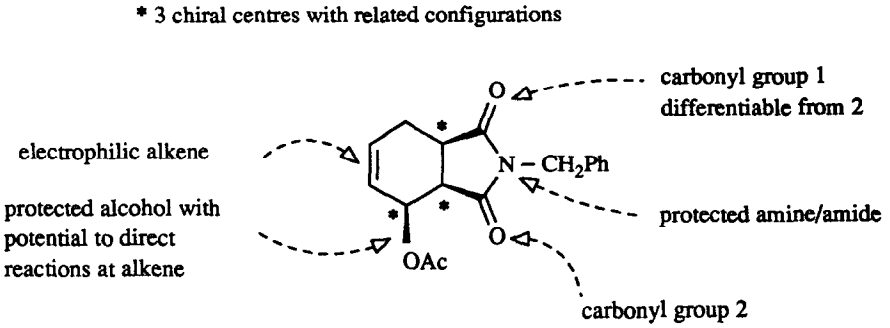

Figure 1

The Diels Alder reaction has been recognised to proceed through a product-like transition state. If the hapten to be selected for raising antibodies were too closely related to the product of the reaction, the tight binding of the transition state would be reflected in product binding as well as in catalysis and product inhibition would be expected to occur. Both Hilvert's and Schultz's group designed haptens that differed significantly from the final product. We limited the difference between product and hapten to the N-alkyl group of the maleimide

through which the hapten was coupled to the immunogenic protein. This strategy is not without risk but, as shown below, was successful.

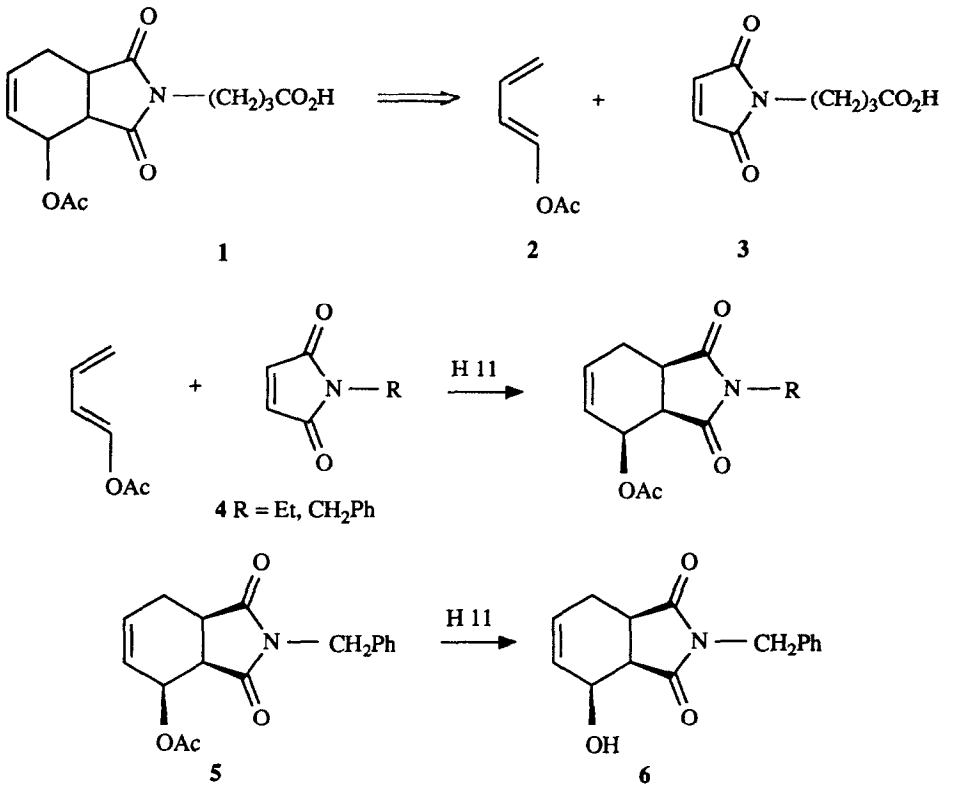

Figure 2.

As described previously[3], mice were immunised with the bovide serum albumin conjugate of the Diels Alder adduct **1** and antibodies were obtained using hybridoma technology. Those that bound the hapten were selected by ELISA using the transferrin conjugate of the hapten. Four clones that catalyse the Diels Alder reaction have been identified of which one, designated H11, has been studied most extensively. H11 accepted both N-ethyl and N-benzyl maleimides as substrates. However it was selective for acetoxybutadiene as substrate; neither reaction of the intrinsically more reactive diene, methoxybutadiene, nor the less reactive pentan-1,3-diene was catalysed. H11 displayed saturation kinetics and the constants k_{cat} = 0.055 s^{-1}, K_m (N-ethylmaleimide) = 8.3 mM were obtained. Acetoxybutadiene underwent slow hydrolysis to afford crotonaldehyde under the reaction conditions and a value for K_m was consequently not obtained. The pseudo first order rate constant of the uncatalysed reaction with a ten fold excess of acetoxybutadiene under the same buffer conditions was determined to be 3.18 x 10^{-5} s^{-1}. From these values k_{cat}/K_m = 6.6 $M^{-1}s^{-1}$ and the rate enhancement was approximately 1,600 fold. H11 can thus be seen to be a moderately effective catalyst for the Diels Alder reaction.

The question of product inhibition was investigated using three compounds, the acetoxybutadiene adducts of N-ethyl-, N-benzyl-, and N-(4-butanoyl)maleimide. In the presence of 31 nM H11, 0.9 mM N-ethylmaleimide, and 0.45 mM N-ethylmaleimide adduct, the rate of cycloaddition of N-ethylmaleimide and acetoxybutadiene was not measurably reduced. Similar behaviour was found for the other two adducts. This result is important because it indicates that with careful selection through screening, antibodies should be available to catalyse useful reactions without the need to invest time in extensive synthesis of complex haptens.

Two unexpected features were also found in studying H11. Firstly, the reaction was found to be pH dependent (figure 3); it was extremely slow at pHs less than 7 but the rate increased rapidly between pH 7 and 8. A pH dependence is not expected for neutral substrates in the Diels Alder reaction although it is well known that the reaction is catalysed by Lewis acids. Two explanations seemed possible for this observation. One possibility is that the antibody undergoes a significant conformational change at above pH 7 moving from an unfavourable to a favourable conformation. To examine this possibility, the CD curves of H11 were measured at 260 and 320 nm over the pH range 5 to 9 but no significant changes were observed. A change in the intrinsic fluorescence of H11 over this pH range was observed which probably reflects local changes in the environment of tryptophan residues. These results do not support a substantial conformational change that might influence catalysis but do not rule out subtle changes involving specific regions of the antibody. A second possibility is that the deprotonation of an acidic group is required for catalysis to occur; in the protonated form, this group could be interacting with acetoxybutadiene reducing its reactivity by protonation. Although such a group has not yet been identified, its presence is consistent with the second unexpected feature of H11, namely catalysis of hydrolysis.

During experiments to isolate the product of the reaction of acetoxybutadiene with the N-benzylmaleimide adduct **5** it was found that the expected ester was difficult to isolate; the major product after an incubation of several hours was identified as the hydroxy product **6** by hplc and ^{1}H nmr spectroscopy in comparison with an authentic sample. Control reactions showed the acetoxy adduct **5** to be completely stable under the reaction conditions. Kinetic experiments then established that H11 was capable of catalysing the hydrolysis of the Diels Alder adduct with the following kinetic constants $k_{cat} = 9.2 \times 10^{-4}$ s^{-1} and $K_m = 1.1$ mM. Thus the rate of hydrolysis was approximately 1/70 of the rate of cycloaddition. To correlate this observation with the unexpected pH dependence of the Diels Alder reaction, the pH dependence of hydrolysis was also measured and a very similar profile emerged but with a maximum at pH 7 (figure 3b). The relationship between the hydrolysis and cycloaddition reactions was investigated further by testing the ability of the initial reactants to inhibit hydrolysis using N-benzylmaleimide. N-Benzylmaleimide (32 mM) was found to inhibit essentially completely the hydrolysis of the corresponding acetoxybutadiene adduct **5** (0.5 mM) in the presence of H11; similarly in the presence of **5** (2 mM), the concentration of N-benzylmaleimide required for 50% inhibition of hydrolysis was approximately 30 mM. A further extensive kinetic analysis of the relationship between hydrolysis and cycloaddition awaits the production of further supplies of H11. These results suggest that we have the first example of a catalytic antibody with dual catalytic activity.

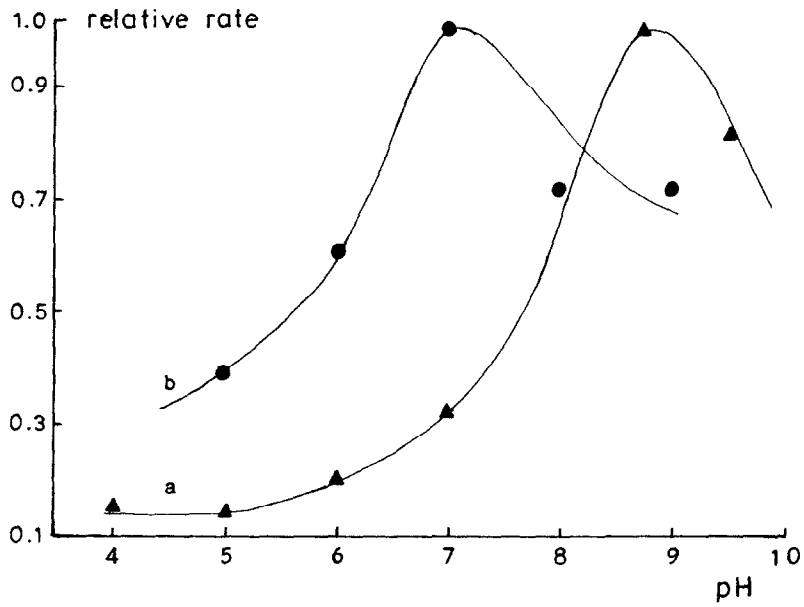

Figure 3

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