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Antibody Catalysis of Some Organic and Biochemical Reactions

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ACADEMIC DISSERTATION

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Abstract

Antibodies are immunoglobulins that bind to stable ground-state molecules and recognise their respective antigens with high affinity and high specificity. Enzymes in turn are natural catalysts that bind and stabilise selectively the transition-state of the reaction and accelerate the rate of a (bio)chemical reaction by lowering the free energy of activation. Enzymes are also able to act as “entropy traps” in reducing the rotational and translational degrees of freedom that are prerequisites for the formation of the activated complex between the reactants. In addition, enzymes use general acid and base catalysis, nucleophiles and co-factors in enhancing the rates of reactions.

It was nearly fifty years ago when Linus Pauling first proposed in his lecture entitled “Chemical Achievement and Hope for the Future” that antibodies binding the ground state molecules might act as enzyme-type catalysts for chemical reactions. Twenty-one years later, William Jencks suggested that it should be possible to obtain an antibody with enzymatic properties by raising it against the antigen that resembles the transition-state of the reaction. It is only eleven years ago that the first reports of the catalytic antibodies emerged from the laboratories of Richard Lerner and Peter Schultz. Indeed, the antibodies elicited against stable, natural or synthetic transition-state analogues of numerous reactions have been found to possess enzyme-like activities. These catalytic antibodies generally display the Michaelian type saturation kinetics, competitive inhibition by the transition-state analogue, selective binding to the transition-state and remarkable substrate specificities.

In the present investigation, structurally different antigens (haptens) were used to study whether it was possible to obtain antibody catalysts for the acyl-transfer, Diels–Alder and peptidyl-prolyl *cis-trans* isomerisation reactions. Acyl-transfer reactions, such as hydrolytic reactions are important transformations both in bio-

chemistry and synthetic organic chemistry; the Diels–Alder reaction is synthetically useful in constructing substituted cyclohexenes; and the peptidyl-prolyl *cis-trans* isomerisation reaction is a highly substantial biochemical reaction which plays a significant role in protein folding, transport and transmembrane signalling.

Monoclonal antibodies were raised against two α-keto amide moiety containing antigens. They were anticipated to induce antibodies for hydrolytic acyl-transfer reactions, *i.e.* ester and amide hydrolyses. α-Keto amide substructures found in natural macrolides such as FK506, rapamycin and cyclotheonamide A are known to mimic the twisted amide bond that is one possible transition-state for the amide bond hydrolysis. During the study, a new, synthetically useful concurrent alkylative de-carbonylation and decarboxylation reaction of methoxy-substituted 3-phenyl-2-oxo-propanoic acids was discovered. It turned out to be a viable method for the preparation of isopropyl anisoles and veratroles, producing them in high yields.

The elicitation of antibodies against the freely-rotating, lipophilic and highly aromatic ferrocene haptens as loose transition-state mimics was successful. Both *endo* and *exo* selective antibodies catalysing the Diels–Alder reaction between 4-carboxy-benzyl *trans*-1,3-butadiene-1-carbamate and *N*,*N*-dimethylacrylamide were found. High regio-, diastereo- and enantioselectivities and no product inhibition were observed. Moreover, the found Diels–Alderases had effective molarities comparable to those of antibodies elicited against the constrained bicyclo[2.2.2]octene haptens.

The dicarbonyl moiety in natural products FK506 and rapamycin and less complex pyruvylamides adopts an orthogonal conformation and possibly serves as a twisted-amide mimic. The α-keto Val–Pro–Phe hapten was anticipated to induce anti-body binding sites that were complementary to the twisted α-keto amide functionality and of hydrophobic character. Indeed, two antibodies were found to catalyse the *cis* to *trans* isomerisation of the fluorophoric tripeptides and the 4-nitroanilide substrates as characterised using both direct fluorescence quench and chymotrypsin-coupled assays, respectively. Both catalyst showed competitive inhibition by the antigen derivative, and the product inhibition, *i.e.* binding to the *trans* isomer, did not appear to be significant. In catalysis and binding the peptide substrates, factors other than simple hydrophobic interactions are possibly involved, such as transition-state stabilisation and ground-state destabilisation.

Preface

The experimental part of this investigation was carried out at the Departments of Molecular Biology and Chemistry of The Scripps Research Institute (TSRI, La Jolla, California, USA) during the years 1992–1995, and at Chemical Technology, Technical Research Centre of Finland (VTT) during the years 1992–1996.

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Helsinki, February 1997,

Contents

List of original publications

This thesis consists of the following papers (Appendices I–IV in this publication), referred to in the text by the Roman numerals given below.

I Yli-Kauhaluoma, J. and Janda, K. D. 1994. Twisted α-Keto Amides as Transition-State Analogues for Acyl-Transfer Reactions: Synthesis of the Immunoconjugates. *Bioorg. Med. Chem.* **2**, pp. 521 – 528.

II Yli-Kauhaluoma, J. and Janda, K. D. 1994. An Expedient Synthesis of Isopropyl Anisoles and Veratroles. *Tetrahedron Lett.* **35**, pp. 4509 – 4510.

III Yli-Kauhaluoma, J. T., Ashley, J. A., Lo, C.-H., Tucker, L., Wolfe, M. M. and Janda, K. D. 1995. Anti-Metallocene Antibodies: A New Approach to Enantioselective Catalysis of the Diels–Alder Reaction. *J. Am. Chem. Soc.* **117**, pp. 7041 – 7047.

IV Yli-Kauhaluoma, J. T., Ashley, J. A., Lo, C.-H., Coakley, J., Wirsching, P. and Janda, K. D. 1996. Catalytic Antibodies with Peptidyl-prolyl *Cis-trans* Isomerase Activity. *J. Am. Chem. Soc.* **118**, pp. 5496 – 5497, + 26 suppl. p.

Abbreviations

ABTS	2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)
Abz	2-aminobenzoyl
Ac	acetyl
BICINE	*N*,*N*-bis(2-hydroxyethyl)glycine
Boc	butoxycarbonyl
BOPCl	bis(2-oxo-3-oxazolidinyl)phosphinic chloride
B.p.	boiling point
BSA	bovine serum albumin
Bz	benzoyl
Bzl	benzyl
CDR	complementarity determining region
Cp	cyclopentadienyl
CsA	cyclosporin A
cy	cytosolic
Cyp	cyclophilin
d.e.	diastereomeric excess
DMEM	Dulbecco’s Modification of Eagle’s Medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulphoxide
EC	Enzyme Commission
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
EDIA	*N*,*N*-diisopropylethylamine
e.e.	enantiomeric excess
ELISA	enzyme-linked immunosorbent assay
EPPS	4-(2-hydroxyethyl)piperazine-1-propanesulphonic acid
eu	entropy unit [cal mol^{-1} K^{-1}]
FAB	fast atom bombardment
FKBP	FK506 binding protein
HAT	hypoxanthine, aminopterin and thymidine
HOMO	highest occupied molecular orbital
Ig	immunoglobulin
KLH	keyhole limpet hemocyanin
L.	Linné, Carl von, Swedish biologist (1707 – 1778)
LUMO	lowest unoccupied molecular orbital
mAb	monoclonal antibody
mito	mitochondrial
MPL	monophosphoryl lipid A
mRNA	messenger ribonucleotide acid
NBA	nitrobenzyl alcohol
N. D.	not determined
NHS	*N*-hydroxysuccinimide
NMO	*N*-methylmorpholine *N*-oxide
PBS	phosphate buffered saline
PCC	pyridinium chlorochromate
PDC	pyridinium dichromate

Ph	phenyl
PMA	phosphomolybdic acid
pNA	*para*-nitroaniline
PPIase	peptidyl-prolyl *cis-trans* isomerase
salen	*N,N'*-bis(salicylidene)ethylenediamine dianion ligand
Suc	succinyl, succinic, $-CO(CH_2)_2CO_2H$
TDM	trehalose dicorynomycolate
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran
TMS	tetramethylsilane
TPAP	tetra-*n*-propylammonium perruthenate
trien	triethylenetetramine
TRIS	tris(hydroxymethyl)aminomethane
Xaa	any of the naturally occurring amino acids
Å	ångström [1 Å = 10^{-10} m]

Amino acid	**S Symbol**	**Amino Acid**	**Symbol**
Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine	Asn	Methionine	Met
Aspartic acid	Asp	Phenylalanine	Phe
Cysteine	Cys	Proline	Pro
Glutamine	Gln	Serine	Ser
Glutamic acid	Glu	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

Kinetic parameters

k_{cat}	catalytic constant, turnover number
k_{uncat}	rate constant for the uncatalysed and thermal background reaction
K_M	Michaelis constant
K_i	competitive inhibition constant
K_d	dissociation constant
k_{cat}/K_M	specificity constant, second-order rate constant
[S]	substrate concentration

1 Introduction

Antibodies are glycoproteins generated by the immune defence system to recognise foreign molecules and neutralise or intiate their removal. The immune system is capable of producing antibodies against proteins, nucleic acids, carbohydrates and even small organic molecules, provided that they are linked to an immunogenic carrier protein. A pool of antibodies can be regarded as a diverse library consisting of a variety of affinities and specificities towards their antigens (Lerner and Benkovic, 1988, Schultz, 1989, Lerner *et al.*, 1991). The antibody molecules resemble natural enzyme catalysts in that they also bind their respective ligands with high affinity, specificity and selectivity. Despite these similarities, most antibodies are not catalytic. In 1986, the first examples of antibody catalysts were published by Lerner and Schultz laboratories (Tramontano *et al.*, 1986, Pollack *et al.*, 1986). Until today, over 80 chemical reactions have been successfully catalysed by specific, catalytic antibodies with the rate accelerations of up to approximately 10^8. These reactions also include such thermodynamically disfavoured transformations that do not seem to occur in biological chemistry.

1.1 Antibodies as catalysts for organic reactions

It was nearly fifty years ago when Pauling (1948) proposed that antibodies binding the ground state molecules might act as enzyme-type catalysts for chemical reactions. Twenty-one years later, Jencks (1969) suggested that it should be possible to obtain an antibody with enzymatic properties by raising it against an antigen that resembles the transition-state of the reaction. It is only eleven years ago that the first reports of the catalytic antibodies emerged from the Lerner and Schultz groups (Tramontano *et al.*, 1986, Pollack *et al.*, 1986). Indeed, the antibodies elicited against the stable, synthetic transition-state analogues of numerous reactions have been found to possess enzyme-like activities. These catalytic antibodies generally display the Michaelian type saturation kinetics, competitive inhibition by the transition-state analogue, selective binding to the transition-state and remarkable substrate

specificities. In this chapter, the structure, properties and generation of monoclonal antibodies are presented. In addition, examples of the antibody-mediated reactions catalysed by different mechanisms will be discussed. The enzymes typically utilise transition-state stabilisation as one of their major catalytic mechanism. General acid–base or nucleophilic catalysis, proximity effects (entropic effects), co-factors and desolvation mechanisms also play a significant role in the enzymatic catalysis. Not surprisingly, the same mechanisms are generally exploited by catalytic antibodies, and often the catalytic antibodies may utilise complex multi-step reaction mechanisms.

1.1.1 Structure and properties of antibodies

There exist five different classes of antibodies in nature. Their heavy chains are designated as α, δ, ε, γ and μ and the corresponding classes of antibodies as IgA, IgD, IgE, IgG and IgM, respectively. Generally, the antibodies belonging to the antibody classes IgG, IgA and IgM have been used in studying their catalytic properties. The molecular weight of a typical immunoglobulin G class antibody (IgG) is approximately 150 kD. Antibodies are capable of recognising their respective antigens with high affinity and high specificity. The binding constants of the antibodies to their antigens range typically from 10^{-14} to 10^{-4} M^{-1}.

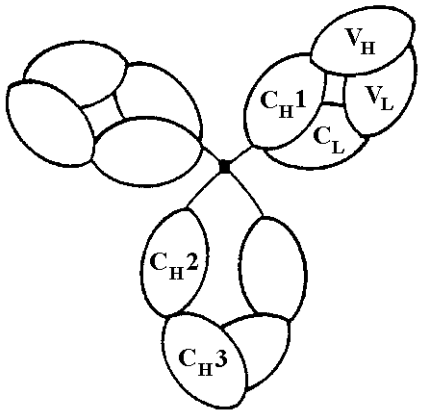


Figure 1. The structure of an IgG molecule. The common antibody fragments are: Fab = (V_HC_H1)(V_LC_L), Fc = 2(C_H2C_H3), Fd = (V_HC_H1) and Fv = (V_HV_L).

The IgG antibodies consist of four polypeptide chains: two identical heavy chains and two identical light chains (Fig. 1). The light chains can be divided into variable (V_L) and constant (C_L) domains, while the heavy chains consist of four domains: a variable (V_H) and three constant domains (C_H1, C_H2 and C_H3). The light and heavy chains of the same antibody class possess a constant amino acid sequence in the C_L and C_H regions, while the unique V_L and V_H regions are highly polymorphic for each immunoglobulin molecule. The folding of constant regions is characterised by seven antiparallel β-strands and the folding of variable regions by nine antiparallel β-strands that form closely packed β-sheets.

The antigen binding site consists of approximately 110 amino acid residues in the *N*-termini of the heavy and light chains of the antibody molecule (Amzel and Poljak, 1979). Three complementarity determining regions (CDR) are highly variable segments which are responsible for antigen recognition. In mice, more than 10^8 antibody molecules are available through the recombination of V_L and V_H genes, making the antibody pool an attractive source of various molecular specificities. After the encounter of an antigen, additional diversity occurs by somatic mutations that produce approximately ten thousand new binding affinities (Tonegawa, 1983).

Although there are over two thousand complete antibody sequences available, currently only about forty structures of antibodies or antibody fragments determined by X-ray crystallography are deposited in the Brookhaven Protein Data Base (Wilson and Stanfield, 1994). The reason for the small number of structures determined could be the difficulty in crystallisation of the antibodies due to the extreme conformational flexibility of an antibody molecule.

The IgG molecule can be cleaved enzymatically into different antibody fragments (Fig. 1). For example, the proteolytic enzyme papain cleaves an IgG molecule into two Fab fragments and one Fc fragment. The Fab fragment consists of the antibody light chain (V_LC_L) that is covalently connected to the V_H and C_H1 by a disulphide bond. The remaining Fc fragment consists of two C_H2 and two C_H3 domains (Goding, 1996). The Fab fragment is responsible for antigen binding and the Fc fragment for effector functions. The Fab and Fv fragments generally retain binding properties similar to those of their parent IgGs and are considered to be equivalent to the whole antibody.

1.1.2 Generation of monoclonal antibodies

A polyclonal mixture of antibodies contains a variety of antibody molecules with different affinities and specificities against the same antigen. On the other hand, a monoclonal antibody is a single molecule with a defined activity and specificity for a hapten. The reproducible production of the antibodies with tailor-made affinities and specificities, and the ease of their production on a gramme scale are the major reasons for their utilisation as diagnostic reagents in medicine and, for example, as catalysts in organic chemistry (Schultz, 1989, Shokat and Schultz, 1990, Goding, 1996).

The hapten (antigen) is first conjugated to the carrier protein because small organic molecules are far too small to elicit an immune response *in vivo*. A hapten is usually coupled to keyhole limpet hemocyanin (KLH) for immunisation and to bovine serum albumin (BSA) for use in ELISA assays to identify hapten-specific antibodies. Coupling is usually carried out as a formation of an amide bond between the carboxyl group of the antigen and ε-amino groups of the surface lysine residues of the carrier protein (Nishima *et al*., 1974, Erlanger, 1980). A diazo linkage can also be formed to the surface tyrosine residues of the carrier protein via an electrophilic aromatic substitution reaction. The disulphide exchange reaction and reductive amination are other means of linking the hapten to its carrier protein (Erlanger, 1980). To exclude the steric interactions of the hapten with the surface of a carrier protein, the length of the spacer or linker is generally longer than 8 Å (Nishima *et al*., 1974).

Mice of BALB/c or Swiss Wistar strain are immunised with the hapten-carrier protein conjugate, their spleen is removed after the immune response, and the antibody producing splenocytes are fused (hybridised) with an immortal SP2/0 cell line by polyethylene glycol treatment (Sugasawara *et al*., 1983, Harlow and Lane, 1988, Goding, 1996). The fused hybridoma cells are grown in a selective medium so that only the hybridised cells maintain their ability to divide. As the antibody producing cells can be cultured and myeloma cells multiply indefinitely, the hybridised cells possess the ability to produce monoclonal antibodies, and they can be cultured indefinitely.

The hybridised cells are cloned and separated in colonies of the cells which produce a single antibody. The antibodies are screened by ELISA for binding with a hapten-BSA conjugate, for inhibition of binding to a hapten-BSA conjugate by free hapten, and for lack of cross-reactivity with KLH (Engvall, 1972).

The produced monoclonal antibody is purified by means of standard protocols (Harlow and Lane, 1988, Goding, 1996). It is precipitated by ammonium sulphate and purified by any of the following methods or their combinations: affinity chromatography, cation exchange chromatography or anion exchange chromatography. Rigorous purification of monoclonal antibodies is tremendously essential, especially when the antibodies are exploited as diagnostic tools or catalysts for organic reactions. During the studies of antibodies as catalysts for the organic reactions, the most difficult contaminants have turned out to be adenosine deaminase and various ribonucleases and glycosidases (Schultz, 1989). Exclusion of numerous esterases and lipases is important when the antibody-catalysed acyl-transfer reactions are studied.

1.1.3 Stabilisation of the transition-state in antibody catalysis

One of the major mechanisms of enzymatic catalysis is stabilisation of the transition-state of a reaction. Enzymes recognise selectively the transition-state of the reaction, and stabilise it by lowering the free energy of activation ($\Delta G^{\ddagger}$) with the subsequent acceleration in the rate of a reaction (Pauling, 1948, Jencks, 1969). Specific electrostatic interactions, *e.g.* hydrogen bonding and complementary structure of the transiton-state in the enzyme's active site, are the most significant factors contributing to stabilisation of the transition-state.[1] Antibodies are macro-molecules that bind the stable ground-state molecules. There exist specific natural compounds that mimic the transition-state of the reaction and bind very tightly to specific enzymes. These tight-binding transition-state analogues inhibit the enzymatic

[1] The transition-state stabilisation plays an important role in the enzymatic catalysis as is evidenced by the study of the hydrolytic enzyme subtilisin. The triple mutation of its catalytic triad, His-Ser-Asp to unfunctional alanines gave the mutant with a k_{cat}/k_{uncat} of approximately 3,000 for the hydrolysis of 4-nitroanilide peptide substrate demonstrating the stabilisation of the transition-state by binding factors other than the catalytic triad (Carter and Wells, 1988).

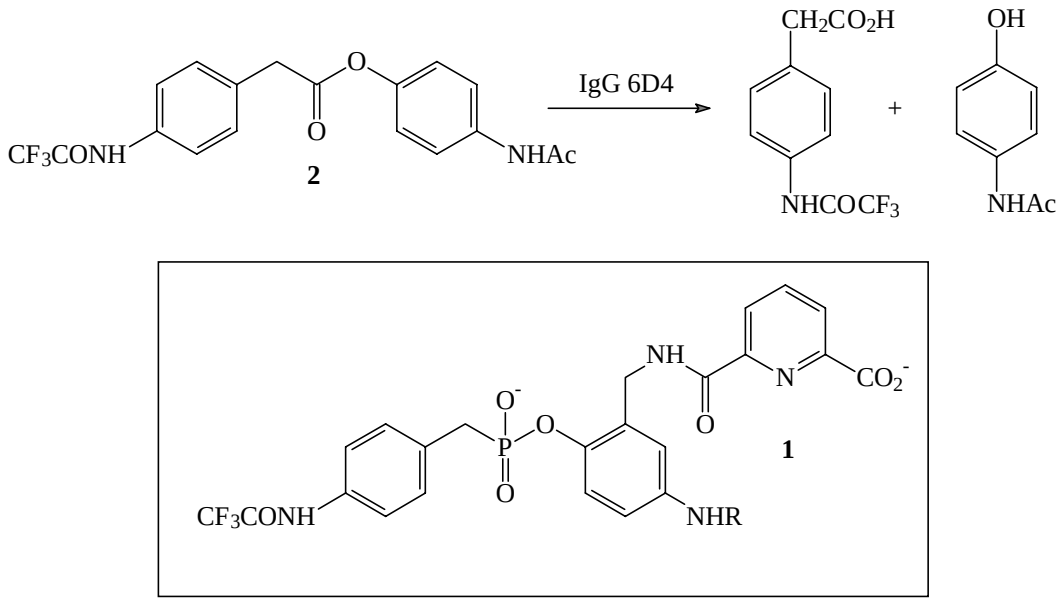
reaction by preventing the enzyme to sequester the respective substrates into the active-site. Moreover, theoretical chemistry provides us with the approximation of a transition-state of the reaction. The appropriate stable transition-state analogues can be designed and synthesised. Therefore, the antibodies raised against the transition-state analogues[2] are expected to be capable of recognising, binding and stabilising the actual transition-state and to accelerate the rate of the reaction.

Not all antibodies generated against the transition-state analogues are catalytic. There are several factors that determine if the antibody will act as a catalyst: (1) the conformation of the antibody's binding site, (2) the binding affinity of the antibody for the transition-state, and (3) the specific (or catalytic) amino acid residues of the binding pocket.

The first examples of hydrolytic antibody catalysts based on the transition-state stabilisation approach came from the laboratories of Lerner and Schultz in 1986. In both studies, the negatively-charged, tetraco-ordinated phosphorus compounds were used as haptens to generate monoclonal antibodies (Pollack *et al*., 1986, Tramontano *et al*., 1986). Although the reactants can geometrically be regarded as being almost planar and uncharged, the transition-state of the hydrolytic acyl-transfer reaction is tetrahedral and negatively charged. It is also known that some phosphates and phosphonates are potent inhibitors of proteinases (Jacobsen and Bartlett, 1981, Bartlett and Marlowe, 1983). Taken together, phosphates, phosphonates or phosphonamidates are generally used as transition-state analogues to elicit antibodies for the catalysis of acyl-transfer reactions such as carbonate, ester and amide hydrolyses, amide bond formation and lactonisation.

Tramontano *et al.* (1986) used tetraco-ordinated phosphonate hapten **1**, R = $CO(CH_2)_4CO{-}NHS$, to raise antibodies to catalyse the hydrolysis of phenyl esters of 4-substituted phenylacetic acids **2** (Scheme 1). Three of the pool of 18 hybridomas produced catalytically active hydrolytic antibodies. In this study, the mAb 6D4 proved to be the fastest with $k_{cat} = 2.7 \times 10^{-2}$ s^{-1}, $K_m = 1.9 \times 10^{-6}$ M, $K_i = 1.6 \times 10^{-7}$ M and $k_{cat}/k_{uncat} = 9.6 \times 10^2$ at 25 °C and pH 8.0.

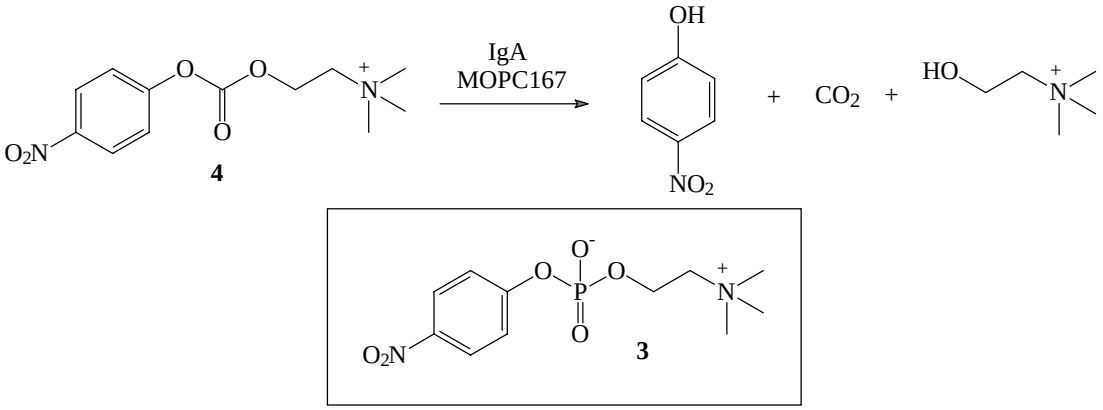
[2] Both naturally occurring and synthetic transition-state analogues are stable, ground-state molecules.



Scheme 1

Pollack *et al*. (1986) found that the naturally occurring class IgA antibody, which binds the tetraco-ordinated 4-nitrophenylphosphorylcholine **3**, hydrolyses the corresponding carbonate **4** to 4-nitrophenol, carbon dioxide and choline (Scheme 2). The antibody binding site contains both specific amino acid residues to recognise and bind the substrate and the appropriately oriented amino acid residue(s) to accelerate the hydrolysis of **4**. The mAb MOPC167 catalysed hydrolysis of the carbonate **4** with $k_{cat} = 6.7 \times 10^{-3}$ s^{-1}, $K_m = 2.1 \times 10^{-4}$ M, $K_i = 5.0 \times 10^{-6}$ M, and $k_{cat}/k_{uncat} = 7.7 \times 10^2$ at 30 °C and pH 7.0. In both reactions, the antibody catalysts followed the Michaelis–Menten type saturation kinetics, competitive inhibition by the transition-state analogue, selective binding to the transition-state and remarkable substrate specificity. Unlike the hydrolytic enzymes that generally exploit cysteine, zinc, a dyad of aspartate or the catalytic triad of histidine, serine and aspartate in the catalysis of the acyl-transfer reactions,[3] tyrosine and/or arginine residues were assumed to be responsible for the catalytic activity in these cases (Pollack *et al*., 1986, Tramontano *et al*., 1986, 1988, Jacobs *et al*., 1987).

[3] In the catalytic step, aspartic (Subramanian *et al*., 1977) and cysteine (Drenth *et al*., 1968, Kamphuis *et al*., 1985) proteinases employ the presence of a specific aspartate or cysteine residue in the enzyme's active site. In carboxypeptidase A, a zinc ion is co-ordinated to two histidine side chains and a glutamate side chain in the active site. The active site of chymotrypsin contains in turn the classical catalytic triad of histidine, serine and aspartate (Blow and Steitz, 1970).



Scheme 2

1.1.4 Reduction of the entropy barrier in antibody catalysis

The entropy of activation ($\Delta S^{\ddagger}$) can contribute significantly to the Gibbs free energy of the activation ($\Delta G^{\ddagger}$) of some organic reactions. Intramolecular macro-cyclisations and unimolecular lactonisations, unimolecular Claisen rearrangements, intermolecular Diels–Alder reactions and bimolecular peptide bond formations provide some examples of entropy-controlled reactions.

The effect of entropy in enzymatic reactions has been studied in several instances. Jencks has proposed that effective molarities up to 10^8 M represent the upper limit for enzyme-catalysed reactions compared to their uncatalysed bimolecular counterparts (Page and Jencks, 1971, Jencks, 1975). Proximity effects play a significant role in the enzyme-catalysed reactions (Bruice and Pandit, 1960, Westheimer, 1962, Dafforn and Koshland, 1973). In analogy, it should be possible to generate rate-enhancing antibody catalysts to act as “entropy traps” for organic reactions with unfavourable entropies of activation. The binding energy of the antibodies might be utilised in reducing the rotational and translational degrees of freedom that are required to form an activated complex between the reactants. Hence, an antibody could be able to sequester the reactants into the favourable reacting conformation within the antibody’s binding site, which provides a complementary environment to the conformationally restricted transition-state of the reaction (Lerner *et al*., 1991, Schultz and Lerner, 1995). Examples of these entropy-controlled reactions are presented in Schemes 3 to 5 and in Table 1.

Table 1. Kinetic parameters for some entropy-controlled antibody-catalysed reactions.

	Reaction	**mAb** **pH, T [°C]**	k_{cat} **[s^{-1}]**	K_M **[M]**	k_{cat}/K_M **[M^{-1} s^{-1}]**	k_{cat}/k_{uncat}
1	Claisen rearrangement	11F1-2E11; 7.0; 10	4.5×10^{-2}	2.6×10^{-4}	1.7×10^{2}	1.0×10^{4}
2	Claisen rearrangement	1F7; 7.5; 14*	3.9×10^{-4}	4.9×10^{-5}	8.0	2.5×10^{2}
3	Trans-esterification	21H3; 9.0; 23	7.5×10^{-2}	6.9×10^{-3} (**9**) 2.0×10^{-3} (**10**)	11 (**9**) 38 (**10**)	N. D.
4	Lactonisation†	24B11; 7.0; 25	8.3×10^{-3}	7.6×10^{-5}	1.1×10^{2}	1.7×10^{2}
5	Amide formation	24B11; 7.0; 25	1.1×10^{-3}	4.9×10^{-3} (**13**) 1.2×10^{-3} (**14**)	0.22 (**13**) 0.92 (**14**)	16 M¶

*The rate constant for the uncatalysed thermal rearrangement (k_{uncat}) was extrapolated from the data of Andrews *et al*. (1973).

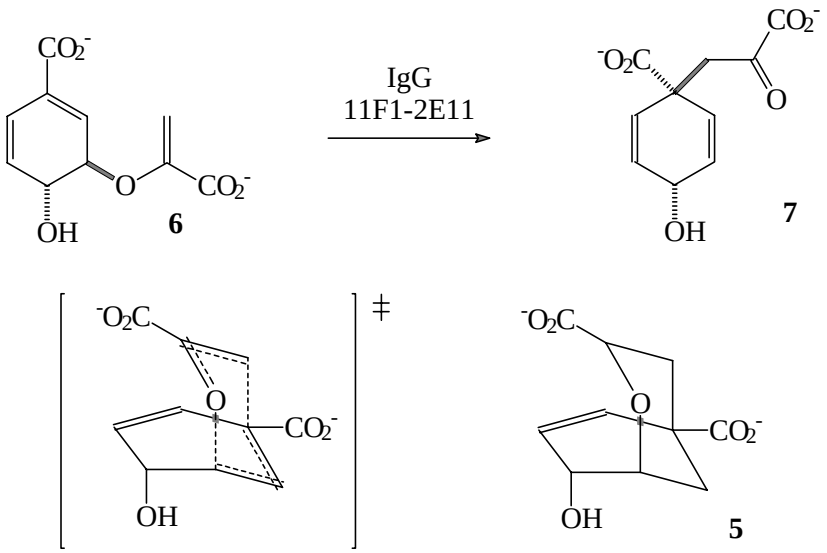
†In the absence of binding by the unreactive enantiomer, $K_M \approx 38$ μM for the reactive substrate.

¶For the rate of uncatalysed background reaction, the value of k_{uncat} is divided by 2 to allow for the two amino groups in 1,4-phenylenediamine **14** capable reacting with the lactone **13**.

The Claisen rearrangement is a thermal [3,3] sigmatropic rearrangement of allyl vinyl ethers proceeding via an asymmetric chair-like transition-state (Bennett, 1977, Ziegler, 1977, Bartlett, 1980, Ganem, 1996).

Monoclonal antibodies were generated against the bicyclic, conformationally restricted transition-state analogue **5** (Bartlett and Johnson, 1985, Jackson *et al*., 1988, 1992) which is known as the most potent inhibitor of the natural enzyme *Escherichia coli* chorismate mutase (EC 5.4.99.5) with $K_i = 0.15$ μM. Indeed, the mAb 11F1-2E11 exhibited 10,000-fold rate enhancement in catalysing the rearrangement of chorismic acid **6** to prephenic acid **7** (Scheme 3, Table 1, Entry 1). Interestingly, the natural enzyme *E. coli* chorismate mutase catalyses the reaction 3.0×10^{6} -fold over the uncatalysed reaction *in vivo* (Koch *et al*., 1972, Ganem, 1978, Görisch, 1978). For the

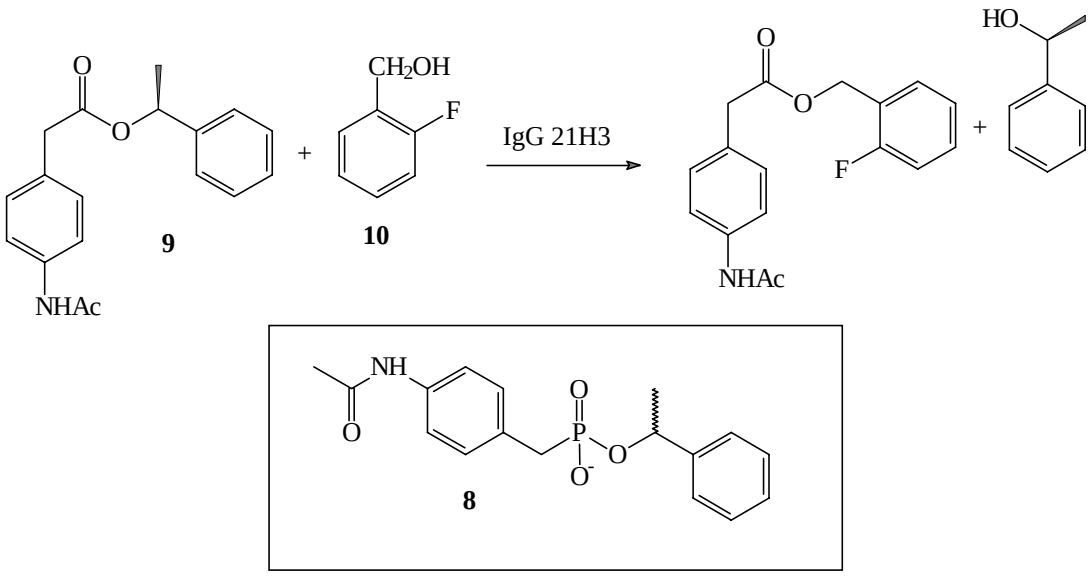
antibody-catalysed reaction, the entropy of activation is approximately zero eu, whereas the $\Delta S^{\ddagger}$ of background reaction is –13 eu (Andrews *et al*., 1973). Since no solvent-D_2O isotope effect was observed, the contribution of general acid and base catalysis in the rate-determining step was ruled out. Similarly, cationic substrate effects were ruled out, leaving the plausible catalytic mechanism of entropic proximity effects for this antibody (Jackson *et al*., 1988, 1992, Wiest and Houk, 1995). Hilvert has also characterised another antibody catalysing the same reaction (Hilvert *et al*., 1988, Hilvert and Nared, 1988, Shin and Hilvert, 1994). The mAb 1F7 accelerated the rearrangement of **6** to **7** at a lower rate through reducing the enthalpy of activation ($\Delta H^{\ddagger}$), obviously by a different mechanism (Table 1, Entry 2).



Scheme 3

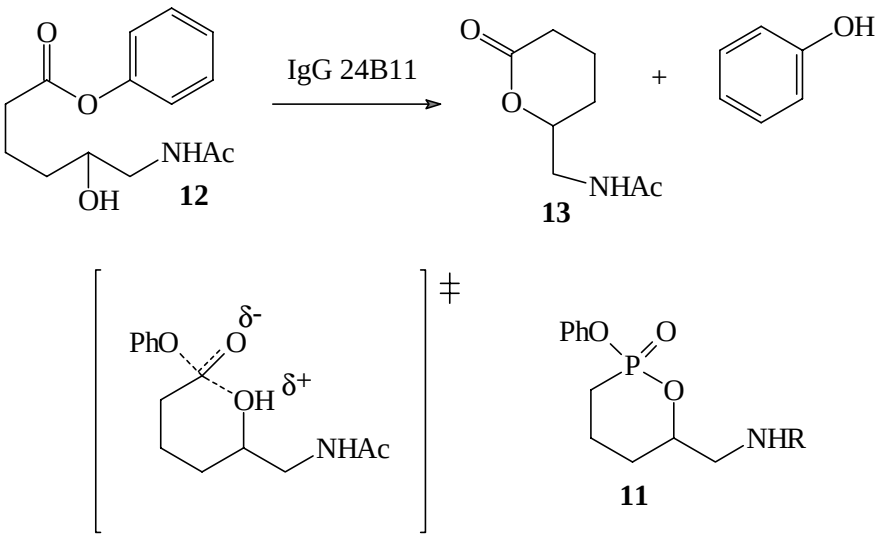
Another example of a pericyclic reaction proceeding through a highly ordered transition-state is the Diels–Alder reaction. It is a reaction between a diene and dienophile, forming an unsaturated six-member ring and simultaneously generating two new carbon–carbon bonds (Sauer, 1967, Carruthers, 1990, Oppolzer, 1991). An unfavourable entropy of activation –40 to –30 eu is generally observed. The examples of antibody catalysis and the minimisation of entropic factors of the Diels–Alder reactions are presented later in chapter 1.3.6.

The tetrahedral, negatively-charged phosphonate hapten **8** was used to elicit monoclonal antibodies for catalysis studies of a bimolecular transesterification reaction (Wirsching *et al*., 1991). The mAb 21H3 was found to catalyse stereo-selectively the reaction between *sec*-phenethyl ester **9** and 2-fluorobenzyl ester **10** in an aqueous environment (Scheme 4, Table 1, Entry 3). In addition, the mAb 21H3 is a highly efficient catalyst with effective molarities ranging from 10^6 to 10^8 M, which corresponds to a reduction in the entropy of activation ($\Delta S^{\ddagger}$) of approximately 35 eu (Page and Jencks, 1971, Jencks, 1975).



Scheme 4

Lactonisation is an example of the intramolecular cyclisation reaction, whose course is regulated essentially by entropic factors. Napper and co-workers (1987) raised antibodies against the cyclic phosphonate hapten **11**, R = $CO(CH_2)_3CO$–NHS, as the transition-state analogue representative of a six-member ring lactonisation (Scheme 5, Table 1, Entry 4). The mAb 24B11 catalysed stereoselectively the lactonisation of **12** to **13** with 170-fold rate over the uncatalysed background reaction, most likely by reducing rotational entropy of the reaction.



Scheme 5

An example of bimolecular amide formation, in which a phosphonamidate-specific mAb catalysed the reaction is presented in Table 1, Entry 5 (Benkovic *et al.*, 1988). In addition to being capable of catalysing the above mentioned lactonisation reaction (Scheme 5), the mAb 24B11 also accelerated the stereospecific formation of an amide from the racemic lactone **13** with 1,4-phenylenediamine.

1.1.5 General acid-base and nucleophilic antibody catalysis

The contribution of a transition-state stabilisation is often insufficient to be the sole mechanism in antibody catalysis; other mechanisms also play a remarkable role in the overall contribution to the catalysis. It is conceivable that an appropriately positioned catalytic functional group containing amino acid residues could be induced to the antibody binding pocket by rational hapten design, chemical modification or genetic engineering (Lerner *et al.*, 1991). Enzymes typically exploit the carboxylate of aspartic acid and glutamic acid, thiol of cysteine, hydroxyl of serine or tyrosine, and imidazole of histidine as either a general base or a nucleophile in catalysing various hydrolytic reactions. These catalytic amino acid residues have high pK_a values in the hydrophobic active sites of enzymes (Walsh, 1979). Some examples of antibodies exploiting general acid-base catalysis are presented in Schemes 6 to 9 and in Table 2.

Table 2. Kinetic parameters for some antibody-mediated reactions catalysed by acid-base and nucleophilic mechanisms.

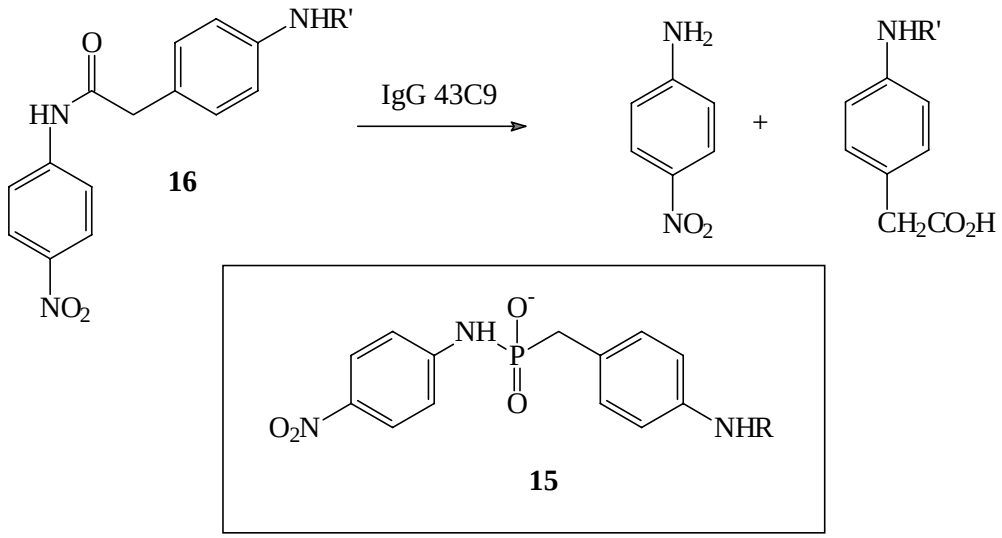
	Reaction	mAb pH, T [°C]	k_{cat} [s^{-1}]	K_M [M]	k_{cat}/K_M [M^{-1} s^{-1}]	k_{cat}/k_{uncat}
1	Amide Hydrolysis*	43C9; 9.0; 37	1.3×10^{-3}	5.6×10^{-4}	2.3	2.5×10^{5}
2	β-Elimination†	43D4-3D3; 6.0; 37	3.2×10^{-3}	1.8×10^{-4}	18	1.5×10^{3}
3	*syn*-Elimination	1D4; 9.0; 37	4.9×10^{-5}	2.1×10^{-4}	0.23	N. D.¶
4	*cis-trans* Isomerisation	DYJ10-4; 7.5; 25	8.0×10^{-2}	2.2×10^{-4}	3.6×10^{2}	1.5×10^{4}

*The background rate of hydrolysis is very slow ($k_{uncat} = 5.7 \times 10^{-9}$ s^{-1}) and does not contribute to the mAb-catalysed reaction. DMSO and MeCN were used as co-solvents.

†The rate constant for acetate-catalysed conversion of **18** to the olefin is 1.9×10^{-4} M^{-1} s^{-1}.

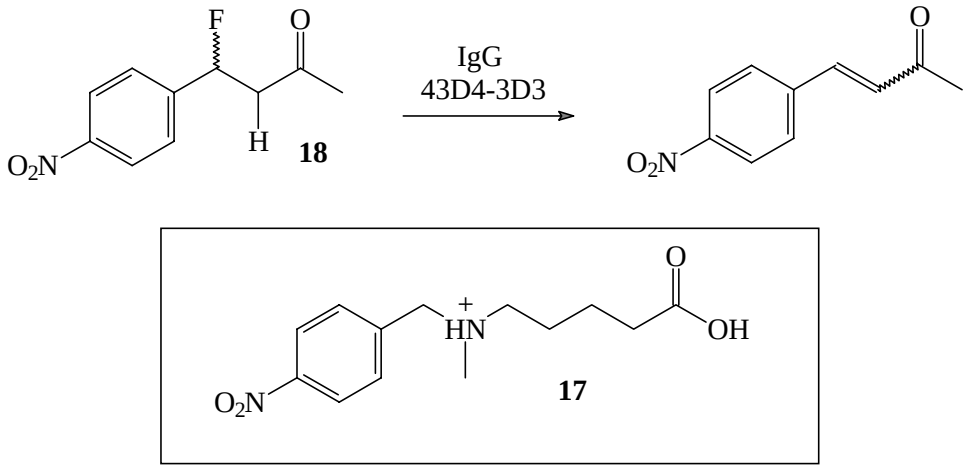
¶The rate acceleration could not be determined because the uncatalysed background reaction was immeasurably slow.

To accelerate the hydrolysis rate of an amide bond, several enzymes use catalytic amino acid residues to stabilise the oxyanion transition-state and to protonate the amide nitrogen. Janda *et al*. (1988b) generated antibodies against the tetraco-ordinated, negatively-charged phosphonamidate hapten **15**, R = $CO(CH_2)_3CO$–NHS, and found a catalytic antibody which was capable of hydrolysing the 4-nitroanilide substrate **16**, R' = $CO(CH_2)_3CO_2H$, with the rate enhancement of 250,000 (Scheme 6, Table 2, Entry 1). The transition-state stabilisation alone could not account for the catalytic mechanism, and the experimental evidence such as the pH dependence of the catalysis (optimum pH 9.0), and the inhibition of the antibody catalyst by 150 mM NaCl led to the conclusion that the general acid-base catalysis was contributing significantly to the rate acceleration.



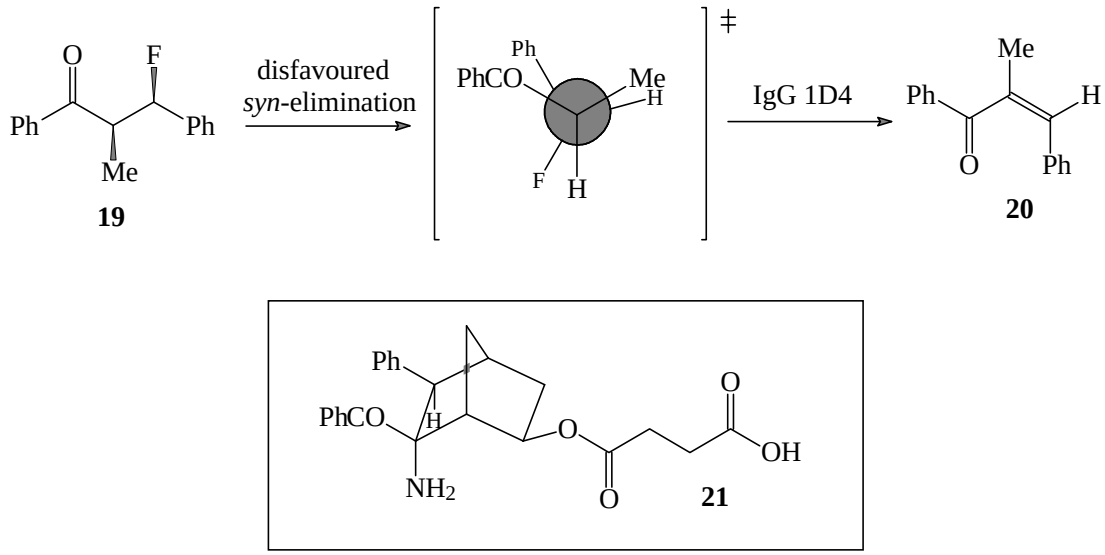
Scheme 6

Shokat and co-workers (1989, 1994) used the hapten **17** with appropriately positioned positive charge to induce a complementary negative charge in the binding pocket of the hapten-specific antibodies. A positively charged ammonium group was anticipated to induce a carboxylate in the binding site to act as a general base and to abstract an α-proton of the fluoroketone substrate **18** with concurrent elimination of the β-fluoride (Scheme 7). Four out of six monoclonal antibodies catalysed the β-fluoride elimination, probably by means of acid-base catalysis (Table 2, Entry 2). The pK_a of the catalytic carboxylate tends to rise in the hydrophobic binding site of the antibody, since a salt bridge interaction is lost when the hapten is replaced by a substrate. The introduction of a carboxylate residue in the binding site of the antibody increased the rate of the β-elimination approximately 90,000-fold compared to the elimination of **18** in an acetate-free solution.



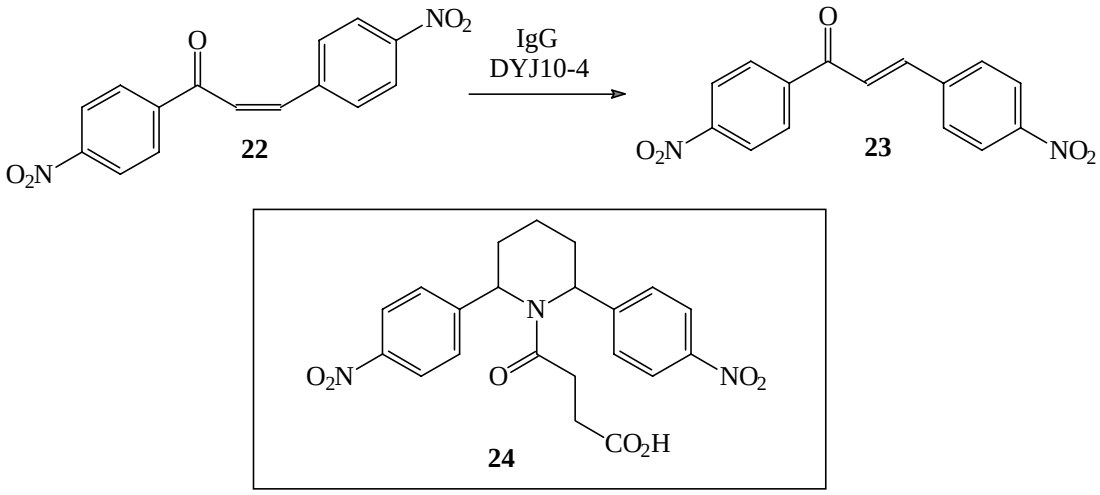
Scheme 7

In another study, a thermodynamically difficult *syn*-elimination of the β-fluoro ketone **19** to the *(Z)* olefin **20** was observed (Cravatt *et al.*, 1994). The rigid bicyclo-[2.2.1]heptane hapten **21** with equatorial benzoyl and phenyl substituents and an axial amino group was synthesised, and monoclonal antibodies raised against it (Table 2, Entry 3). The bicyclic hapten mimicked the eclipsed *syn* co-planar transition-state, and its primary amino group was expected to induce an amino acid side chain in the antibody's binding site to act as a general base to abstract an α-keto proton from the substrate (Scheme 8).



Scheme 8

α,β-Unsaturated *cis* ketone **22** was isomerised to the *trans* ketone **23** by the mAb DYJ10-4 which possessed an active-site nucleophile to react in the Michael addition to the starting enone (Jackson and Schultz, 1991). The antibody also facilitated the rotation around the formed α,β-single bond and the collapse of the intermediate to the isomerised product. A positively charged amino group of the *trans* disubstituted piperidine hapten **24** induced an active-site carboxylate to act as a nucleophile or a general base, while the *trans* configuration of aryl groups of the hapten mimicked the 90° transition-state geometry of a rotated α,β-bond (Scheme 9, Table 2, Entry 4).



Scheme 9

1.1.6 Co-factors in antibody catalysis

Co-factors are used by enzymes to facilitate the catalysis of various energetically demanding hydrolytic or redox reactions. Typically, co-factors are metal ions, hemes or vitamins. For example, cytochrome P_{450} enzyme exploits iron-heme as a co-factor, α-ketoacid dehydrogenases thiamine pyrophosphate (vitamin B_1), amino acid oxidase flavin, alanine racemase pyridoxal phosphate and alcohol dehydrogenase zinc(II) ions. Metal hydrides, transition metals, Lewis acids, hemes, periodate, peroxycarboximidic acid and even monochromatic light have been used as co-factors of monoclonal antibody catalysts as shown in Table 3 (Schultz, 1989, Lerner *et al*., 1991, Schultz and Lerner, 1995).

The hydrolysis of an amide bond is an energetically disfavoured reaction. Enzymes utilise transition metals as co-factors by three distinct mechanisms: (1) transition metal co-ordinates with the oxygen atom of the amide bond carbonyl group polarising it and facilitating the nucleophilic attack to the amide carbonyl, (2) transition metal binds to the hydroxyl group of a nucleophile and delivers it to the carbonyl group of the amide carbonyl and (3) a mechanism that combines both the transition metal assisted polarisation of the carbonyl group towards a nucleophilic attack and a binding of the transition metal to the attacking hydroxyl group. An antibody with proteolytic activity should bind both the co-factor and peptide substrate in a favourable conformation to facilitate the hydrolysis of an amide bond and the release of the hydrolysis products from the antibody's binding sites.

Table 3. Kinetic parameters of some co-factor-assisted antibody-catalysed reactions.

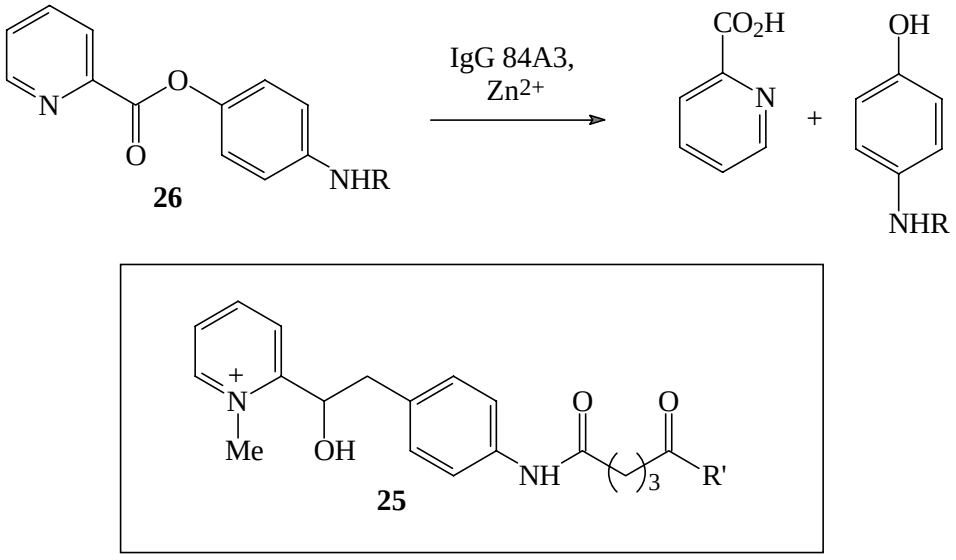
	Reaction	**mAb** **pH, T [°C]**	k_{cat} **[s^{-1}]**	K_M **[M]**	k_{cat}/K_M **[M^{-1} s^{-1}]**	k_{cat}/k_{uncat}
1	Amide Hydrolysis	28F11-Zn^{2+}; 6.5; 37	6.0×10^{-4}	N. D.	N. D.	2.0×10^{5}
2	Ester Hydrolysis*	84A3-Zn^{2+}; 7.0; 25	4.5×10^{-2}	8.4×10^{-4}	54	1.2×10^{3}
3	Reduction of α-Keto Amide	A5; $NaBH_3CN$; 5.0; 22	1.7×10^{-3}	1.2×10^{-3} (**30**)	1.4×10^{3} (**30**)	2.9×10^{2}
4	Oxidation of Sulphide	28B4.2; $NaIO_4$; 5.5; 22.5	8.2	4.3×10^{-5} (**34**)	1.9×10^{5} (**34**)	2.2×10^{5} (**34**)†
5	[2π + 2π] Cycloreversion	15F1-3B1; 7.5; 18	2.0×10^{-2}	6.5×10^{-6}	3.1×10^{3}	2.2×10^{2}¶

*The Zn^{2+}-catalysed background rate was 3.7×10^{-5} s^{-1} (Wade *et al.*, 1993).

†The second-order rate constant $k_{uncat} = 8.5 \times 10^{-1}$ M^{-1} s^{-1}.

¶The first-order rate constant for unsensitised dimer cleavage was 9.2×10^{-5} s^{-1} (Cochran *et al.*, 1988).

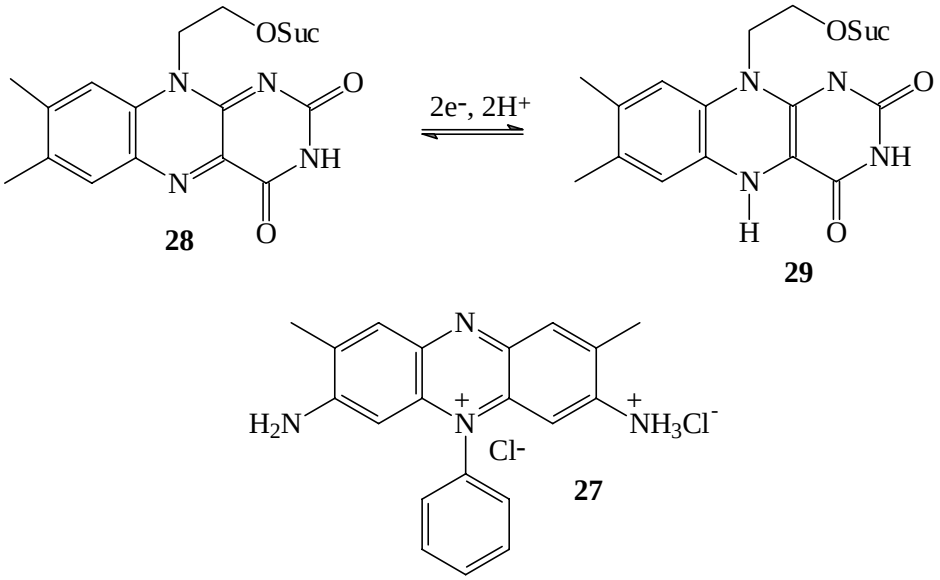
A Co^{3+} triethylenetetramine (trien) hapten complexed to the tetrapeptide Gly–Phe–β-Ala–Gly was used to induce a hapten-complementary binding site to monoclonal antibodies (Table 3, Entry 1). The antibody catalysts were not found to be specific towards the cobalt(III) co-factor. Interestingly, the hydrolysis of the scissile Gly–Phe peptide bond at neutral pH took place even when trien complexes of Mg^{2+} or the transition metals Zn^{2+}, Ga^{3+}, Fe^{3+}, In^{3+}, Cu^{2+}, Ni^{2+}, $Lu^{3+}$and Mn^{2+} were used instead of Co^{3+} (Iverson and Lerner, 1989). These sequence-specific peptide-bond hydrolysing catalytic antibodies can be regarded as analogues of the natural restriction enzymes that hydrolyse specific nucleotide sequences (Schultz, 1989). In another example, it was demonstrated that a metal ion or a co-ordination complex need not be included within the hapten **25**, R' = NHS, for the induction of antibodies that bind a metallo complex and provide a favourable environment for the antibody and zinc(II)-mediated hydrolysis of pyridine carboxylic acid ester **26**, R = $CO(CH_2)_3CO$–$NHCH_2CHOHCH_2OH$, (Scheme 10, Table 3, Entry 2) (Wade *et al*., 1993).



Scheme 10

Currently, three examples of co-factor-assisted reductions by catalytic antibodies are known. Safranine T substrate **27** could be reduced by the complex formed by oxidised 1,5-dihydroflavin **28** and the antibody generated to it, but not by reduced 1,5-dihydroflavin **29** itself (Scheme 11). The three rings in the oxidised flavin are co-planar, whereas the reduced form of flavin, *i.e.* 1,5-dihydroflavin, has a remarkably

different electron distribution, and its ring system adopts a distorted conformation compared to the oxidised flavin. Specific binding-features differentiating the oxidised and reduced forms include dipole moment, hydrogen bonding, changed pK_a and non-planarity. The antibody's binding energy is used to destabilise the reduced form of flavin, generating a more potent donor of electrons to the substrate (Shokat *et al*., 1988). If the substrate-binding site within the antibody binding pocket could be assembled (or engineered) adjacent to that of flavins, the reaction should proceed in a stereocontrolled fashion.

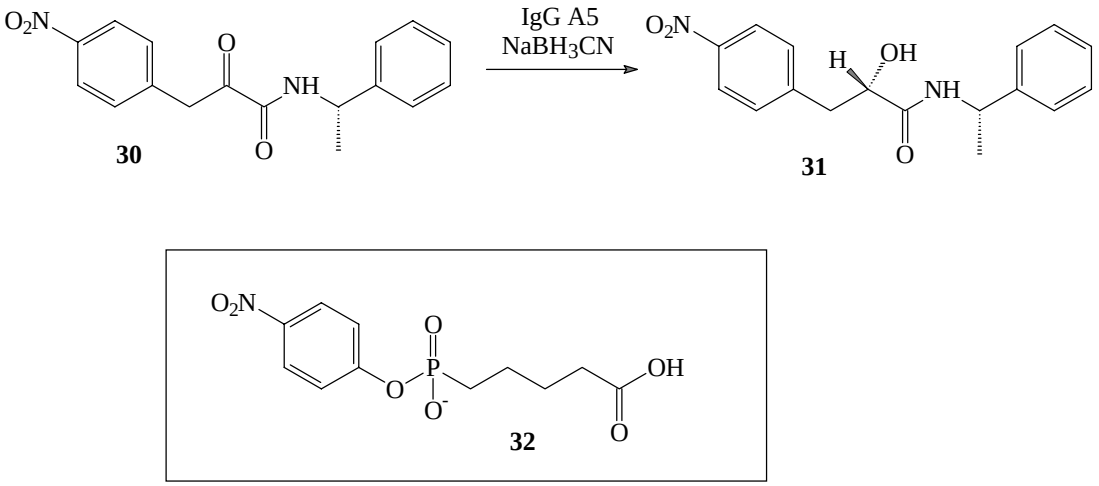


28 **29** **27**

Scheme 11

The α-keto amide **30** could be catalytically reduced to the respective α-hydroxy amide **31** by the mAb A5 using sodium cyanoborohydride as a co-factor (Scheme 12). Monoclonal antibodies were raised against the negatively-charged tetraco-ordinated phosphonate hapten **32** which mimicked the target carbonyl group. This hapten elicited a complementary binding site in the antibody and polarised the target carbonyl group towards the attack by a small hydride ion. The mAb A5 catalysed the reduction of the α-keto amide **30** in a highly stereospecific fashion, producing the dia-stereomer **31** in 99% d.e. (Nakayama and Schultz, 1992).

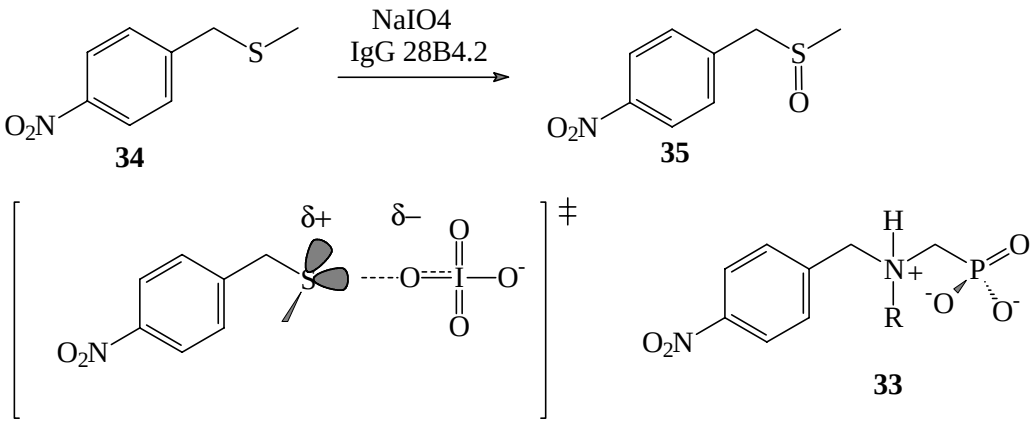
In addition, the monoclonal antibody for catalysing the regioselective and stereoselective reductions has been elicited against the tetrahedral *N*-oxide hapten. In this study, sodium cyanoborohydride was also employed as a co-factor (Hsieh *et al*., 1993). In comparison, the enzymatic reduction of carbonyl groups to secondary alcohols requires the co-factor nicotineamide adenine dinucleotide, NADH, or its phosphate, NADPH.



Scheme 12

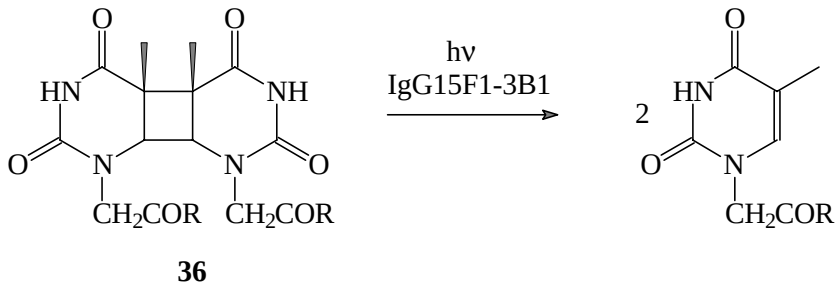
Monoclonal antibodies against *N*-methyl mesoporphyrin IX bound iron(III)-mesoporphyrin IX (Cochran and Schultz, 1990a). This complex catalysed the oxidation of several substrates such as pyrogallol, hydroquinone and *o*-dianisidine by hydrogen peroxide. Only on binding to the co-factor, the antibody created a favourable environment for these oxidation reactions (Cochran and Schultz, 1990b).

In another example, monoclonal antibodies raised against the aminophosphonic acid **33**, R = $(CH_2)_4CO_2H$, with a protonated secondary amino group catalysed the oxidation of the sulphide **34** to the corresponding sulphoxide **35**, employing sodium periodate as a co-factor (Scheme 13, Table 3, Entry 4). No product inhibition could be observed and the turnover was comparable to that of the oxidising enzymes (Hsieh *et al*., 1994, Hsieh-Wilson *et al*., 1996). The ability of monoclonal antibodies to catalyse the epoxidation of unfunctionalised alkenes by peroxyimidic acid co-factor was demonstrated a few years ago (Koch *et al*., 1994).



Scheme 13

Surprisingly, monochromatic light is a co-factor in the catalytic [2π + 2π] cyclo-reversion reaction of the *cis-syn* thymine dimer **36**, R = OH, which is responsible for intrachain linkage of adjacent thymines *in vivo* (Blackburn and Davies, 1967, Cochran *et al*., 1988).[4] The bacterial photoreactivating enzyme *E. coli* DNA photolyase (EC 4.1.99.3) employs the active-site tryptophan residues and visible light (λ > 300 nm) in the cleavage of the thymine dimer (Sutherland, 1981, Jorns *et al*., 1985). A planar, aromatic derivative of the dimer **36**, R = $NHCH_2CO_2H$, was used to elicit antibodies for the catalytic photosensitised cleavage of a thymine dimer (Scheme 14). The aromatic π-electron system of the hapten was expected to induce π-stacking of aromatic amino acid residues in the binding sites. The antibody-catalysed photolysis involved tryptophan residues which used light quanta to generate a thymine-dimer radical anion whose breakdown was partitioned by the antibody. The kinetic parameters and the quantum yield were comparable to those of the natural enzyme.



Scheme 14

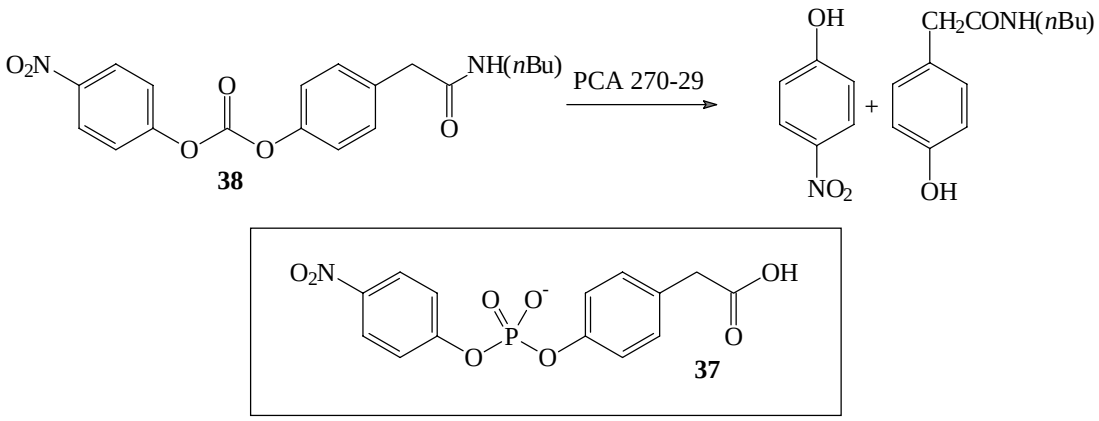
[4] Jacobsen *et al*. (1995) have isolated the mAb UD4C3.5 which catalysed a similar photocleavage reaction of uracil dimers with $k_{cat}/K_m = 28\ M^{-1}\ s^{-1}$ and $k_{cat}/k_{uncat} = 3.8 \times 10^2$.

1.2 Antibody catalysis of acyl-transfer reactions

1.2.1 Hapten design

Tetraco-ordinated phosphorus in phosphates, phosphonates and phosphonamidates has turned out to be a suitable transition-state mimic for the hybridisation change $sp^2 \rightarrow sp^3$ of the planar substrates proceeding through the tetrahedral transition-state to the products. These haptens have been used in carbonate, carbamate, ester and amide bond hydrolyses, amide bond formation and transesterification as well. The antibody catalysts raised against the phosphonate or phosphonamidate haptens show generally rather modest k_{cat}/K_M values 1 to 40 M^{-1} s^{-1}, with a couple of exceptions displaying k_{cat}/K_M values in the range of 10^3 to 10^4 M^{-1} s^{-1} (Thomas, 1994).

The only reports of using the phosphate or phosphorothioate haptens are for production of polyclonal antibodies (Gallacher *et al*., 1991, 1992, Wilmore and Iverson, 1994) or generation of catalytic antibodies by *in vitro* immunisation (Ståhl *et al*., 1995). Gallacher *et al*. (1992) obtained the polyclonal antibody catalyst PCA 270-29 that was raised against the diphenyl phosphate hapten **37** (Scheme 15). It hydrolysed the activated 4-nitrophenyl carbonate **38** with following kinetic constants at 25 °C and pH 8.0: $k_{cat} = 0.14$ s^{-1}, $K_M = 4.0 \times 10^{-6}$ M, and $k_{cat}/k_{uncat} = 6.8 \times 10^2$. The same monoclonal antibody also hydrolysed the 4-nitrophenyl amide at 25 °C and pH 9.0 with $k_{cat} = 6.0 \times 10^{-5}$ s^{-1}, $K_M = 5.4 \times 10^{-6}$ M, and $k_{cat}/k_{uncat} = 3.0 \times 10^2$.



Scheme 15

Phosphonate haptens have mostly been employed in acyl-transfer reactions. Tramontano *et al*. (1986) generated the first monoclonal antibody catalysts of ester hydrolysis reaction from the immunisation by a phosphonate hapten **1** (Scheme 1). Van Vranken *et al*. (1994) obtained the mAb 33B4F11 that hydrolysed 4-nitrophenyl carbamate at 25 °C and pH 7.0 with $k_{cat} = 2.5 \times 10^{-2}$ s^{-1}, $K_M = 5.5 \times 10^{-6}$ M and $k_{cat}/k_{uncat} = 2.7 \times 10^2$. The 4-nitrophenyl phosphonate hapten was used to generate the antibody catalysts.

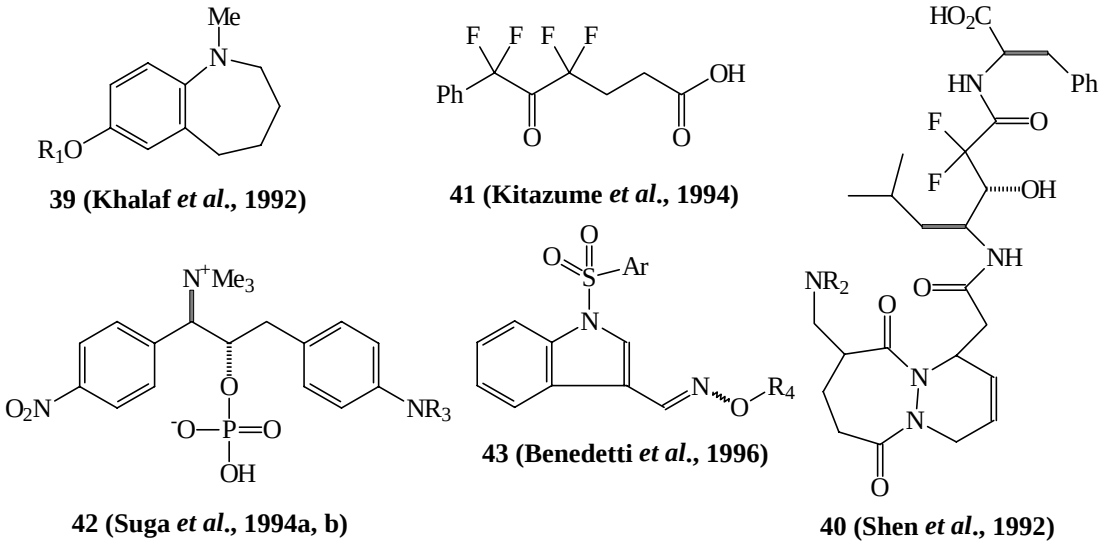
The phosphonate haptens have also been used in the production of antibody catalysts for the amide-bond forming reactions. The phosphonate diester haptens produced antibodies that catalysed the coupling of a phenylalanyl amine group and an acyl azide derived from L-alanine (Jacobsen and Schultz, 1994), and the formation of a dipeptide amide bond between 4-nitrophenyl esters of *N*-acetyl leucine, phenyl-alanine, and valine with tryptophan amide (Hirschmann *et al*., 1994).

Aryl phosphonamidates have been successfully employed both in the formation and hydrolysis of an amide bond. Janda *et al*. (1988b) synthesised the hapten **15** for the production of hydrolytic antibodies (Scheme 6). The mAb NPN43C9 catalysed the hydrolysis of an activated amide substrate **16** at 37 °C and pH 9.0. In another study, the mAb 17G8 raised against the quinaldine phosphonamidate catalysed the bimolecular amide-bond formation between phenyl ester and benzylamine. The catalyst followed the Michaelis–Menten saturation kinetics, and it was found to provide an effective molarity of 10.5 M (Janda *et al*., 1988a).

Janda *et al*. (1990b) presented a new strategy which they termed “bait and switch” catalysis. In this approach, an amino acid is elicited in the antibody’s binding site to assist in an acyl-transfer reaction. The positive point charge of the *N*-methyl-pyridinium hapten **25** was expected to elicit a complementary negative charge (aspartate, glutamate) to the binding site close to the acyl functionality to be hydrolysed (Scheme 10). The carboxylates of the amino acid would then act as general bases or nucleophiles to catalyse the reaction.[5] The mAb 30C6 hydrolysed phenyl benzoate at 37 °C and pH 7.2 with $k_{cat} = 8.3 \times 10^{-5}$ s^{-1}, $K_M = 1.1 \times 10^{-3}$ M and $K_i = 8.3 \times 10^{-5}$ M.

[5] The hydroxyl group of the hapten **25** serves as a surrogate of the tetrahedral transition-state.

There are scattered reports of employing other antigens than phosphorus or "bait and switch" based haptens in raising the antibodies to catalyse acyl-transfer reactions (Fig. 2). A protonated form of the benzazepine hapten **39** has been used to elicit an antibody catalyst for the hydrolysis of a 4-nitrophenyl ester (Khalaf *et al*., 1992). The difluorostatine transition-state analogue **40**, and the exclusively hydrated[6] tetrafluoro-ketone hapten **41** have been used to generate antibody catalysts for the hydrolysis of an ester (Shen *et al*., 1992) and a carbonate (Kitazume *et al*., 1994), respectively. The zwitterionic 1,2-amino alcohols **42** as surrogates for the ester or amide moiety of the substrates have also been suggested and used as transition-state analogues (Suga *et al*., 1994a, b). Benedetti *et al*. (1996) characterised an *N*-*p*-toluoylindole hydrolysing antibody that was elicited against the sulphonamide hapten **43**.



39 (Khalaf *et al*., 1992) **41 (Kitazume *et al*., 1994)**

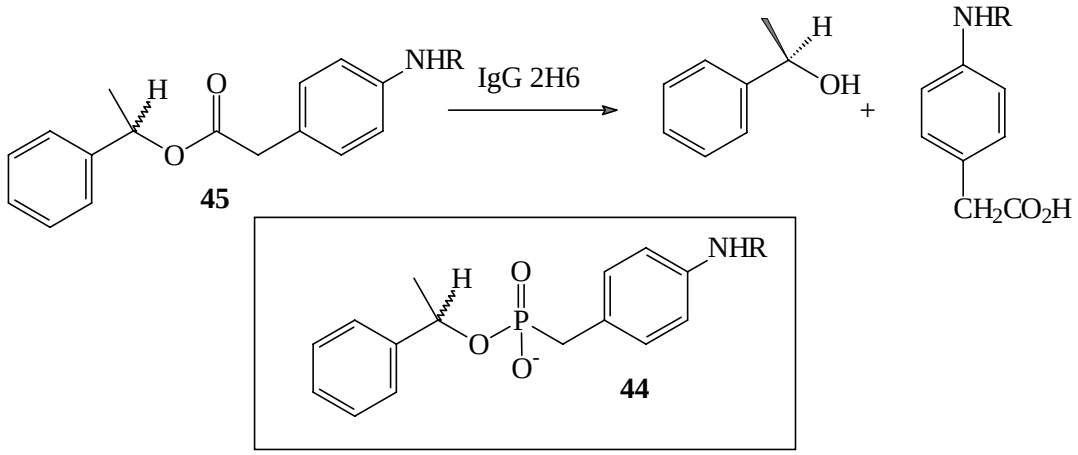
42 (Suga *et al*., 1994a, b) **43 (Benedetti *et al*., 1996)** **40 (Shen *et al*., 1992)**

Figure 2. Various transition-state analogues for the hydrolytic acyl-transfer reactions. R_1 = $(CH_2)_3CO_2H$, R_2 = $CH_2(C_6H_4)NHCO(CH_2)_4$–maleimide, R_3 = $CO(CH_2)_3CO_2H$, R_4 = CH_2CO_2H and Ar = *p*-tolyl.

[6] The *gem*-diol structure should act as an excellent structural mimic of the tetrahedral intermediate.

1.2.2 Stereochemistry of the acyl-transfer reactions

The hydrolysis of unactivated esters (kinetic resolution) by antibodies was achieved by Janda *et al*. (1989). The racemic phosphonate antigen **44** was used to elicit antibodies that exclusively bound to either the *(R)*-**45** or *(S)*-**45** α-methylbenzyl ester, R = $CO(CH_2)_3CO_2H$, (Scheme 16). Two of the 18 antibody catalysts hydrolysed stereoselectively the *(S)*-**45** ester, and 9 of the 18 catalysts hydrolysed the *(R)*-**45** α-methylbenzyl ester. For example, the mAb 2H6 hydrolysed the *(R)*-**45** ester at 21 °C and pH 9.0 with $k_{cat} = 7.7 \times 10^{-2}$ s^{-1}, $K_M = 4.0 \times 10^{-3}$ M, and $k_{cat}/k_{uncat} = 8.3 \times 10^5$. The competitive inhibition of $K_i = 2.0 \times 10^{-6}$ M by the hapten **44** was observed.



Scheme 16

Pollack *et al*. (1989) reported the stereoselective hydrolysis of alkyl esters by monoclonal antibodies. Eighteen of the 31 class IgG antibodies were found to catalyse the hydrolysis of **46** with exquisite specificity for the *(R)*-phenylalanine-containing diastereomer. The tetraco-ordinated tripeptide phosphonate as its diastereomeric mixture **47** was used as a transition-state mimic for the reaction. The mAb 2H12E4 hydrolysed the *(R)*-phenylalanine isomer **46** at 24 °C and pH 8.0 with the following kinetic parameters: $k_{cat} = 3.1 \times 10^{-4}$ s^{-1}, $K_M = 1.5 \times 10^{-5}$ M, $K_i = 2.4 \times 10^{-6}$ M and $k_{cat}/k_{uncat} = 2.7 \times 10^2$ (Fig. 3). Tyrosine was suggested to be one of the catalytic amino acids of the mAb 2H12E4 as evidenced by the destruction of its catalytic activity by the chemical modification of active-site tyrosine by tetranitromethane.

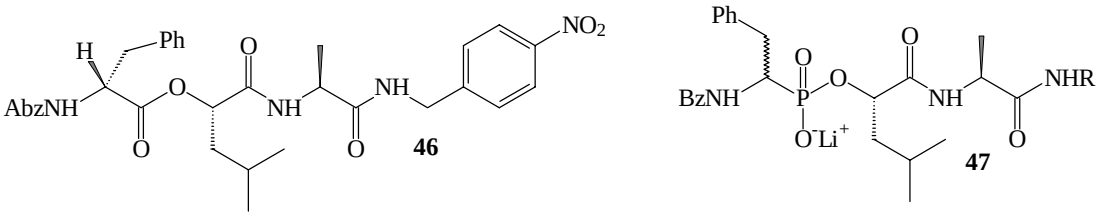
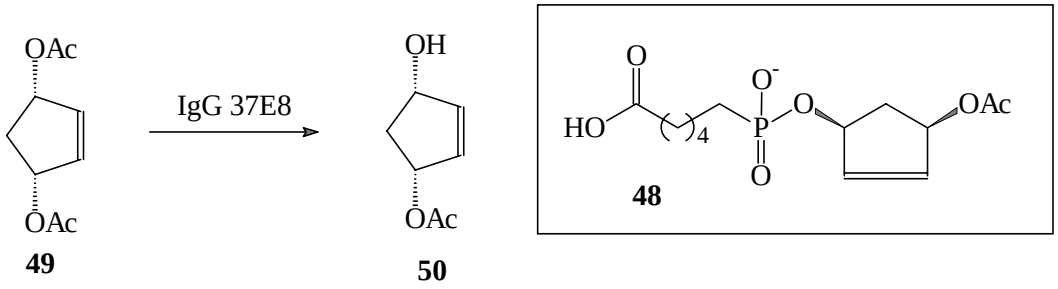


Figure 3. The structures of peptidyl alkyl ester hydrolysed by the mAb 2H12E4 and the phosphonate hapten. R = $CH_2(C_6H_4)NH–Suc$.

Ikeda *et al*. (1991) observed enantioselectivities greater than 98% e.e. when they used the phosphonate enantiomer **48** as antigen to induce monoclonal antibodies that hydrolysed stereospecifically the *meso* substrate **49** to *(1R,4S)*-(+)-4-hydroxy-2-cyclopentenyl acetate **50** (Scheme 17).[7] The mAb 37E8 catalysed the hydrolysis of the *meso* ester **49** at 37 °C and pH 8.0 with $k_{cat} = 1.2 \times 10^{-4}$ s^{-1}, $K_M = 1.8 \times 10^{-4}$ M, and $k_{cat}/k_{uncat} = 88$. The competitive inhibition of $K_i = 7.0 \times 10^{-6}$ M by **48** was observed.



Scheme 17

The use of catalytic antibodies to produce chiral fluorinated *(R)*- or *(S)*-1-(fluoroalkyl)alkanols or an allylic alcohol containing a trifluoromethyl group has been reported: Enantioselectivities greater than 98% e.e. were observed by Kitazume *et al*. (1991). Additionally, the mAb 1C7 has been found to catalyse stereospecifically the hydrolysis of *(R)*-isomer of an ethyl ester (Nakatani *et al*., 1994). Several monoclonal antibodies that were raised against the racemic phosphonate hapten displayed both high enantioselectivities and broad substrate specificities in catalysing the hydrolysis of various *N*-carboxybenzyl-protected amino acid esters (Tanaka *et al*., 1996).

[7] *(1R,4S)*-(+)-4-Hydroxy-2-cyclopentenyl acetate **50** is used as starting material in the total synthesis of prostaglandin $F_{2\alpha}$ (Danishefsky *et al*., 1989).

1.2.3 Chemical means of improving acyl-transfer antibody catalysts

To improve the efficiency of the monoclonal antibody catalysts for the acyl-transfer reactions, various chemical methods exist, such as introduction of chemical catalysts into the antibody binding pocket, immobilisation of the catalytic antibodies, and the use of reverse micelles or lipid-coated antibodies.

The introduction of nucleophiles (thiols, imidazoles) into the antibody's binding pocket (or near it) could enhance the rate of the acyl-transfer reactions because the introduced groups can act either as general bases or nucleophiles. Thiol groups have been covalently attached to the tyrosine and lysine residues into the binding pocket of the Fab fragment of the IgA MOPC 315 by using cleavable affinity labels (Pollack *et al.*, 1988). The hapten was attached covalently to the affinity-labelling, cross-linking reagent, such as an electrophilic aldehyde or α-bromoketone. The affinity-labelled hapten was incubated with the antibody, which resulted in the covalent attachment of the label to the antibody binding site or its vicinity. After the reductive cleavage of the cross-link and the dissociation of the free hapten from the binding pocket, the antibody binding pocket was site-specifically labelled with a free thiol. The introduced thiol functionality acted as a nucleophile to enhance the rate of the ester thiolysis 60,000-fold compared to the background rate. The free thiol also provided a handle that was used to introduce other bases or nucleophiles to the binding pocket, *e.g.* imidazole (Pollack and Schultz, 1989). In this case, the rate acceleration of the hydrolysis was 1,100-fold compared to the uncatalysed background rate.

Several enzymes have been found to retain their catalytic properties in organic solvents (Chen and Sih, 1989, Klibanov, 1990), in solubilised form in the hydrophilic core of reverse micelles (Luisi, 1985) or in water-miscible or water-immiscible organic solvents (Kirchner *et al.*, 1985). The same holds generally for the catalytic antibodies. Durfor *et al.* (1988) showed that antibodies can carry out reactions on water insoluble substrates in reverse micelles. The hydrolysis rates of phenylacetate at 35 °C and pH 8.5 for the mAb 20G9 were 0.31 s^{-1} (no micelles) and 6.5×10^{-2} s^{-1} (in micelles, at a Wo of 23).[8] The K_m values were 1.6×10^{-4} M and 5.7×10^{-4} M, respectively.

[8] Wo is the molar ratio of water to detergent.

Janda *et al*. (1990a) immobilised catalytic antibodies on solid supports. The subsequent studies of the catalytic properties of the hydrolytic antibodies 2H6 and 21H3 showed that they lost 10% and 31%, respectively, of their activities after the immobilisation. However, both of the antibodies retained their stereospecificities (Janda *et al*., 1989). One of the immobilised antibodies retained a modest activity in 40% aqueous DMSO. In another study, the water requirement of the mAb 21H3 in octane was found to be approximately 15% (v/v) to act as an effective catalyst in transesterification of vinyl acetate (Ashley and Janda, 1992). The excellent catalytic activity was observed in as low as 2% (v/v) water.

The mAb 6D9 coated with a synthetic glycolipid was shown to have a remarkable reactivity for hydrolysis of lipophilic esters in a buffer solution. The lipid-coated antibody 6D9 hydrolysed chloramphenicol at 30 °C and pH 8.0 in 20% (v/v) DMSO: $k_{cat} = 3.5 \times 10^{-3}$ s^{-1}, $K_m = 7.5 \times 10^{-5}$ M, $k_{cat}/K_m = 47$ M^{-1} s^{-1} and $k_{cat}/k_{uncat} = 1.2 \times 10^3$. The kinetic constants for the native antibody 6D9 were: $k_{cat} = 8.3 \times 10^{-5}$ s^{-1}, $K_m = 1.2 \times 10^{-4}$ M, $k_{cat}/K_m = 0.69$ M^{-1} s^{-1} and $k_{cat}/k_{uncat} = 29$ (Okahata *et al*., 1995).

1.2.4 Biochemical means of improving acyl-transfer antibody catalysts

Site-directed mutagenesis

Site-directed mutagenesis is a genetic modification technique used to alter any amino acid residue of the protein to the desired one. Since the catalytic residues of the antibodies are located in the antibody's combining site, it is conceivable that the catalytic efficiency of hydrolytic antibodies could, in principle, be enhanced by introducing more efficient catalytic amino acid residues to the binding pocket.

Baldwin and Schultz (1989) performed the site-directed mutagenesis of a Fv fragment[9] of 7-hydroxycoumarin ester hydrolysing IgA class antibody MOPC315 by reconstituting a recombinant variable light chain (V_L) produced in *E. coli* with a variable heavy chain (V_H) derived from MOPC315. To introduce a catalytic

[9] The Fv-fragment is a heterodimer of V_H and V_L peptide chains with molecular weights of 14 kD and 12 kD, respectively (Fig. 1). It contains all the amino acid sequences required for the proper folding of the antigen-binding domain and recognition of the 2,4-dinitrophenyl hapten.

nucleophile or general base into the antibody binding pocket, the tyrosine-34 residue of V_L was substituted by a histidine residue. The mutant Fv enhanced the hydrolysis rate of 7-hydroxycoumarin ester of 5-(2,4-dinitrophenyl)aminopentanoic acid 90,000-fold compared to the reaction catalysed by 4-methylimidazole in the same conditions. The initial rate was 45 times as great as that obtained with the wild-type Fv. The following kinetic parameters were determined to the Fv(Tyr34His) mutant at 25 °C and pH 6.8: $k_{cat} = 3.0 \times 10^{-3}$ s^{-1}, $K_m = 2.2 \times 10^{-6}$ M and $k_{cat}/K_m = 1.4 \times 10^{3}$ M^{-1} s^{-1}.

In another study, site-specific mutations were generated in the genes encoding the variable region of the heavy chain (V_H) of the mAb S107 (Jackson *et al*., 1991). The mAb S107 belongs to a family of homologous antibodies that bind phosphoryl-choline esters (Scheme 2) and catalyses the hydrolysis of 4-nitrophenyl *N*-trimethyl-ammonioethyl carbonate to 4-nitrophenol, carbon dioxide and choline at 25 °C and pH 7.5: $k_{cat} = 1.2 \times 10^{-2}$ s^{-1} and $K_m = 0.71 \times 10^{-3}$ M. Three active-site arginine-52 mutants (Arg52Lys, Arg52Gln, Arg52Cys) and four active-site tyrosine-33 mutants (Tyr33His, Tyr33Phe, Tyr33Glu, Tyr33Asp) were generated by *in vitro* site-directed mutagenesis, and the S107 mutant antibodies were subsequently expressed in myeloma cells. The heavy chain mutations at tyrosine-33 showed little effect on the catalytic activity of S107, except the mAb S107(Tyr33His) which had 8-fold higher rate than the wild-type antibody in the hydrolysis of the substrate. The kinetic parameters were determined as follows: $k_{cat} = 9.5 \times 10^{-2}$ s^{-1} and $K_m = 1.6 \times 10^{-3}$ M at 25 °C and pH 7.5. The relative rate compared to the same reaction catalysed by 4-methylimidazole was 7,000. In addition, the heavy chain arginine-52 mutations had no significant effect on the rate. However, the results suggest that the arginine-52 residue was responsible for stabilising the negatively charged transition-state of the hydrolytic acyl-transfer reaction.

Antibody gene libraries

The recent development of phage display techniques has simplified the selection of peptides and recombinant antibody fragments (Burton, 1991, 1993). Antibody libraries that are based on the expression and display of large repertoires of antibody fragments on the surface of bacteriophage have been used in some cases to find

antibodies with improved binding affinities to a given antigen. The antibody gene libraries typically provide antibodies with higher specificities and affinities compared to the monoclonal antibodies obtained by the use of conventional hybridoma techniques. The antibody fragment obtained from the library can be subjected to site-directed mutagenesis that might afford an antibody with improved recognition of the respective transition-state analogue and with enhanced specificity. For the reactions utilising the transition-state stabilisation as a catalysis mechanism, these antibodies should also accelerate the rate of the reaction.

Huse (1989) and Sastry (1989) with their respective co-workers cloned and expressed a combinatorial phage-display library of the Fd heavy chain fragment and the light chain in *E. coli*. The library was based on the initial Fab expression library, whose mRNA was isolated from the mouse immunised with the 4-nitrophenyl phosphoramidate hapten **15** to raise antibodies for the hydrolysis of 4-nitrophenyl-amide **14** (Scheme 6, Janda *et al*., 1988b). First, the separate Fd heavy and light chain genes were amplified, and the libraries were constructed. Then, these two libraries were combined resulting in a random library of approximately 2.5×10^7 clones that co-expressed a heavy and a light chain of the antibody fragment in *E. coli*. The highly diverse bacteriophage λ immunoglobulin library expressed a population of functional antibody fragments (Fab) on the surface of bacteriophages, and the high-affinity antibodies expressing bacteriophages were selected by affinity chromatography for the subsequent activity assays.

Gibbs *et al*. (1991) converted the Fab fragment 7A4-1/212 from the combinatorial library into a single-chain antibody fragment that retained the catalytic properties of the corresponding Fab fragment and the parent mAb 43C9. The single-chain catalytic antibody hydrolysed 4-nitrophenyl amide with $k_{cat} = 1.1 \times 10^{-4}$ s^{-1} and $K_m = 9.6 \times 10^{-4}$ M. In comparison, the parent mAb 43C9 catalysed the same reaction: $k_{cat} = 1.4 \times 10^{-4}$ s^{-1} and $K_m = 1.1 \times 10^{-3}$ M. Chen *et al*. (1993) also found a catalyst from the antibody gene library. The Fab 1D hydrolysed the 4-nitrophenyl ester with $k_{cat} = 4.2 \times 10^{-3}$ s^{-1} and $K_m = 1.2 \times 10^{-4}$ M at pH 7.2.

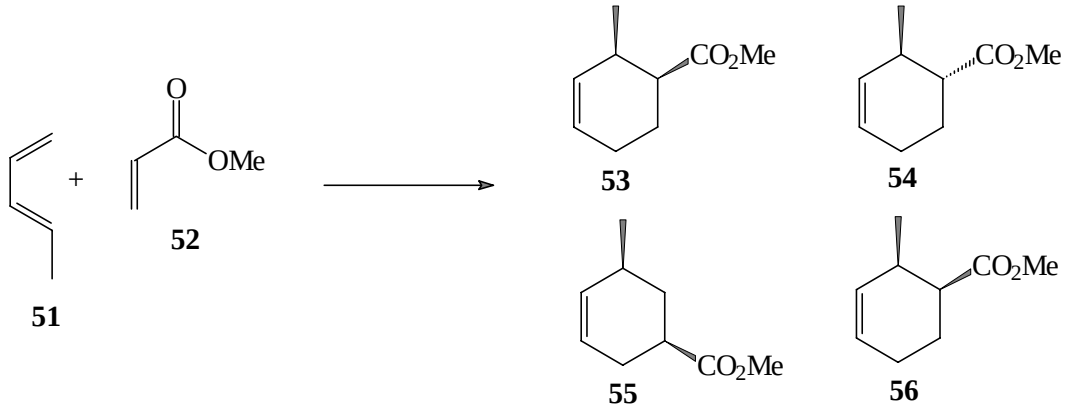
1.3 Catalysis of the Diels–Alder reaction

The Diels–Alder reaction is a versatile method for forming substituted cyclohexenes. This transformation is a concerted [4π + 2π] cycloaddition of a conjugated diene and a dienophile, and belongs to the larger class of pericyclic reactions. In the overall reaction, two new σ-bonds are formed at the expense of two π-bonds in an allowed concerted process in terms of the Woodward–Hoffmann frontier orbital symmetry classification (Diels and Alder, 1928, Sauer, 1967, Carruthers, 1990, Oppolzer, 1991). Theoretically, the Diels–Alder reaction between a monosubstituted diene and dienophile could yield eight possible isomeric products. Thus, to obtain one stereoisomeric product exclusively, the reaction must proceed not only regio- and diastereoselectively, but also enantioselectively. In recent years, several physical, chemical and catalytical methods have been developed to enhance the stereoselectivity in the Diels–Alder reaction. Indeed, asymmetric catalysis of the Diels–Alder reaction has now been achieved, for example by chiral Lewis acids, supramolecular catalysts, proteins, and monoclonal antibodies (Oppolzer, 1984, Narasaka, 1991, Kagan and Riant, 1992, Pindur *et al*., 1993).

1.3.1 Lewis acid promoted Diels–Alder reactions

Yates and Eaton (1960) found that $AlCl_3$ efficiently catalyses the Diels–Alder reactions of *p*-benzoquinone, dimethyl fumarate and maleic anhydride with anthracene. There are copious examples of using Lewis acids such as $EtAlCl_2$, Et_2AlCl, $TiCl_4$, BF_3 and its etherate, $SnCl_2$, $SnCl_4$, $ZnCl_2$, $MgBr_2$ and lanthanide complexes, among others, to accelerate Diels–Alder reactions, allowing them to be run in very mild conditions, often below 0 °C (Oppolzer, 1984, Carruthers, 1990, Pindur *et al.*, 1993). Lewis acid co-ordinates to the C=O or C=N of the dienophile increasing the rate, regioselectivity (*ortho/meta*), diastereoselectivity (*endo/exo*), and π-face selectivity of the reaction. The frontier orbital theory has been applied to explain the role of the Lewis acid catalysts in the [4π + 2π] cycloaddition reactions (Fleming, 1976). The interaction of the dienophile with the catalyst reduces the energy of its HOMO and the LUMO. Hence, in the case of the Diels–Alder reaction with

normal electron demand, the separation between the molecular orbitals will decrease and the transition-state will be stabilised. The cycloaddition between *trans*-1,3-pentadiene **51** and methyl acrylate **52** serves as an example of the influence of $AlCl_3$ on the regioselectivity and diastereoselectivity of the Diels–Alder reaction (Inukai and Kojima, 1967, Oppolzer, 1991) (Scheme 18, Table 4).



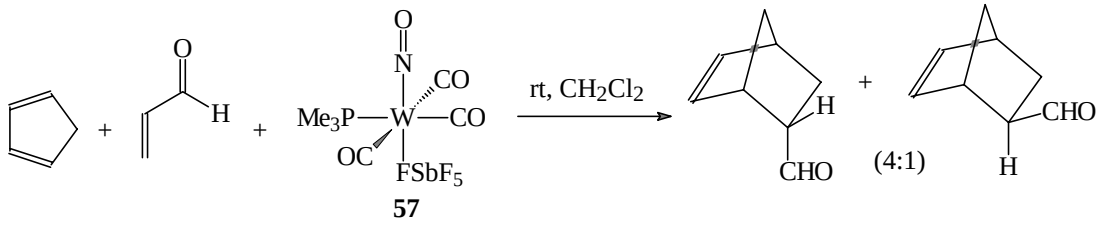
51 **52** **53** **54** **55** **56**

Scheme 18

Table 4. Effect of $AlCl_3$ on the regioselectivity and diastereoselectivity of the Diels–Alder reaction between *trans*-1,3-pentadiene and methyl acrylate in benzene.

Temperature [°C]	**Time [h]**	**$AlCl_3$ [mol equiv.]**	**Yield (%) 53+54+55+56**	**Ratio 53/54/55/56**
120	6	0	53	45/39/11/5
25	1680	0	39	51/39/7.3/2.7
10 – 20	3	0.15	50	93/4.9/1.9/0.1

Organotransition metal complexes acting as Lewis acids have been frequently used to catalyse Diels–Alder cycloaddition reactions (Beck and Sünkel, 1988, Bonnesen *et al.*, 1989). The octahedral tungsten nitrosyl Lewis acid **57** has been employed at only 0.1 mol-% catalytic loading to enhance the rate and the selectivities in the Diels–Alder reaction between butadiene or cyclopentadiene and α,β-unsaturated enones (Scheme 19). According to X-ray crystal structural data, the mode of catalysis might involve activation of the α,β-unsaturated enone by η^1-carbonyl activation (Honeychuck *et al.*, 1987).



Scheme 19

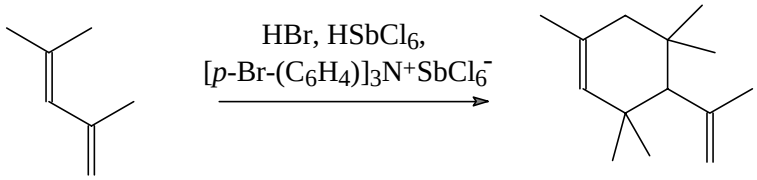
Two further examples of the plethora of organotransition metal complexes of Lewis acids include $[Ti(Cp)_2(H_2O)_2](CF_3SO_3)_2$ and $[Ru(salen)(NO)(H_2O)]SbF_6$ that catalyse the Diels–Alder reaction between various aliphatic dienes and enones (Hollis *et al.*, 1992), and the reactions of aldehyde- and ketone-containing dienophiles with aliphatic 1,3-dienes (Odenkirk *et al.*, 1992), respectively. The previous catalyst is stable to air and very insensitive to the presence of water, contrary to the more traditional Lewis acids such as BX_3, AlX_3 and TiX_4 (X = Br, Cl), which are extremely sensitive to water. The latter catalyst was used at low 1 to 2 mol-% loadings under homogeneous catalysis, and rate accelerations of more than 100,000 were observed in several cases.

1.3.2 Brønsted acid and radical catalysis in Diels–Alder reactions

Brønsted acids such as acetic acid, dichloroacetic acid and trichloroacetic acid have been used to catalyse the [4π + 2π] cycloadditions between cyclopentadiene and ethyl acrylate, methyl methacrylate, methyl vinyl ketone, acrylonitrile and dimethyl maleate. The kinetic behaviour and the rate enhancement of these reactions have been extensively studied (Kasper and Zobel, 1975, Bischoff and Kasper, 1986). The enhancement of the reaction rates is believed to be due to the protonation of the electron-poor dienophile and therefore, to the decrease of the LUMO energy of a dienophile (Sauer and Sustmann, 1980).

An example of the mixed aminium cation radical and Brønsted acid induced Diels–Alder reaction is provided by the tris(4-bromophenyl)ammoniumyl hexachloro-antimonate mediated cyclodimerisation of 2,4-dimethyl-1,3-pentadiene **58** to **59** (Scheme 20). The same reaction is also catalysed by acidic catalysts such as

hexachloroantimonic acid and hydrogen bromide (Gassman and Singleton, 1984b). The aminium radical cation is believed to act primarily as an indirect source of protons which are subsequently used in an acid-catalysed cycloaddition reaction (Gassman and Singleton, 1984a).



58 **59**

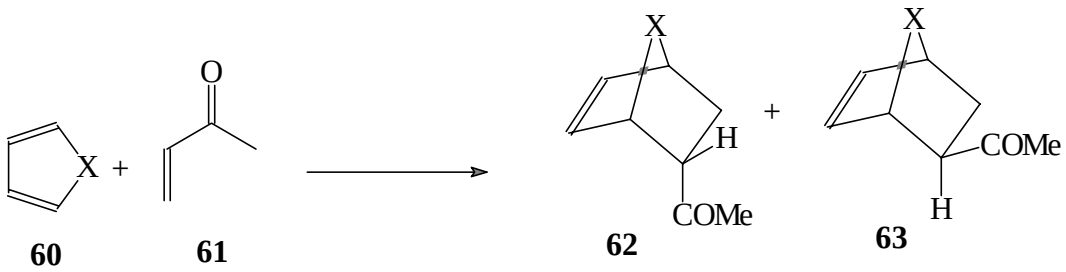
Scheme 20

The conversion of a neutral or electron-rich dienophile into the equivalent cation radicals generally accelerates the Diels–Alder reaction. For example, the dimerisation of 1,3-cyclohexadiene under cation radical catalysis is a symmetry-allowed, stereo-specific reaction that occurs under mild conditions. The cation radical catalysed reaction can be executed at 0 °C in 15 minutes in the presence of tris(4-bromophenyl)-ammoniumyl hexachloroantimonate, whereas the uncatalysed thermal reaction requires treatment at 200 °C for 20 hours (Bellville *et al*., 1981, Bellville and Bauld, 1982, Bauld *et al*., 1983, Harirchian and Bauld, 1987).

1.3.3 Heterogeneous inorganic catalysts in Diels–Alder reactions

Zeolites, clays and silica gel have been used to accelerate numerous Diels–Alder reactions either alone or in combination with a co-catalyst (Laszlo and Lucchetti, 1984a-c, Ipaktschi, 1986, Laszlo, 1986, Veselovsky *et al*., 1988). Molecular sieves are zeolites and can be characterised as sodium aluminium silicates that possess several water-containing cavities. The exact catalytic mechanism of acceleration of the peri-cyclic [4π + 2π] cycloaddition reactions is not known. However, it is hypothesised that the transition-state shape selectivity plays a significant role in the catalysis (Ipaktschi, 1986, Hölderich *et al*., 1988). The cycloaddition takes place only if the

transition-state structure of the reaction is smaller than the diameter of the pores. Montmorillonite is an alumina hydrosilicate mineral whose lamellar structures contain many accesible cations such as Fe^{3+}, Cu^{2+} and Zn^{2+} (Cornelis and Laszlo, 1985, Laszlo, 1986, Cabral and Laszlo, 1989, Laszlo and Moison, 1989, Cativiela *et al.*, 1991). The effect of silica gel (SiO_2) and SiO_2·MgO on the acceleration of the Diels–Alder reaction between cyclopentadiene, isoprene, butadiene or 1,3-cyclohexadiene and various acyclic dienes has been studied (Veselovsky *et al.*, 1988). Some examples of clay, zeolite or silica gel mediated acceleration of the Diels–Alder reaction between furan or cyclopentadiene **60** and methyl vinyl ketone **61** are presented in Scheme 21 and Table 5 (Mellor and Webb, 1974, Laszlo and Lucchetti, 1984a-c, Ipaktschi, 1986, Adams *et al.*, 1987, Veselovsky *et al.*, 1988).



60 **61** **62** **63**

Scheme 21

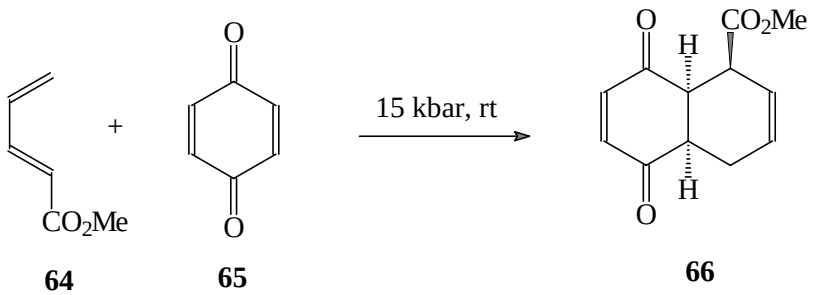
Table 5. Effect of clay, zeolite or silica gel catalyst on the Diels–Alder reaction between methyl vinyl ketone and cyclopentadiene or furan.

X (60)	Solvent	Catalyst	T [°C]	Time [h]	Yield (%) 62+63	Ratio 62/63
O	CH_2Cl_2	K10–Fe^{3+}	–43	6	60	31:69
O	CH_2Cl_2	Cu^{+} zeolite	0	48	73	29:71
O	–	SiO_2·MgO	20	70	48	20:80
CH_2	benzene	–	80	16	80	83:17
CH_2	CH_2Cl_2	K10–Fe^{3+}	–24	4	96	95:5
CH_2	CH_2Cl_2	Cr^{3+} clay	22	0.3	91	90:10
CH_2	–	SiO_2	–20	1	90	96:4

1.3.4 Other non-biological means to accelerate the Diels–Alder reaction

In addition to the Diels–Alder catalysts already discussed, there are several other non-biological means of accelerating the rate or selectivities of the inter-molecular Diels–Alder reactions. These include methods such as high pressure, sonication, solvophobic effects or molecular aggregation. Two examples are given below.

Intermolecular Diels–Alder cycloaddition reaction is characterised by large negative activation volumes (–25 to –45 cm^3 mol^{-1}) and large negative volumes of reaction (Matsumoto and Sera, 1985). Consequently, the intermolecular Diels–Alder reaction can be accelerated by applying a high pressure of 1 to 25 kbar to the reaction mixture. The thermal reaction of methyl 2,4-pentadienoate **64** and 1,4-benzoquinone **65** at normal pressure and at 80 ºC for 24 hours gave the quinone **66** in a 28% yield (Scheme 22). Under 15 kbar at room temperature for 18 hours, the same reaction afforded the quinone **66** in a 64% yield (Dauben and Baker, 1982).

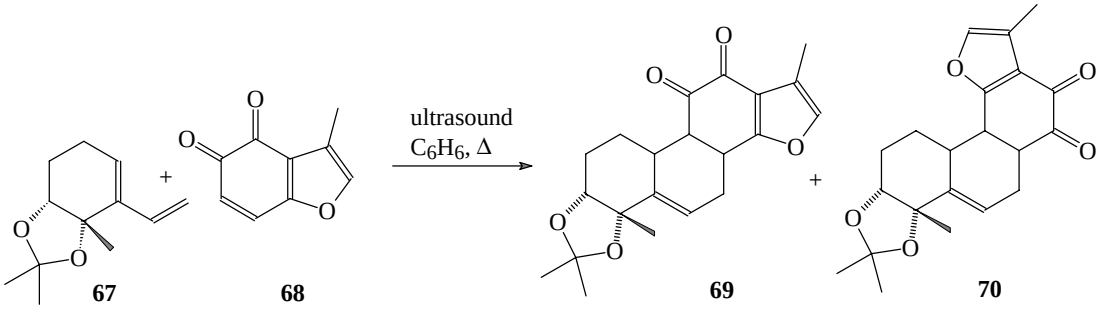


64 **65** **66**

Scheme 22

The application of ultrasonic radiation (20 to 850 kHz) has been found to enhance both the rate and selectivity of various organic reactions (Ley and Low, 1989). The cavitation effect is responsible for the high local pressure, temperature and electrostatic potential differences in the reaction mixture. The cycloaddition between the acetal protected diene **67** and 3-methyl-4,5-benzofurandienone **68** is an example of

the use of sonication in accelerating the Diels–Alder reaction. The background reaction in refluxing benzene for 8 hours affored a 50:50 mixture of the regioisomers **69** and **70** in a total yield of 15% (Scheme 23). Interestingly, the same reaction under sonication at 45 ºC for 2 hours gave an 83:17 mixture of the regioisomers **69** and **70** in a total yield of 76% (Lee and Snyder, 1989, 1990).[10]



Scheme 23

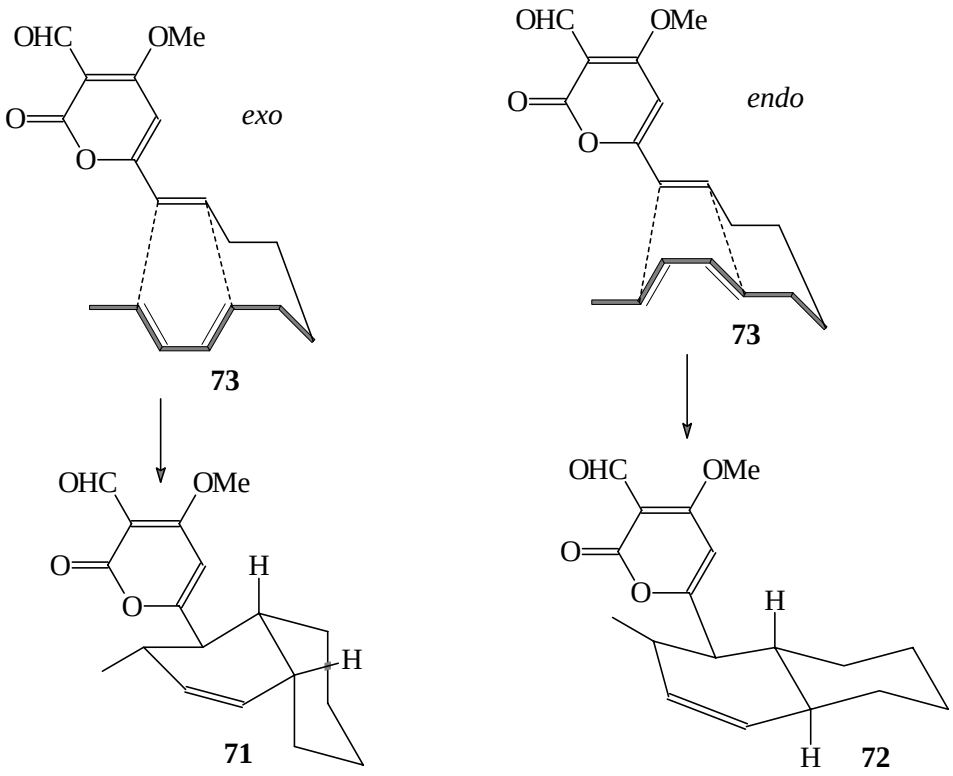
1.3.5 Diels–Alder reactions in nature

Several studies have been published on the putative occurrence of the biosynthetic enzyme-catalysed pericyclic Diels–Alder reactions. Some of these conversions presumably take place spontaneously and do not require the participation of an enzyme. Instead, the reaction may be catalysed by Lewis acids present in the plant tissue. Other biosynthetic Diels–Alder conversions are believed to proceed enzymatically by a cyclase enzyme which yields optically active cycloadducts (Stipanovic, 1992, Laschat, 1996).

The biosynthesis of solanapyrones A **71** and D **72** is presented as an example of a possible enzyme-catalysed Diels–Alder reaction (Scheme 24). Oikawa *et al.* (1989a, b, 1994) have reported the biosynthesis of these phytotoxins produced by the pathogenic fungus *Alternaria solani* and shown unambiguously that the decalin system of solanapyrones is formed by an intramolecular [4π + 2π] cyclo-addition. Incubation of prosolanapyrone III **73** with a cell-free extract of *A. solani* afforded a mixture of the

[10] The major regioisomer **69** can be converted to (±)-tanshindiol after oxidative aromatisation and removal of the acetal protective group (Lee and Snyder, 1989, 1990).

exo cycloadduct solanapyrone A **71** and the *endo* adduct solanapyrone D **72** with an *exo/endo* ratio of 53:47. In the absence of the cell-free extract under the same reaction conditions or in a control experiment in the presence of denatured enzyme, prosolanapyrone III **73** was converted to solanapyrone A **71** and D **72** with an *exo/endo* ratio of 3:97. Hence, the enzyme-related *exo/endo* ratio was calculated to be 87:13 (Oikawa *et al.*, 1995). However, it should be noticed that these investigations were carried out in the presence of a cell-free extract and not in the presence of an isolated, purified and characterised enzyme.



Scheme 24

Other studies of the tentative occurrence of natural Diels–Alderases include, among others, the biosynthetic studies of brevianamide mycotoxins by *Penicillium brevicompactum* (Sanz-Cervera *et al*., 1993), an antibiotic nargenicin by *Nocardia argentinensis,* Huang (Cane *et al*., 1993), betaenone B phytotoxin produced by *Phoma betae,* Frank (Oikawa *et al*., 1988), a hypocholesterolemic agent mevinolin by *Aspergillus terreus* (Moore *et al.*, 1985) and optically active prenylchalcones chalcomoracin and kuwanon J in *Morus alba,* L. cell cultures (Hano *et al.*, 1990, 1992).

1.3.6 Antibody-catalysed Diels–Alder reactions

The Diels–Alder reaction has proven to be among the most popular pericyclic antibody-catalysed reactions. (Hilvert *et al.*, 1989, Braisted and Schultz, 1990, Suckling *et al.*, 1992, Gouverneur *et al.*, 1993). The reason is the crucial importance of this [4π + 2π] cycloaddition reaction in synthetic organic chemistry, in addition to its interesting physico-chemical properties and mechanistic aspects.[11] The antibody-mediated Diels–Alder reactions studied so far are presented in Table 6.

Table 6. Kinetic parameters of the antibody-catalysed Diels–Alder reactions.

	Reaction	**mAb, pH, T [°C]**	k_{cat} **[s^{-1}]**	K_M **[M]**	k_{cat}/K_M **[M^{-1} s^{-1}]**	k_{cat}/k_{uncat}
1	**75** + **76** → **78***	1E9; 6.0; 25	7.2×10^{-2}	2.1×10^{-2} (**76**)†	3.4	1.1×10^{2}
2	**80** + **81** → **82**	39A11; 7.5; 25	0.67	1.1×10^{-3} (**80**) 7.4×10^{-4} (**81**)	6.1×10^{2} (**80**) 9.0×10^{2} (**81**)	0.35 M
3	**83** + **84** → **86**; R = Et¶	H11; 8.0; 18	5.5×10^{-2}	8.3×10^{-3} (**84**)	6.6 (**84**)	1.7×10^{3}‡
4	**87** + **88** → **90** (*exo*)	22C8; 7.4; 37	5.3×10^{-5}	7.0×10^{-4} (**87**) 7.5×10^{-3} (**88**)	7.6×10^{-2} (**87**) 7.1×10^{-3} (**88**)	18 M
5	**87** + **88** → **92** (*endo*)	7D4; 7.4; 37	5.7×10^{-5}	9.6×10^{-4} (**87**) 1.7×10^{-3} (**88**)	5.9×10^{-2} (**87**) 3.4×10^{-2} (**88**)	4.8 M

*The second-order rate constant for the uncatalysed cycloaddition was 6.7×10^{-4} M^{-1} s^{-1} (Hilvert *et al.*, 1989).

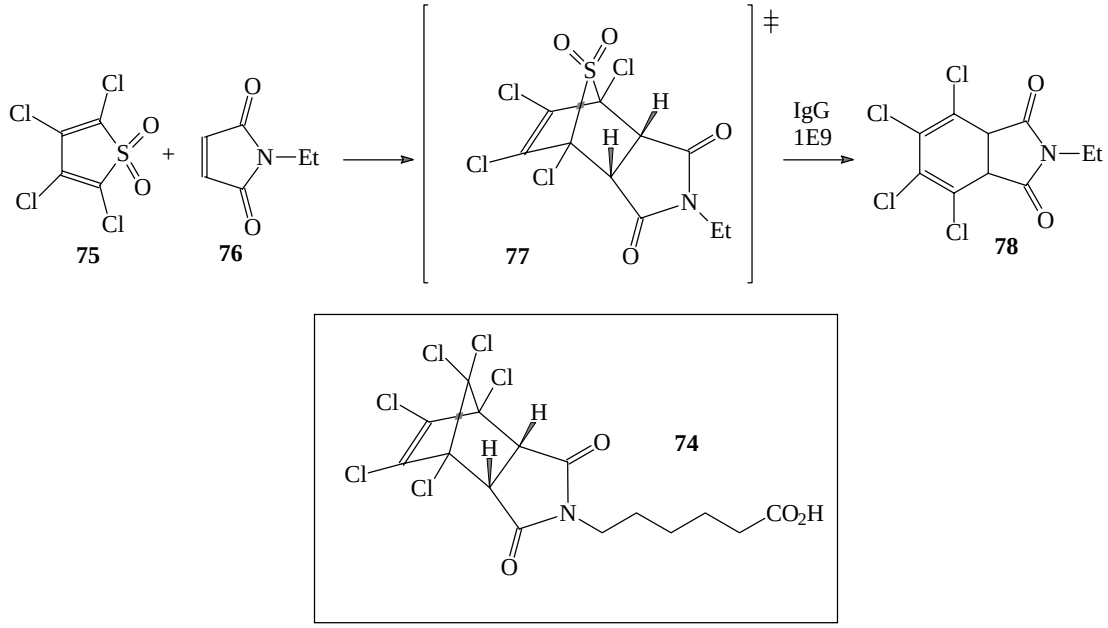
†Low solubility of **75** prevented the determination of its K_m value (Hilvert *et al.*, 1989).

¶Acetoxybutadiene **83** underwent slow hydrolysis to crotonaldehyde under the reaction conditions and a value for K_m was not obtained (Suckling *et al.*, 1992).

‡The pseudo first-order rate constant $k_{uncat} = 3.2 \times 10^{-5}$ s^{-1} (Suckling *et al.*, 1992).

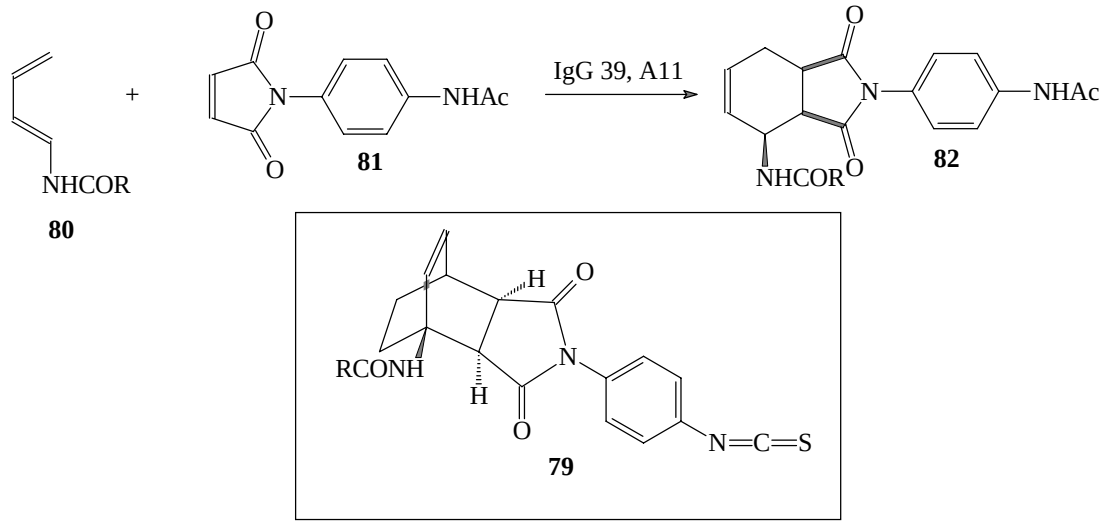
[11] Presently, there is only one example of catalysis of the retro Diels–Alder reaction by an antibody with nitroxyl synthase activity (Bahr *et al.*, 1996).

In Hilvert's approach, the bicyclic adduct **74** was used as a stable transition-state mimic for the [4π + 2π] cycloaddition between tetrachlorothiophene dioxide **75** and *N*-ethylmaleimide **76** (Hilvert *et al.*, 1989). The heterocyclic diene **75** and the dienophile **76** form an unstable bicyclic adduct **77** that spontaneously extrudes sulphur-dioxide affording a dihydrophthalimide product **78** that binds only weakly to the antibody. The hapten takes into account both the proximity effect of the catalysis by lowering the entropy of activation ($\Delta S^{\ddagger}$) and the diminishing of the product inhibition. The hapten-specific mAb 1E9 catalysed the reaction with an apparent effective molarity of 110 M per antibody binding site (Scheme 25).



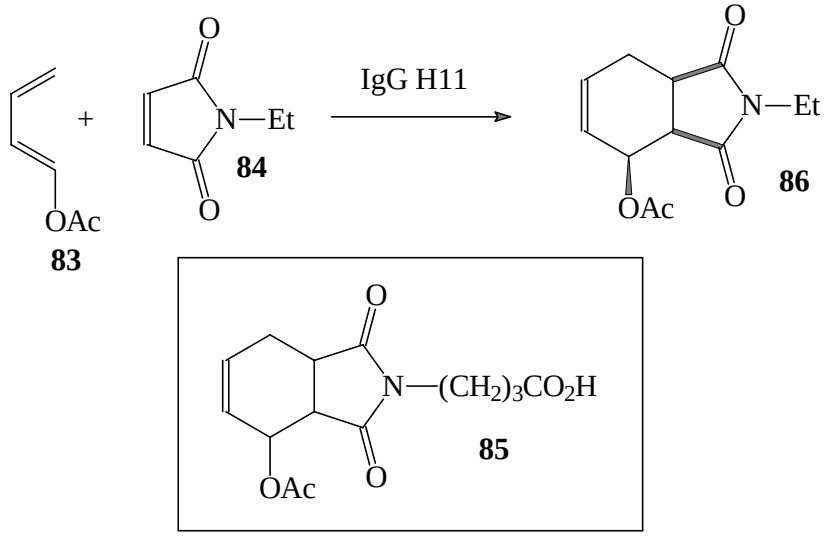
Scheme 25

Schultz's group prepared a KLH conjugate of isothiocyanate derivative of bicyclo[2.2.2]octene **79**, R = OCH_2CO_2H, to be used as a hapten to produce antibodies against this transition-state mimic of the Diels–Alder reaction between aminoacyl-butadiene **80** and *N*-phenylmaleimide **81** (Braisted and Schultz, 1990). The ethano bridge locks the cyclohexane ring of the hapten **79** into a similar *s-cis* type conformation to that of the substrate diene **80** in the transition-state of Diels–Alder reaction (Scheme 26). Hence, the hapten-specific antibodies were believed to act as entropy traps in lowering the translational and rotational entropy of activation of the reaction, and to harvest the diene **80** and dienophile **81** into a reactive conformation in the hydrophobic binding pocket of the antibody. In fact, the mAb 39, A11 catalysed the formation of **82** with k_{cat}/K_M of 6.1×10^2 $M^{-1}s^{-1}$ (**80**) and 9.0×10^2 $M^{-1}\,s^{-1}$ (**81**).



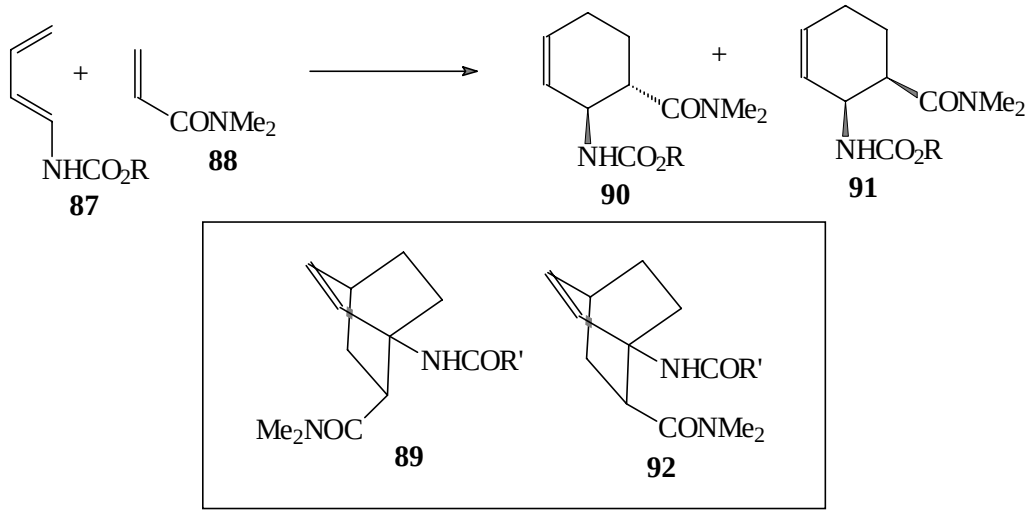
Scheme 26

The Diels–Alder reaction between 1-acetoxybutadiene **83** and *N*-ethylmaleimide **84**, was studied by Suckling's group (Suckling *et al*., 1992, 1993). Monoclonal antibodies were generated against a tetrahydroisoindole dione hapten **85** that resembled more the reaction product **86** than the actual transition-state of the reaction (Scheme 27). The mAb H11 and its Fab fragment were found to catalyse the reaction with k_{cat}/K_M of 6.6 M^{-1} s^{-1} for the dienophile **84**.



Scheme 27

Gouverneur *et al*. (1993) introduced the concept of selectivity to the antibody-catalysed Diels–Alder reactions by considering the secondary orbital interactions in the transition-state of a reaction between *trans*-1-*N*-carbamoylamino-1,3-butadiene **87**, R = $CH_2(C_6H_4)CO_2H$, and *N*,*N*-dimethylacrylamide **88.** The secondary orbital interactions determine the diastereoselectivity of the Diels–Alder reactions, which is displayed in different *endo/exo* ratios of the products (Scheme 28). By using the boat-shaped bicyclo[2.2.2]octene mimic **89**, R = $(CH_2)_3CO$–NHS, for the pericyclic *exo* transition-state, the mAb 22C8 was found to produce diastereoselectively the *exo* cycloadduct **90** with an effective molarity of 18 M. Not surprisingly, immunisation with the hapten **92** mimicking an *endo* transition-state gave the mAb 7D4, which catalysed exclusively the formation of the *endo* adduct **91** with an effective molarity of 4.8 M.

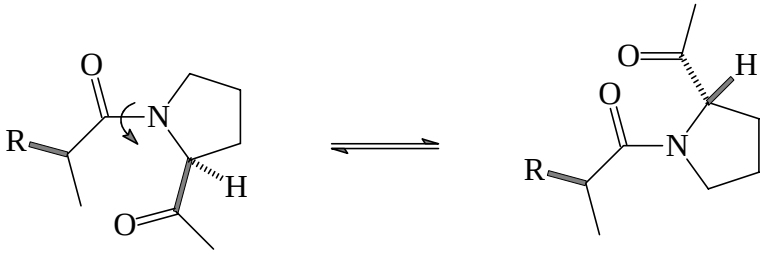


Scheme 28

Both of these reactions were also enantioselective with enantiomeric excesses of > 98%. The antibody's binding energy was used to control the relative energy of the transition-states of various possible reaction pathways and to ensure that the antibodies harvest reactants into the reactive conformation in their binding sites. The bicyclic hapten with its boat-shaped cyclohexene ring proved once more to be a reliable mimic for the pericyclic transition-state of the Diels–Alder reaction.

1.4 Peptidyl-prolyl *cis-trans* isomerisation reaction

Peptidyl-prolyl *cis-trans* isomerisation is a slow conformational inter-conversion of the *cis* conformation **93** of an *N*-terminal amide bond of the amino acid proline to the corresponding *trans* conformation **94** (Scheme 29). Peptidyl-prolyl *cis-trans* isomerase enzymes (EC 5.2.1.8) exist to enhance the isomerisation rates *in vivo* (Galat, 1993).



93 **94**

Scheme 29

Isomerisation of the peptide bond (both catalysed and uncatalysed) plays an important role in such biochemical processes as protein folding in ribosomal protein synthesis (Kiefhaber *et al.*, 1992, Shalongo *et al.*, 1992, Texter *et al.*, 1992), regulation of the activation or breakdown of peptide hormones (Yaron and Naider, 1993), recognition of peptide antigens (Richards *et al.*, 1990), transport of polypeptides through lipid bilayer membranes, disposal of malfunctioning proteins, oligomerisation of proteins (Jaenicke, 1984), and triggering receptor-mediated trans-membrane signalling (Williams and Deber, 1991, Vogel *et al.*, 1993). Chemically, the peptidyl-prolyl *cis-trans* isomerisation is a rotation around the single bond and can thus be regarded as one of the simplest chemical “reactions” (Fischer, 1994).

1.4.1 Proline and peptide conformation

Among the twenty naturally occurring, common amino acids, proline is a unique protein building block as its α-nitrogen atom is part of the secondary amino group in the rigid pyrrolidine ring and at the same time it is covalently bound to the

preceding amino acid (McArthur and Thornton, 1991). Common amino acids assume *trans* conformation in peptides and proteins. However, proline amides display an equal tendency to assume both the stable *cis* and stereoisomeric *trans* amide conformation, and both forms occur in peptides, as in bradykinin (London *et al.*, 1979) and proteins, such as ribonuclease (Lewis *et al.*, 1973). Protein folding is slow when proline residues exist in the polypeptide. Rotation about the formally single bonds in the peptide backbone and in its sidechains is responsible for the flexibility of the polypeptide chain. Slow conformational changes arise primarily from delocalisation of the electrons in the amide bond and additional steric constraints imposed by the proline ring.

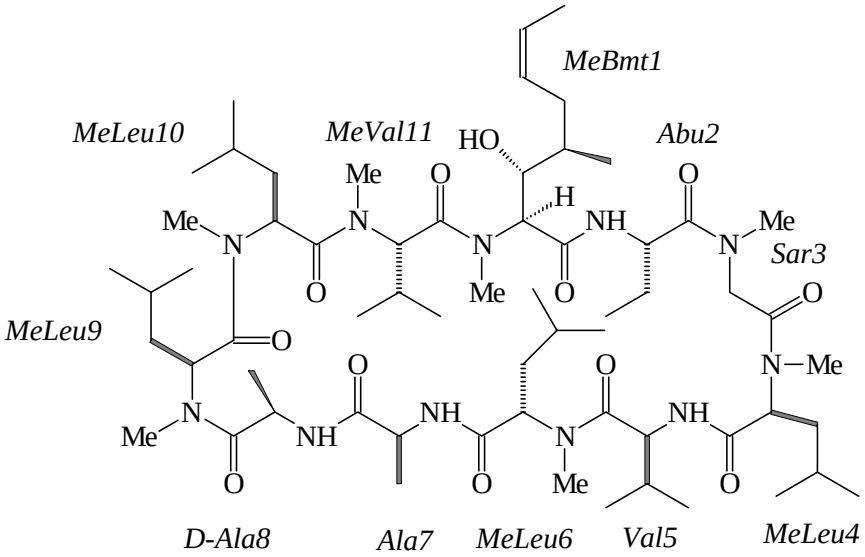
1.4.2 Peptidyl-prolyl *cis-trans* isomerases

The peptidyl-prolyl *cis-trans* isomerases (PPIases) are enzymes with rotamase activity in catalysing the interconversion of the peptidyl-prolyl *cis* conformers to the corresponding *trans* conformers (Galat, 1993, Fischer, 1994, Table 7). Peptidyl-prolyl *cis-trans* isomerases cannot significantly enhance the isomerisation rate of open-chain *N*-alkyl peptide bonds in oligopeptides (Schmid *et al.*, 1993). Currently, three protein families with peptidyl-prolyl *cis-trans* isomerase activity are known to exist in nature: cyclophilins (Cyp), FK506 binding proteins (FKBP) and parvulins (Kay, 1996). The PPIases can be found in a wide variety of prokaryotic and eukaryotic organisms.

Table 7. Characteristics of the three PPIase families (Kay, 1996).

	FKBPs	**CYCLOPHILINS**	**PARVULINS**
Domain size	approx. 108 residues	approx. 165 residues	92 residues
PPIase activity	– to ++	++ to +++	? to +++
Distribution	mammals, plants, lower eukaryotes, prokaryotes	mammals, plants, lower eukaryotes, prokaryotes	prokaryotes
Natural inhibitors	FK506, rapamycin	cyclosporins	?
Known in humans	5 (FKBP12, 12A, 13, 25, 52)	6 (CypA, B, C, D, 40, NK)	?

The first two families of PPIases are inhibited by cyclosporin A **95** and macrolides (Fig. 4). The cyclophilins are high-affinity PPIases for the naturally occurring immunosuppressive undecapeptide cyclosporin A (CsA) **95** which also specifically inhibits the rotamase activity of the cyclophilins (Handschumacher *et al*., 1984, Fischer *et al*., 1989, Takahashi *et al*., 1989).



95

Figure 4. Structure of the cyclophilin undecapeptide inhibitor cyclosporin A.

Furthermore, the FK506 binding proteins are specific binding proteins for two other naturally occurring secondary metabolites, FK506 **96** (Harding *et al*., 1989, Siekierka *et al*., 1989) and rapamycin **97** (Bierer *et al*., 1990a, b, Somers *et al*., 1991). The PPIase activity of the FKBPs is likewise completely inhibited by these immuno-suppressive macrolides (Fig. 5). Interestingly, no cross-inhibition of the cyclophilins by FK506 or rapamycin, nor the FKBPs by cyclosporin A has been observed. The parvulins are PPIases that are not members of either the cyclophilins or the FKBPs. Their rotamase activity is comparable to that of the cyclophilins, and it is higher than that of any FKBPs. In addition, the PPIase activity of parvulins cannot be inhibited by cyclosporin A, FK506 or rapamycin (Rahfeld *et al*., 1994a, b, Rudd *et al*., 1995).

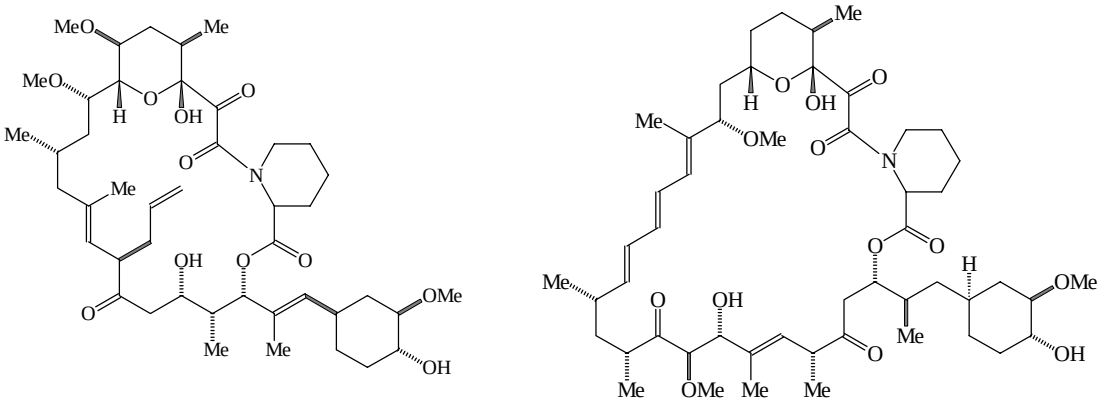


Figure 5. Stuctures of the FK506 binding protein inhibitors FK506 and rapamycin.

The amino acid sequence of the cyclophilins is very different from that of the FKBPs (Wiederrecht *et al*., 1991), and the structural similarity of their tentative active sites is not remarkable (Denesyuk *et al*., 1993). However, within the cyclophilin and FKBP families the primary amino acid sequences of the enzymes are highly conserved (Fischer, 1994).

The peptidyl-prolyl *cis-trans* isomerases generally follow the Michaelis–Menten kinetics, especially when the PPIase concentration is low during the isomerisation (Fischer, 1994). When $[S]_0 << K_m$, the rate enhancement for the *cis* to *trans* isomerisation is linearly dependent on the enzyme concentration. The *cis/trans* isomer distribution of the peptide substrate can be regarded independent of the PPIase concentration when [PPIase] << $[S]_0$. However, the isomer ratio can be dramatically changed if [PPIase] = $[S]_0$ or if there is an excess of the PPIase (Fischer *et al*., 1984b, London *et al*., 1990, Schönbrunner *et al*., 1991).

1.4.3 The peptidyl-prolyl isomerisation reaction mechanism

The determination of a peptidyl-prolyl *cis-trans* interconversion mechanism of both cyclophilins and FK506 binding proteins is a difficult task because neither bond formation nor bond breaking takes place during the reaction. Moreover, the PPIases exist in multiple enzyme forms with different catalytic characteristics (Fischer, 1994). Three different mechanisms for the *cis-trans* isomerisation can be suggested: (1)

nucleophilic attack to the amide bond carbonyl by the enzyme's nucleophile, (2) kinetic protonation of the nitrogen atom of the amide proline, and (3) twisting the amide bond out of conjugation (Fischer, 1994). The intent is to reduce the double bond character of the amide and to convert it into a single bond as the loss of amide bond resonance lowers the barrier of rotation. It must also be noted that water must be excluded from the active site of the enzyme to prevent hydrolysis of the amide bond. The isomerisation reactions of the dialkyl amides have been found to be faster in organic, non-polar solvents. The ground state of the isomers is destabilised and the electronic delocalisation of the amide bond is reduced (Drakenberg *et al*., 1972).

Both enzymatic nucleophilic and general base catalysis have been ruled out as the PPIase mechanisms by mutagenesis studies. Initially, it was observed that the PPIase activity of the human cyclophilin Cyp18cy can be suppressed by adding thiol modifying reagent 4-hydroxymercurybenzoic acid to the reaction mixture (Fischer *et al*., 1989). It was believed that the active-site cysteine residue plays a role in the isomerisation reaction. However, the mutagenesis of all four cysteines near the binding site of the Cyp18cy to alanines did not affect the PPIase activity of the enzymes significantly (Liu *et al*., 1990). Analogously, the mutation of such potential hydrogen bond donors and nucleophiles as cysteine, serine and threonine to alanines in the active site of FKBP12 did not result in loss of activity or affinity to FK506 and rapamycin (Park *et al*., 1992). Moreover, the secondary deuterium isotope effect of $[k_{cat}/K_M]_{\mathbf{H}}/[k_{cat}/K_M]_{\mathbf{D}} \approx 1.1$ of the Gly–Pro peptide substrate deuterated at the α-position is considered as evidence of that no hybridisation change $sp^2 \rightarrow sp^3$ of the amide bond carbonyl takes place (Harrison *et al*., 1990a). Thus, no nucleophilic addition of an enzyme nucleophile or hydroxide (water) occurs to the amide carbonyl during catalysis.

The mutations in the Cyp18cy active site arginine-55, phenylalanine-60 and histidine-125 suppressed the PPIase activity without eliminating the binding to cyclosporin A. Taken together with the structural studies of Cyp18cy and FKBP12, it could be conceivable that FKBP12's guanidine proton of arginine-55 (Zydowsky *et al*., 1992) and the C-terminal amide nitrogen proton of the substrate (Fischer *et al*., 1993**)** could act as proton donors in the kinetic protonation of the proline nitrogen atom. The arginine-55 of Cyp18cy cannot possibly participate in the protonation of the

proline nitrogen atom as its pK_a is about 12. However, it could stabilise the hydrogen bonding either directly or via a water molecule to the lone electron pair of proline nitrogen (Texter *et al*., 1992). The kinetic protonation of the proline nitrogen has been ruled out because the solvent deuterium isotope effects $[k_{cat}/K_M]_{\mathbf{H2O}}/[k_{cat}/K_M]_{\mathbf{D2O}} \approx 1$ and no pH effects on the isomerisation rate have been observed (pH 5 to 9) (Stein, 1993). Generally, the Cyp18cy and FKBP mediated *cis-trans* isomerisation reactions exhibit low enthalpies of activation and high entropies of activation (Harrison and Stein, 1992, Stein, 1993) when compared to uncatalysed *cis-trans* isomerisation reactions.

The current model of PPIase mechanism is a combination of substrate distortion by the PPIase and stabilisation of the transition-state by the hydrogen bonding to the lone electron pair of proline nitrogen (Fischer, 1994). The FKBP ligand FK506 adopts the orthogonal dihedral angle of the α-keto carbonyl both in solution (Petros *et al*., 1993) and when bound to FKBP (Van Duyne *et al*., 1991). FKBP twists the amide bond of the peptide to enhance the rate of the *cis-trans* isomerisation. The transition-state binds strongly to the enzyme which decreases the enthalpy of activation and at the same time the Gibbs free enery of activation (Harrison and Stein, 1992).

In FKBP12, the major driving force of the peptidyl-prolyl *cis-trans* isomerisation reaction is substrate destabilisation by twisting the amide carbonyl out of the plane with the nitrogen atom, and the subsequent repulsion of the amide carbonyl oxygen lone pairs of the substrates by the active site tyrosine-82 and aspartate-37. Autocatalysis is an additional factor in the FKBP catalysis; the lone electron pair of proline is stabilised by the substrate's amide proton of the residue C-terminal to proline (Fischer *et al*., 1993).

In cyclophilin Cyp18cy, the peptide substrate does not bind properly for the autocatalysis to take place (Kallen *et al*., 1992, Fischer *et al*., 1993). The active site arginine-55 residue is responsible for the hydrogen bond stabilisation of the proline lone electron pair. Additionally, X-ray crystallographical data suggest that the tetrapeptide substrates exhibit the distorted dihedral angles of *cis*-Xaa–Pro when bound to Cyp18cy (Kallen and Walkinshaw, 1992).

1.4.4 FK506 binding proteins

FK506 binding peptidyl-prolyl *cis-trans* isomerases (FKBPs) belong to the smallest enzymes known as their polypeptide chain consists of approximately 110 amino acids and their molecular weight is around 12 kDa. They are strongly and specifically inhibited by lipid soluble macrolides FK506 **96** and rapamycin **97** (Fig. 5) but not by cyclosporin A **95** (Fischer, 1994, Kay, 1996). FK506 **96** is a 21-member macrolactone with a pyranose ring and a masked tricarbonyl hemiketal structure (Tanaka *et al*., 1987). Rapamycin **97** in turn is a 31-member ring lactam with lactone linkages, an interesting *(E,E,E)* triene moiety and α,β-keto amide masked via C-10 hemiketal formation (Swindells *et al*., 1978, Findlay and Radics, 1980). There are currently approximately thirty FKBPs from a wide variety of prokaryotes and eukaryotes that are known at the genetic level (Kay, 1996).

Cytoplasmic FKBP12cy is a primary target for the immunosuppressive agents FK506 and rapamycin in human cells. It also possesses PPIase activity (Fischer *et al*., 1989, Takahashi *et al*., 1989). It is an unexceptionally stable protein widely distributed in mammalian tissues, especially in the brain (Steiner *et al*., 1992, Asami *et al*., 1993), lymphocytes (Sigal *et al*., 1990, Siekierka *et al*., 1991) and the regions around cell nuclei (Kobayashi *et al*., 1993). The amino acid sequence of FKBP12cy has no significant similarity to that of the cyclophilins (Kay, 1996).

FKBPs have also been isolated in other eukaryotes, such as the yeasts *Saccharomyces cerevisiae* (Heitman *et al*., 1991, Koltin *et al*., 1991) and *Candida albicans* (Ferrara *et al*., 1992), and the fungi *Neurospora crassa* (Tropschug *et al*., 1990) and *Tolypocladium inflatum* (Lee *et al*., 1992).[12] The presence of FKBPs in plants is not common. There is some evidence that a low-level PPIase activity detected in pea *Pisum sativum*, L. could be effected by a FKBP-family protein. The found PPIase activity was partially inhibited by rapamycin, but completely inhibited by CsA (Breiman *et al.*, 1992). In prokaryotes, FKBPs have been characterised in, for example, *Neisseria meningitidis* (McAllister and Stephens, 1993, Sampson and Gotschlich, 1992) and from three streptomycetes *Streptomyces chrysomallus, S.*

[12] Very interestingly, *T. inflatum* is a cyclosporin A (CsA) producing fungus that is a potent inhibitor of other main group of PPIases cyclophilins (Lee *et al*., 1992).

hygroscopicus and *S. hygroscopicus* subsp. *ascomycetius* (Pahl and Keller, 1992).[13] However, it is still very uncertain whether *E. coli* is able to produce FKBPs with PPIase activity (Kay, 1996).

FK506 binding proteins generally show high specificity for the amino acid at position P_1 of the peptide substrate (Albers *et al*., 1990, Harrison and Stein, 1990c, Fischer *et al*., 1992, Nielsen *et al*., 1992).[14] High second-order rate constants (or specificity constants k_{cat}/K_m) are observed when the amino acid residue in the P_1 position of the substrate has a relatively large hydrophobic side chain. Compared to cyclophilins, the FKBPs are less effective in catalysing the *cis-trans* isomerisation reactions (Fischer, 1994). Even with the optimum peptide substrate of FKBP12cy, Suc–Ala–Leu–Pro–Phe–*p*NA, the k_{cat}/K_m is approximately an order of magnitude smaller than with Cyp18cy (Park *et al*., 1992).

The inhibition of FKBPs by FK506 or rapamycin is regularly stoichiometric, for example, hFKBP12cy forms a 1:1 complex. The hydrophobic interactions are important in complex-formation as is evident from the enthalpy of formation of these complexes and the X-ray crystallographic structure of the FK506–FKBP. All the amino acid residues (Phe36, Ile56, Tyr82, Ile91, Leu97, Phe99) close to the binding site are hydrophobic (Van Duyne *et al*., 1991, Connelly and Thomson, 1992).

The complexation of FK506 in its FKBP receptor appears to be entirely reversible, neither does it involve a covalent binding of either of the electrophilic carbonyl groups of FK506. A *cis* conformation of the FK506 prevails in the solid state, a combination of *cis* and *trans* conformers are present in an aqueous solution, and after binding to its receptor FKBP, FK506 acquires the *trans* conformation. Despite the great electrophilic nature of the α-keto carbonyl, there is no experimental evidence that hemiketal or hydrate formation would be taking place in the α-keto amide carbonyl of either FK506 or rapamycin (Rosen *et al*., 1990).[15] Based on X-ray

[13] *S. hygroscopicus* produces rapamycin and *S. hygroscopicus* subsp. *ascomycetius* produces another FKBP inhibitor ascomycin. It is obvious that these streptomycetes have an efficient self-protection mechanism (McAllister and Stephens, 1993).

[14] P_1 refers to the amino acid Xaa whose carboxyl terminus is coupled to the isomerising proline ring.

[15] The related compounds, cyclotheonamides isolated from marine organisms are known as human α-thrombin proteinase inhibitors having similar α-keto amide moiety in their rings. Contrary to rapamycin and FK506, cyclotheonamides form a covalent tetrahedral adduct with the serine hydroxyl in the active site of α-thrombin and act thus as suicide inhibitors of the enzyme (Fusetani *et al*., 1990, Maryanoff *et al*., 1993).

crystallographical and NMR studies, it is known that the dihedral angle about the α-keto amide carbonyls of FK506 and rapamycin is 95° in the solid state (Findlay and Radics, 1980, Tanaka *et al.*, 1987). It is also known that the twisted peptidyl-prolyl amide bond of the substrate in the FKBP's active site adopts the dihedral angle of approximately 90° (Rosen *et al*., 1990). α-Keto amide carbonyls adjacent to the homoprolyl amide bond of FK506 and that of rapamycin are considered a substitute for the twisted amide carbonyl of a bound peptide substrate, since their ground-state geometry is similar to the transition-state structure of the peptide structure. *Hence, both FK506 and rapamycin can be regarded as stable transition-state analogues of the peptidyl-prolyl cis-trans isomerisation* (Rosen *et al*., 1990).

1.4.5 Cyclophilins

Cyclophilin Cyp18cy (previously known as cyclophilin A, CypA) is the major cytoplasmic peptidyl-prolyl *cis-trans* isomerase isoform abundantly expressed in all mammalian tissues. It has typically a molecular mass of 18 kDa. Cyp18cy binds the undecapeptide immunosuppressant cyclosporin A (CsA) **95** with high nanomolar affinity (K_d = 6 nM), and it appears to facilitate protein folding by enhancing the *cis-trans* interconversion of peptidyl-prolyl bonds (Fischer *et al*., 1989). Moreover, its PPIase activity is potently inhibited by cyclosporin A. All the eukaryotic cyclophilins seem to possess a high affinity to CsA (Fischer, 1994). Prokaryotic cyclophilins tend to bind CsA to a much smaller extent (Liu and Walsh, 1990, Hayano *et al*., 1991).

The amino acid sequence of cyclophilins contains highly conserved areas, which is believed to be an evidence of evolutionary conservation of protein-folding catalysis (Maki *et al*., 1990, Schönbrunner *et al*., 1991). The cyclophilins are chemically and thermally stable enzymes. However, due to their highly lipophilic character cyclophilins are easily denaturated by contact to hydrophobic plastic surface (McDonald *et al*., 1992). Cyp18cy is present in tissues and various cell types in elevated concentrations, exceptionally high concentrations have been observed in lymphocytes, kidney tubules, endothelial cells, T cells, and neuron-rich areas of the brain (Ryffel *et al*., 1988, Mihatsch *et al*., 1989, Lad *et al*., 1991). There also exist

cyclophilins that are secreted through the cell membrane in response to certain stimuli to the cell. For example, Cyp18cy related IL-1 is secreted through the cell membrane after stimulation of growth factors (Davis *et al*., 1991, Sherry *et al*., 1992).

Cis-trans isomerisation of the peptidyl-prolyl bond appears to play an important role in membrane channels, as is evidenced by blockade of the transport proteins in the inner membrane of mitochondria by cyclosporin A (McGuinness *et al*., 1990). It has been demonstrated with CsA-resistant unfunctional Cyp24mito mutants of *Neurospora crassa* that it is the formation of the CsA–Cyp complex and not the inhibition of PPIase activity by CsA alone that exerts the cytotoxic effects (Tropschug *et al*., 1989).

Cyp18cy is a highly effective enzyme whose catalytic efficiency approaches to the diffusion limits with its k_{cat}/K_m close to 2×10^8 M^{-1} s^{-1} (Fersht, 1985, Fischer, 1994). Additionally, the k_{cat}/k_{uncat} is approximately a million times higher with the already fast thermal and uncatalysed background reaction (Kofron *et al*., 1991). Interestingly, the nature of the Xaa of the optimal peptide substrates, Suc–Ala–Xaa–Pro–Xaa–*p*NA does not have a great effect on the k_{cat}/K_m of the isomerisation. The difference is only less than 20-fold when comparing the second-order rate constants of the best uncharged side chain Xaa-containing substrates to the "worst" charged side chain Xaa-containing peptide substrates (Harrison and Stein, 1990b, c, Bergsma *et al*., 1991, Liu *et al*., 1991, Compton *et al*., 1992).

Cyclophilins are considered to be the true catalysts in dynamic protein folding processes (Bächinger, 1987, Lang *et al*., 1987, Lang and Schmid, 1988).[16] The catalytic effect of cyclophilins on the protein refolding reactions is suppressed by cyclosporin A. When compared to the *cis-trans* isomerisation reactions of the simple peptide substrates, the catalytic efficiencies of cyclophilins with the macromolecular substrates are generally at least ten times less, provided that $[S] << K_m$ (Fischer, 1994). However, there are currently only a few examples providing quantitative data for the cyclophilin-assisted protein folding *in vivo*. For example, the triple helix formation in fibroblast procollagen was reduced 1.4-fold by CsA (Steinmann *et al*., 1991).

[16]Cyclophilins do not need the stoichiometric amounts of helper proteins or additives in the folding process like the molecular chaperones do. Molecular chaperones do not contain steric information about the correct folding, and they increase the yield but not the rate of the folding (Hartl, 1996).

As noted earlier, cyclophilins bind CsA in a nanomolar to micromolar range, and their PPIase activity is competitively inhibited by CsA.[17] The binding of CsA to Cyp18cy is a kinetically complex, solvent-dependent process (Kofron *et al*., 1991, 1992, Kuzmic *et al*., 1992). To act as cyclophilin inhibitor, CsA must have a *trans* conformation around its ^{9}MeLeu–^{10}MeLeu (Fesik *et al*., 1990, 1992, Weber *et al*., 1991). This required conformation is present in THF in very low concentration. However, in LiCl/THF the active inhibitory *trans* conformation of CsA is the predominant one (Köck *et al*., 1992). CsA cannot be regarded as a suicide inhibitor since Cyp18cy is believed not to be able to catalyse the *cis* to *trans* conversion of CsA. According to X-ray crystallographical (Pflügl *et al*., 1993) and NMR (Thériault *et al*., 1993) studies of the CsA–Cyp18cy, the structure of cyclophilin does not change remarkably during the formation of the complex. Antithetically, the bound conformation of CsA in Cyp18cy is immensely different from that in either the non-aqueous solution (Kessler *et al*., 1990) or the solid state (Loosli *et al*., 1985).

[17]Cyclosporin H (CsH) containing the **D**-form of ^{11}MeVal has neither significant affinity to cyclophilins nor any inhibitory effect on the PPIase activity of the cyclophilins (Fischer, 1994).

2 Aims of the present study

This work represents the field of applied biocatalysis in synthetic organic chemistry and aims at generating antibody proteins as specific catalysts for the organic and biochemical reactions.

The specific aims were:

1. To design and synthesise the appropriate haptens for elicitation of monoclonal antibodies by hybridoma technology for the following chemical and bio-chemical reactions: (a) the twisted α-keto amide haptens for *the acyl-transfer reactions*, (b) the conformationally flexible ferrocenyl haptens for *the Diels–Alder reaction* between aminobutadiene and *N,N*-dimethylacrylamide and (c) the twisted α-keto amide hapten for *the peptidyl-prolyl cis-trans isomerisation* of the prolyl amide substrates.

2. To synthesise the substrates for screening of the monoclonal antibodies for catalytic activity, and to synthesise the appropriate inhibitors for the found antibody catalysts.

3. To screen the monoclonal antibodies obtained for the possible catalytic activity by either spectrometric or chromatographic methods.

4. To study the kinetic behaviour of the antibody catalysts found, and to determine either their binding or inhibition properties.

3 Experimental

3.1 General synthetic methods (I–IV)

Unless otherwise stated, all reactions were carried out in oven-dried glassware with anhydrous solvents under anhydrous conditions under positive atmosphere of argon or nitrogen. Reagents and solvents were transferred with disposable plastic syringes and oven-dried or disposable needles.

Dichloromethane and chloroform were continuously distilled from calcium hydride and phosphorus pentoxide, respectively. Tetrahydrofuran was distilled from sodium–benzophenone. All reagents were purchased from Aldrich Chemical Company, Sigma Chemical Company, Fluka Chemie AG, Pfaltz & Bauer, Bachem, Bachem California, or Tokyo Kasei. All chromatography solvents were obtained from Fisher Scientific and used without further purification.

Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60F glass plates (0.25 mm, E. Merck, Darmstadt, Germany). UV light, 5% ethanolic phosphomolybdic acid, 1% ethanolic ninhydrin or *p*-anisaldehyde solution and heat were used as developing agents. Flash chromatography was performed with silica gel 60 (230–400 mesh, E. Merck, Darmstadt, Germany) as described by Still *et al.* (1978). Yields are for unoptimised procedures and refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise noted.

Melting points are uncorrected and were determined on a Fisher–Johns melting point apparatus. All proton NMR spectra (300 MHz) were obtained in $CDCl_3$, CD_3OD, D_2O, DMF-d_7, DMSO-d_6 or TFE-d_3 solutions at ambient temperature on a Bruker AM-300 spectrometer. The ^{13}C NMR spectra (500 MHz) were recorded on a Bruker AMX-500 instrument. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane as an internal reference at 0.00 ppm. Coupling constants *(J)* are given in Hertz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet), m (multiplet) and br (broad). High and low resolution fast atom bombardment (FAB) mass spectra were provided by Dr. Gary Siudzak of The Scripps Research Institute Mass Spectrometry Facility.

3.2 Preparation of the immunoconjugates (I, III, IV)

The haptens were activated for the protein coupling by adding 1.3 mole equivalents of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and *N*-hydroxysulphosuccinimide aqueous solutions to a solution of haptens (4.0 mg) in *N*,*N*-dimethylformamide (200 μl) (Staros, 1982, Staros *et al*., 1986, Anjaneyulu and Staros, 1987). Activation reactions were incubated in closed Eppendorf tubes at room temperature for 24 h. In the case of the Diels–Alder haptens, conjugation to the carrier proteins was performed in the absence of light, since aqueous solutions of **98** and **99** (Scheme 33) were prone to photo-oxidation.

The keyhole limpet hemocyanin (KLH) conjugate was prepared by adding 100 μl of the activated hapten solution to a solution of 5 mg of KLH in 900 μl of 50 mM sodium phosphate buffer, pH 7.4. The bovine serum albumin (BSA) conjugate was prepared in a similar fashion. The hapten–protein conjugates were incubated at 4 °C for 24 h. The hapten–protein conjugates were used as such in immunisation without further purification.

3.3 Immunisation, hybridoma generation and production of monoclonal antibodies (I, III, IV)

Hybridomas were generated by standard protocols (Köhler and Milstein, 1975, Harlow and Lane, 1988). Two intraperitoneal (i.p.) injections of Ribi adjuvant (in MPL and TDM emulsions) and 100 μg of the hapten conjugated to KLH were administered to four 8-week-old 129GIX$^+$ mice. After two weeks, booster injection was administered to the same mice. One month after the second injection, the mouse with the highest titer (12,800 to 25,600) was injected intravenously (i.v.) with 50 μg of the KLH conjugate (Engvall, 1980); 3 days later, the spleen was taken for the preparation of hybridoma cells. The splenocytes (1.0×10^8) were fused with SP2/0

myeloma cells (2.0×10^7). Cells were plated into 30, 96-well plates; each well contained 150 µl of hypoxanthine, aminopterin, thymidine–Dulbecco's minimal essential medium (HAT–DMEM) containing 1% Nutridoma® and 2% BSA.

After 2 weeks, the antibodies produced in the wells containing macroscopic colonies were assayed by ELISA for binding to the hapten–BSA conjugates. As the haptens **98** and **99** were sensitive to light, the ELISAs in this specific case were carried out in the dark. The colonies that initially produced antibodies, which bound to the hapten–BSA conjugates, were subcloned twice. All monoclonal antibodies producing hybridoma cells were injected into pristane-primed[18] 129GIX$^+$×BALB/c mice to generate ascites (Lacy and Voss, 1986).

The globular fractions from the ascites were precipitated by dropwise addition of saturated $(NH_4)_2SO_4$ at 4 °C, pH 7.2, to achieve the final concentration of 45%. Ammonium sulphate was removed by dialysis against 10 mM TRIS, pH 8.0 (I, III, IV). Next, the concentrated antibodies were purified by the following methods: anion exchange chromatography (DEAE-Sephacel®) followed by stepwise elution with NaCl gradient (50 to 500 mM NaCl) and concentration by ultrafiltration (I, III); cation exchange chromatography on a mono Q column and elution with stepwise NaCl gradient (0 to 500 mM NaCl) followed by ultrafiltration (I, IV); or affinity purification on a protein G Sepharose column with 0.05 M citric acid, pH 3.0 elution followed by collection of the fractions into neutralising 1 M TRIS, pH 9.0 (I, III, IV). The mAbs were determined to be > 95% homogeneous by sodium dodecyl sulphate polyacrylamide gel electrophoresis with Coomassie blue staining (Laemmli, 1970).

[18] Pristane is a trivial name for 2,6,10,14-tetramethylpentadecane.

3.4 Analytical methods

3.4.1 Preliminary screening of the antibodies (I, III)

The acyl-transfer reactions (I)

All the 22 mAbs obtained from the immunisation with hapten **103** and 26 antibodies for the hapten **104** (Fig. 7) were screened for possible catalytic activity using HPLC assay. The mAbs were incubated with the ester and amide substrates **a–k** (Table 8) in 50 mM BICINE, pH 8.5 at 37 °C. The rates of the uncatalysed reactions were determined in the absence of the monoclonal antibodies. A solution of antibody in 50 mM BICINE, pH 8.5 at 37 °C was assayed with substrates **a–k** in the same buffer using 5% DMSO as a co-solvent to give a final solution containing 20 μM of mAbs and 500 μM of substrates. The HPLC assays were performed on a VYDAC 201TP54 C_{18} reverse-phase column with an isocratic programme of 82% water (0.1% TFA) and 18% acetonitrile flowing at 1.5 ml/min. Product formation was quantitated against 3-methoxyphenol as internal standard at the wavelength of 254 nm. Due to the poor solubilities of the substrates **b** and **i**, their reactions were carried out in the presence of 200 μM and 50 μM substrates, respectively. In both of these reactions, the mAb concentrations were 10 μM.

The Diels–Alder reaction (III)

The initial rates of the Diels–Alder reaction between the aminobutadiene **87** and *N*,*N*-dimethylacrylamide **88** were determined in 10 mM phosphate buffered saline (PBS), pH 7.4 both in the absence and presence of the monoclonal antibodies. The accumulation of the diastereomeric *ortho* Diels–Alder adducts **90** (*exo*) and **91** (*endo*) was monitored by reverse-phase HPLC. Interestingly, formation of the *meta* regio-isomers was not detected in any of the antibody-catalysed or background reaction mixtures by either HPLC or ^{1}H NMR.

As the 33 antibodies specific to BSA-**98** were screened, seven antibodies were found to be catalysts. One of these antibodies was *endo* specific and the six other *exo* specific. The KLH conjugate of **99** elicited eight antibody catalysts from the 38 tested. Seven of these antibodies catalysed the formation of *endo* diastereomer **91** and one the formation of *exo* cycloadduct **90**.

3.4.2 HPLC kinetic assays (III)

A solution of antibody in 10 mM PBS, pH 7.4 at 37 °C was assayed with substrates **87** and **88** in the same buffer to give a final solution containing 20 μM of mAb 4D5 or 13G5, 0.67 to 8.0 mM of diene **87**, and 1.0 to 8.0 mM of dienophile **88**. HPLC assays were performed on a VYDAC 201TP54 C_{18} reverse-phase column with an isocratic programme of 85% water (0.1% TFA) and 15% acetonitrile flowing at 2.0 ml/min. Product formation was quantitated against the internal standard (80 μM, *N*-propyl-2-methylbenzamide), at the wavelength of 240 nm. The retention time of the *exo* **90** (t_R = 11.1 min) and *endo* **91** (t_R = 13.0 min) adducts formed in the catalysed reactions were identical with those of the authentic samples of synthesised **90** and **91**.

3.4.3 Spectrophotometric kinetic assays (IV)

To determine the kinetic constants of the monoclonal antibody catalysts of the peptidyl-prolyl *cis-trans* isomerisation reaction, an indirect α-chymotrypsin-coupled assay was used with minor modifications as described by Fischer (1984a,b) and Kofron (1991) with their co-workers. It is known that α-chymotrypsin cleaves the carboxy terminal peptide bond only in the *trans* Xaa–Pro conformer of the chromogenic substrate, Xaa–Pro–Phe–*p*NA. (Fischer *et al*., 1983, 1984a, b). The anhydrous 470 mM LiCl/TFE solvent system for the substrates increases the equilibrium population of the *cis* conformers, which in turn improves the accuracy of the kinetic assay (Kofron *et al*., 1991). Normally, the *trans* conformation of the Xaa–Pro peptide substrates is thermodynamically more favoured in aqueous systems. After the addition

of α-chymotrypsin to the substrate-containing solution, the burst phase due to the cleavage of the *trans* isomer is completed during the mixing time, and the remaining absorbance change is due to the *cis-trans* isomerisation.

The percentages of the *cis* isomer present in the substrate solutions in 470 mM LiCl/TFE was determined both spectrophotometrically and ^{13}C NMR spectrometrically as decribed by Kofron and co-workers (1991). The spectrophotometric determination is based on the rapid cleavage of the *trans* confomer by α-chymotrypsin prior to the addition of the peptidyl-prolyl *cis-trans* isomerase solution (human recombinant cyclophilin, Sigma). The *cis/trans* ratio can be calculated from the difference between the initial and final absorbances. The ^{13}C NMR method is in turn based on observing the peaks of the *cis* and *trans* C(β) and C(γ) proline resonances.

The assay buffer (100 mM EPPS, pH 8.0), co-solvents 2,2,2-trifluoroethanol (25 μl) and DMF (10 μl) and the aliquots of mAb 35C8 solutions in the assay buffer (final concentration of mAb 0.0, 2.5, 5.0 and 10 μM) were pre-equilibrated in the spectrophotometer until the temperature reached 4.0 °C (Lauda recirculating bath containing 50% ethylene glycol in water) for 10 minutes. A stream of dry air was passed through the cuvette chamber to prevent condensation. Immediately before the onset of the assay, a 100 μl aliquot of a 2.00 mM solution of α-chymotrypsin (Sigma, type II from bovine pancreas) in 1 mM HCl was added to the cuvette, followed by an aliquot of peptide substrate solution (final concentration 200 μM) in anhydrous 470 mM LiCl/TFE via a Hamilton syringe. The final composition of all reactions was 87.5% EPPS buffer, 10.0% 1 mM HCl (200 μM α-chymotrypsin) and 2.5% LiCl/TFE. The reaction was thoroughly mixed for 10 seconds with a teflon mixing foot before the acquisiton of data. The progress of the reactions was followed by observing the increase in absorbance due to the formation of 4-nitroaniline at the wavelengths of 392 nm or 426 nm using a HP 8452A diode array spectrophotometer and HP 89500 UV/VIS Chemstation software. The progress curves of the thermal background reactions were determined in the absence of the mAb solution. Absorbance data were collected on a computer and the progress curves were analysed by nonlinear least-squares fit to the integrated rate equation (Kofron *et al.*, 1991).

3.4.4 Spectrofluorometric kinetic assays (IV)

The peptidyl-prolyl *cis-trans* isomerisation of the fluorogenic substrates was followed by observing the increase in fluorescence due to the formation of the *trans* isomer of the peptide in which there is a decrease in collisional quenching, λ_{ex}= 337 nm, λ_{em}=410 nm, using a SPF-5000C spectrofluorometer (SLM Instruments). Typically, reactions were conducted in a volume of 2.0 ml by first equilibrating 100 mM EPPS, pH 8.0; 470 mM LiCl/TFE; DMF; and the antibody (from a stock solution in EPPS) at 4 °C (Lauda recirculating bath containing 50% ethylene glycol in water) for 10 minutes. A stream of nitrogen was passed through the cuvette chamber to prevent condensation. An aliquot of peptide substrate solution in 470 mM LiCl/TFE was added via a Hamilton syringe to initiate reactions. The final composition of all reactions was 96.5% EPPS buffer; 2.5% 470 mM LiCl/TFE; and 1.0% DMF. The reactions were thoroughly mixed for 10 seconds with a teflon mixing foot before the acquisition of data.

Fluorescence data were collected on a computer, and all data from each assay were analysed by nonlinear least-squares regression according to the groups of Rich and Stein (Harrison and Stein, 1990b, c, García-Echeverría *et al*., 1992). The k_{cat}/K_m values were calculated from the pseudo-first-order rate constants derived from the entire progress curve, and the k_{cat} values were obtained from the initial rates of progress curves at varying substrate concentrations.

The values obtained for the antibody kinetic constants can only be considered approximations taking practical experimental limitations into account. Restrictions were imposed by the concentrations of antibody necessary to observe catalysis and the low solubility of substrates in either the 470 mM LiCl/TFE or the final buffer solutions. Hence, adherence to $[S] << K_m$ for determination of k_{cat}/K_m or $[S] >> K_m$ for determination of k_{cat} could not be rigorously met. The K_m values were assessed over a narrow concentration range ($0.5 \times K_m$ to $1.5 \times K_m$) and estimated to be near 100 μM in all cases. The k_{cat}/K_m was obtained from experiments carried out slightly below K_m, and k_{cat} from reactions at the solubility limit of substrates ($1.5 \times K_m$ to $2 \times K_m$).

3.4.5 HPLC studies for determining enantiomeric excess (III)

The four enantiomeric Diels–Alder *ortho* adducts (two *exo* **90** and two *endo* **91**) could be separated simultaneously by HPLC using a normal phase DAICEL Chiralpak AD column with an isocratic mobile phase of 70% hexane (1.5% TFA) and 30% iso-propyl alcohol flowing at 1.0 ml/min, λ = 240 nm (Gouverneur *et al*., 1993). The retention times of the two *exo* adducts **90** were 6.5 and 7.7 min, while the enantiomeric *endo* adducts **91** eluted at 8.8 and 10.7 min. Unfortunately, under these conditions the substrate diene **88** had a retention time of 7.0 min, thereby co-eluting with the enantiomeric *exo* adducts **90**. Thus, it was necessary to separate the enantiomers from the mixtures containing any excess diene prior to their injection onto the chiral column.

The 1 ml reactions were set up. These reactions containing 20 μM mAb 13G5 or 4D5 and 4.0 to 8.0 mM diene **88** and dienophile **89** were allowed to incubate for 3 to 7 days at 37 °C. The formed enantiomeric adducts were isolated by injecting the reaction mixtures onto a reverse phase semipreparative HPLC column (VYDAC 201TP510) and eluting with 85% water (0.1% trifluoroacetic acid) and 15% aceto-nitrile at 6.0 ml/min, λ = 240 nm. Retention times for the *exo* adducts **90**, *endo* adducts **91**, and diene **88** on the semipreparative column were 14.0, 16.0 and 52.0 min, respectively. The mobile phase was removed by a combination of rotary evaporation and high vacuum pump. The *exo* **90** and *endo* **91** adducts were then re-dissolved in a small volume (100 μl) of equimixture CH_2Cl_2/MeOH/*n*-hexanes, and the aliquots were injected onto the analytical chiral column. Individual enantiomer concentrations from antibody-catalysed reactions were compared to those from appropriate controls to determine their relative enantiomeric excesses.

3.4.6 Fluorescence quench experiment (III)

The mAbs were diluted to 0.1 μM in a final volume of 2 ml with 5% DMF in 10 mM PBS, pH 7.4. Fluorescence was measured on a SPF-5000C Aminco (SLM Instruments) spectrofluorometer with the λ_{ex} and λ_{em} of 280 and 338 nm, respectively.

The solution of 1-carboxy-1'-[(dimethylamino)carbonyl]ferrocene **100** prepared in 5% DMF and 10 mM PBS, pH 7.4 was added sequentially to the mAb solution. The background fluorescence quench was measured by adding 5% DMF in 10 mM PBS, pH 7.4 into the same mAb solution. The bound mAb was determined as the percentage of quench $(Q_0/Q_{max}) \times$ [mAb], where Q_0 is the measured decrease from the initial fluorescence, and Q_{max} was experimentally determined as 90 at high concentrations of **100**. Estimates of average intrinsic affinity were calculated from a Scatchard plot (Scatchard, 1949).

3.4.7 Cross-reactivity study of the catalytic antibodies (III)

Each well of a Costar 96-well microtiter plate was precoated with 25 μl of the primary antigen (5 mg/ml each conjugate) at 1:1000 dilution. The plate was dried overnight at 37 °C, and on day two the wells were fixed with 50 μl/well methanol and allowed to incubate for 5 min at 25 °C. Methanol was removed, and the plate was allowed to air dry for 10 min. To prevent non-specific adsorption, 50 μl/well of BLOTTO[19] was added to the wells. After incubating for 5 min, the BLOTTO was shaken out, and 25 μl/well of new BLOTTO was added to facilitate titering. A 25 μl sample of each catalytic antibody was added to each of the designated wells and serially diluted across the plate. The plate was incubated at 37 °C for another hour in a moist chamber. The plate was then washed 20 times with deionised water, and the bound antibody was detected with the addition of 50 μl of developing agent (glucose; 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and horse-radish peroxidase in phosphate buffer pH 6.0) to each well. Thirty minutes later the plate absorbance was read at 405 nm.

[19] BLOTTO is a blocking buffer used to prevent nonspecific adsorption in ELISA assays. BLOTTO consists of 5% (w/v) nonfat powdered milk in TRIS buffered saline.

4 Results and discussion

4.1 Twisted α-keto amides as transition-state analogues of the acyl-transfer reactions (I, III)

Mechanistic investigations of base-induced acyl-transfer reactions have revealed reaction pathways that traverse through the tetrahedral transition-state by the concerted attack of hydroxide ion on the electrophilic carbonyl carbon concomitant with the development of a negative charge on the carbonyl oxygen. The most successful haptens as transition-state analogues of the acyl-transfer reactions in generating catalytic antibodies have been phosphonates. Their success has been attributed to the tetrahedral geometry, the phosphorus-oxygen bond length (typically 10–15% longer than a carbon-oxygen bond) and evenly distributed negative charge between phosphorus and oxygens. This and other transition-state analogue approaches (Chapter 1.2.1) have been successful. However, to overcome even more difficult acyl-transfer reactions, *i.e.* amide bond hydrolysis, either a large array of antibodies must be sampled (Burton, 1993, Chen *et al*., 1993) or new hapten designs must be explored. That is why this study of the use of the twisted α-keto amides as transition-state analogues for the acyl-transfer reactions was undertaken.

4.1.1 Design of the haptens (I)

The design of the α-keto amide haptens for the production of monoclonal antibodies to study their potential catalytic properties in acyl-transfer reactions is based on the findings of the immunosuppressants FK506 **96** and rapamycin **97** (Fig. 5) as well as the serine proteinase inhibitors cyclotheonamide A **101** and B **102** (Fig. 6) which are believed to function as stable ground-state analogues of the transition-state of the acyl-transfer reactions (Rosen *et al*., 1990, Schreiber, 1991, Hagihara and Schreiber, 1992, Rosen and Schreiber, 1992). X-ray crystallographical analyses of FK506 **96** and rapamycin **97** have clearly demonstrated that the keto amide carbonyls of the α-keto amides are oriented orthogonally to one another, here the dihedral angles are 95°.

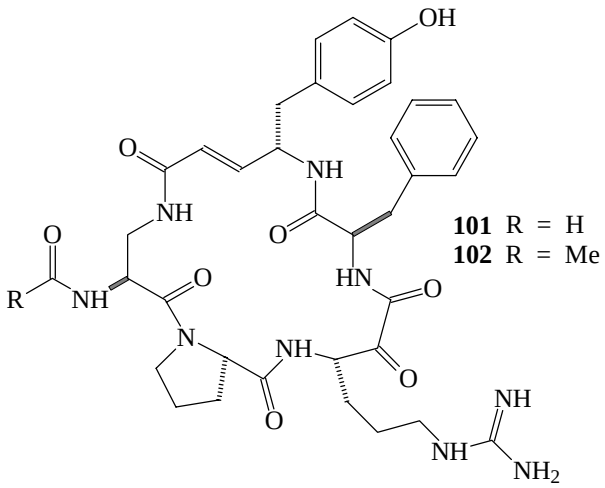


Figure 6. Structures of α-thrombin inhibitors: cyclotheonamides A **101** and B **102**.

Cyclotheonamides A **101** and B **102** are naturally-occurring inhibitors of the human α-thrombin and they possess a characteristic α-keto amide moiety (Fusetani *et al.*, 1990). According to an X-ray crystallographic study, the dihedral angle of the two carbonyl groups of the α-keto amide group is 109° (Maryanoff *et al.*, 1993). Similarly, the two adjacent carbonyl groups of the immunosuppressants FK506 **96** and rapamycin **97** are oriented orthogonally to one another with the dihedral angle of approximately 95° (Findlay and Radics, 1980, Tanaka *et al.*, 1987).

Structurally the roots of hapten **103** can be traced back to the cyclic peptides cyclotheonamide A **101** and B **102**. It has been suggested that the α-keto amide moiety of these peptides may function as an electrophilic mimic of the arginine–Xaa scissile bond of thrombin substrates (Hagihara and Schreiber, 1992). In fact, a study by Maryanoff *et al.* (1993) demonstrated inhibition of human α-thrombin by cyclotheonamide A **101**. Taken together with the dihedral angle of 109° of cyclotheonamide A **101** when bound to α-thrombin, these intermolecular interactions provide an excellent guide to possible structural motifs which the antibody may adopt. It can be anticipated that the α-keto amide substructures found in FK506 **96** and rapamycin **97** mimic a twisted amide bond (*i.e.* one possible transition-state for the amide bond hydrolysis). Analogously, it is conceivable that hapten **104** will adopt an orthogonal conformation, which serves as a transition-state analogue for the acyl-transfer reaction, and should elicit antibodies that twist potential substrates (Fig. 7) into a much more reactive conformation.

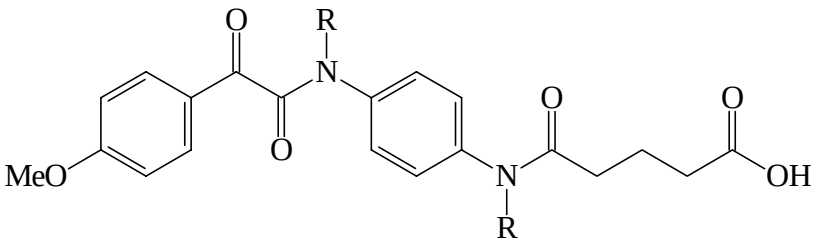
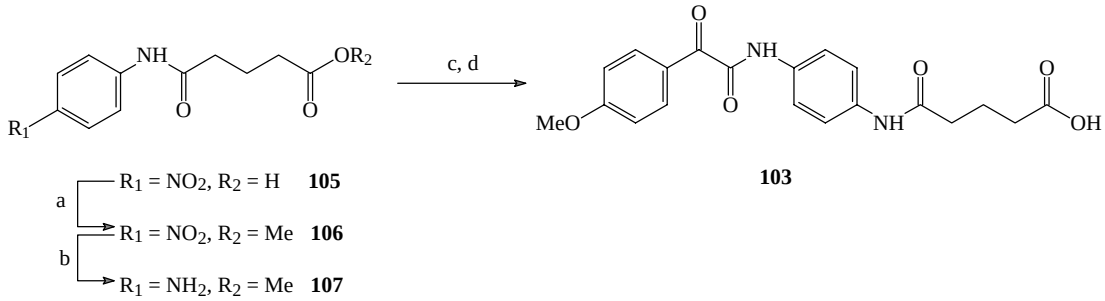


Figure 7. The structure of haptens **103**, R = H, and **104**, R = Me.

4.1.2 Preparation of the haptens (I)

Hapten **103** was prepared from 5-[(4-nitrophenyl)amino]-5-oxopentanoic acid **105** by means of the Fischer esterification reaction to give the ester **106** (MeOH/cat. H_2SO_4, Δ, 2 h, 96%, Scheme 30), catalytic hydrogenation (3 atm H_2, 10% Pd/C, MeOH, rt, 1.5 h, 93%), the amide bond formation between **107** and 4-methoxyphenylglyoxylic acid **108** (BOPCl, TEA, CH_2Cl_2, 0 °C, 1 h, 85%) (Diago-Meseguer *et al.*, 1980) and finally the hydrolysis of the methyl ester (NaOH, H_2O, rt, 20 min, 92%).

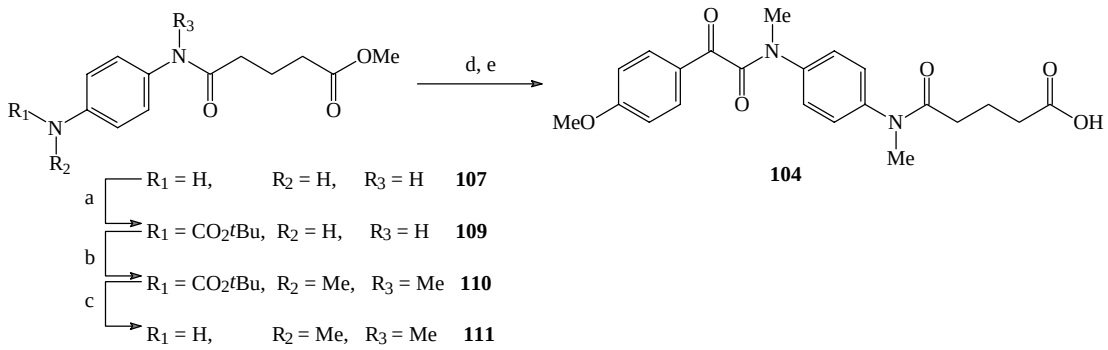


Scheme 30. (a) MeOH/cat. H_2SO_4, Δ, 2 h, 96%; (b) 3 atm H_2, 10% Pd/C, MeOH, rt, 1.5 h, 93%; (c) **108**, BOPCl, TEA, CH_2Cl_2, 0 °C, 1 h, 85%; (d) NaOH, H_2O, rt, 20 min, 92%.

4-Methoxyphenylglyoxylic acid **108** was prepared by the oxidation of (±)-4-methoxymandelic acid with potassium permanganate in NaOH/H_2O at 0 °C (Cornforth, 1951, Fatiadi, 1987). Several attempts to synthesise 4-methoxyphenyl-glyoxylic acid were undertaken. Initial investigations of benzylic oxidation employing pyridinium dichromate, PDC (Corey and Schmidt, 1979) or pyridinium chloro-

chromate, PCC (Corey and Suggs, 1975, Piancatelli *et al*., 1982) at room temperature and MnO_2 at 50 °C (Hudlicky, 1990) gave 4-methoxybenzaldehyde as a major product. Both methods gave very poor yields of 4-methoxyphenylglyoxylic acid (15, 14 and 26%, respectively). The formation of 4-methoxybenzaldehyde occurs via an oxidative cleavage of the carbon–carbon bond of the cyclic chromium or manganese ester. In another attempt, the reaction of (±)-4-methoxymandelic acid with the catalytic oxidant tetra-*n*-propylammoniumperruthenate, TPAP (Griffith *et al*., 1987), using *N*-methylmorpholine *N*-oxide as co-oxidant afforded 4-methoxybenzoic acid as a major product (92% yield). Its formation can also be explained by the oxidative cleavage of the cyclic five-member ruthenium ester.

Similarly, hapten **104** was prepared from methyl 5-[(4-aminophenyl)amino]-5-oxopentanoate **107** by *N*-protection with *tert*-butoxycarbonyl group ($(t\text{-BuOCO})_2O$, 1,4-dioxane, 85 °C, 12 h, 81%, **109**, Scheme 31), *N*-methylation (2.0 equiv. of MeI, KOBu-*t*, THF, rt, 45 min, 90%, **110**), acidic deprotection of the *tert*-Boc group (TFA, CH_2Cl_2, rt, 25 min; then TEA, CH_2Cl_2, 76%, **111**), amide bond formation between **108** and **111** (BOPCl, TEA, CH_2Cl_2, rt, 36 h, 92%) (Diago-Meseguer *et al*., 1980) and finally, by hydrolysis of methyl ester (NaOH, H_2O, 0 °C, 20 h, 76%).



Scheme 31. (a) $(t\text{-BuOCO})_2O$, 1,4-dioxane, 85 °C, 12 h, 81%; (b) 2.0 equiv. of MeI, KOBu-*t*, THF, rt, 45 min, 90%; (c) TFA, CH_2Cl_2, rt, 25 min; then TEA, CH_2Cl_2, 76%; (d) **108**, BOPCl, TEA, CH_2Cl_2, rt, 36 h, 92%; (e) NaOH, H_2O, 0 °C, 20 h, 76%.

As a hydrated keto carbonyl could also be used to mimic the tetrahedral transition-state of the hydrolytic reaction (Rich, 1985, Kitazume *et al*., 1994), ^{13}C NMR studies were undertaken. The studies (DMF-d_7 or DMSO-d_6, 10% D_2O, 1,4-dioxane as an internal standard) of the haptens **103** and **104** showed the absence of

hydration within the α-keto amide moiety (Krois *et al*., 1980, Krois and Lehner, 1982, Rich *et al*., 1982, Angelastro *et al*., 1990, Ocain and Rich, 1992, Patel *et al*., 1993). Based on these findings, it was anticipated that compounds **103** and **104** would exist in their keto forms during the immunisation process rather than a *gem*-diol configuration. The electron-donating methoxy group of the ring provides enhanced electron density to the α-keto carbonyl, thereby retarding any chance of hydration.

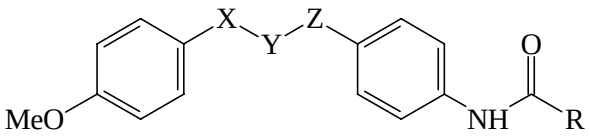
The haptens **103** and **104** feature an α-keto amide moiety as a transition-state functionality, and a 4-methoxyphenyl group for recognition and enhanced immunogenicity. While similar, there is one key structural attribute that differentiates these haptens, namely the *N*-methylation of the α-keto amide in hapten **104**. Such a substitution pattern allows the assessment of the relevance of the dihedral angle which is observed in FK506 **96** and rapamycin **97**, and that of cyclotheonamide A **101**.

4.1.3 Assays

In this study, twisted α-keto amides were used as haptens for the production of monoclonal antibodies to investigate their potential catalytic properties in acyl-transfer reactions. The hapten design was based on experimental findings of the T-cell inhibitors FK506 **96**, rapamycin **97** (Schreiber 1991, Rosen and Schreiber, 1992), serine protease inhibitors (Peet *et al*., 1990, Edwards *et al*., 1992) and thrombin inhibitors cyclotheonamide A **101** and B **102** (Maryanoff *et al*., 1993, 1995).

Twenty-two monoclonal antibodies for the hapten **103** and 26 for the hapten **104** were shown by an ELISA to bind to the α-keto amide haptens of **103** and **104** conjugated to BSA, respectively. All of the antibodies were screened for catalytic activity with the substrates given in Table 8 using HPLC assay. Unfortunately, none of the mAbs elicited against the haptens **103** and **104** showed hydrolytic activity in these assays. However, recent studies with the mAbs obtained from immunization with a structurally related α-keto piperazinyl amide imply that the twisted, non-hydrated α-keto amide moiety can be a viable mimic for the transition-state of the acyl-transfer reactions, eliciting antibodies with hydrolytic properties (M. Taylor, J. Yli-Kauhaluoma, J. Ashley and K. D. Janda, unpublished results).

Table 8. Structures of the substrates for antibody-catalysed hydrolytic reactions.



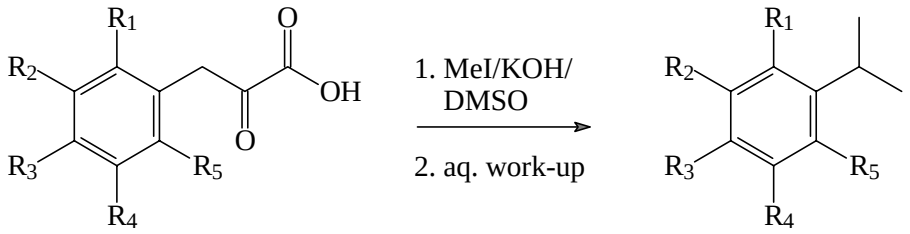
Entry	X	Y	Z	R
a	C=O	NH	CH_2	$(CH_2)_3CO_2H$
b	C=O	O	CH_2	CH_3
c	O	C=O	CH_2	$(CH_2)_3CO_2H$
d	NH	C=O	CH_2	$(CH_2)_3CO_2H$
e	CH_2	C=O	NH	$(CH_2)_3CO_2H$
f	CH_2	C=O	O	$(CH_2)_3CO_2H$
g	C=O	–	NH	$(CH_2)_3CO_2H$
h	C=O	–	O	$(CH_2)_3CO_2H$
i	O	–	C=O	CH_3
j	NH	–	C=O	$(CH_2)_3CO_2H$
k	C=O	NH	CH_2	CH_3

4.1.4 Serendipitous discovery of the concurrent alkylative decarbonylation and decarboxylation reaction of methoxy-substituted phenylpyruvic acids (II)

During the course of the studies on the use of twisted α-keto amides as potential transition-state analogues for acyl-transfer reactions (I) and peptide-prolyl *cis-trans* isomerisation of peptide substrates (IV), several 2-ketocarboxylic acids were prepared as starting materials for the hapten syntheses. Serendipitously, it was found that isopropylanisoles and veratroles can be conveniently obtained from the corresponding methoxy-substituted 3-phenyl-2-oxopropanoic acids.

Methoxy-substituted 3-phenyl-2-oxopropanoic acids **112a–f** were prepared via the classical Erlenmeyer azalactone method (Erlenmeyer, 1893, Carter, 1946). Methoxyphenylaldehydes were transformed to *(E)*-4-arylmethylene-2-oxazolin-5-ones through the condensation reaction of *N*-benzoylglycine in the presence of acetic anhydride and anhydrous sodium acetate. All azalactones obtained were crystalline and were converted to the requisite α-keto acids **112a–f** by refluxing them in aqueous

sodium hydroxide (Snyder *et al.*, 1955). A variety of isopropyl anisoles and veratroles **113a–f** were obtained when α-keto acids **112a–f** were treated with four equivalents of potassium hydroxide, an excess of iodomethane in DMSO and finally with water (Scheme 32, Table 9).[20] The isolated crude products were purified either by silica gel chromatography or fractional distillation.



112 **113**

Scheme 32

Table 9. Reaction of the substituted 3-(methoxyphenyl)-2-oxopropanoic acids with KOH/MeI in DMSO at room temperature.

Compd. 113	R_1	R_2	R_3	R_4	R_5	Yield [%]	B. p. [°C/Pa]
a	H	OMe	OMe	H	H	93	55/270
b	OMe	H	OMe	H	H	89	N. D.
c	H	OMe	H	H	H	92	128/130
d	H	H	OMe	H	H	90	N. D.
e	H	OMe	H	OMe	H	88	N. D.
f	OMe	OMe	H	H	H	91	49/670

This concurrent alkylative decarbonylation and decarboxylation reaction of methoxy-substituted phenylpyruvic acids is devoid of the formation of unwanted regioisomers. The yields typically range from good to excellent. Furthermore, the use of other alkylating agents could expand the scope of this reaction to other alkyl derivatives of anisoles and veratroles.

[20] Upon substituting KOH for caesium carbonate, the same reaction gave methyl 3-(methoxyphenyl)-3,3-dimethyl-2-oxopropanoates in good yields.

4.2 The antibody-catalysed Diels–Alder reaction (III)

In view of its synthetic utility, the Diels–Alder reaction has been seen as an important focus for the catalytic antibody field (Chapter 1.3.6). One of the previous approaches utilised the bicyclo[2.2.2]octene haptens **89** and **92** to induce antibody catalysts to control the product distribution of the reaction between diene **87** and dienophile **88** (Gouverneur *et al*., 1993). This was based on the work reported by Braisted and Schultz (1990) who suggested that the bicyclo[2.2.2]octene system might be a good mimic for the highly-ordered transition-state of the Diels–Alder reaction which was later confirmed by *ab initio* calculations.

In this study, the ability of the immune system to recognise conformationally unrestricted haptens was studied. The dicyclopentadienyl system of ferrocene was proposed to act as a loose mimic for the cyclic, six-member transition-state of the Diels–Alder reaction between 4-carboxybenzyl *trans*-1,3-butadiene-1-carbamate **87** and *N*,*N*-dimethylacrylamide **88**. Clearly, from the vast immune library, antibodies to all stable conformers of the molecules should exist. However, the antibody recognition and the freezing out of a conformer that resembled the Diels–Alder transition-state would be critical to the success of this work.

4.2.1 Hapten design and the synthesis of haptens and substrates

There were three major reasons for the decision to use ferrocene complexes of **98** and **99** as haptens for the Diels–Alder reaction (Fig. 8, Schemes 28, 33). First, these haptens have two pentagonal, delocalised π-electron ring systems stacked upon each other, in a sandwich-type arrangement, with an inter-ring distance of ≈ 3.3 Å (Rosenblum, 1965, Deeming, 1982). Such a network would not only be highly immunogenic but might also elicit antibodies with combining sites which could harvest the diene and dienophile into a reactive, ternary complex. Second, exploitation of rotational freedom was pivotal in this hapten design. The barrier of rotation around the common axis in this type of ferrocenes is low, 2 to 5 kcal mol^{-1}, (Rosenblum 1965, Deeming, 1982). Therefore, the rotation of the cyclopentadienyl rings with

respect to each other should not be restricted. This feature provides an opportunity to mimic the diastereomeric transition-states of the Diels–Alder reaction and the exciting possibility of generating, with a single hapten, monoclonal antibodies that could catalyse formation of all diastereomers. Third, the lipophilic character inherent in the disubstituted η^5-cyclopentadienyl system of haptens **98** and **99** should induce a hydrophobic microenvironment in the antibody's binding site. This should serve to enhance sequestering the diene and dienophile from aqueous solution and improve observed rates.

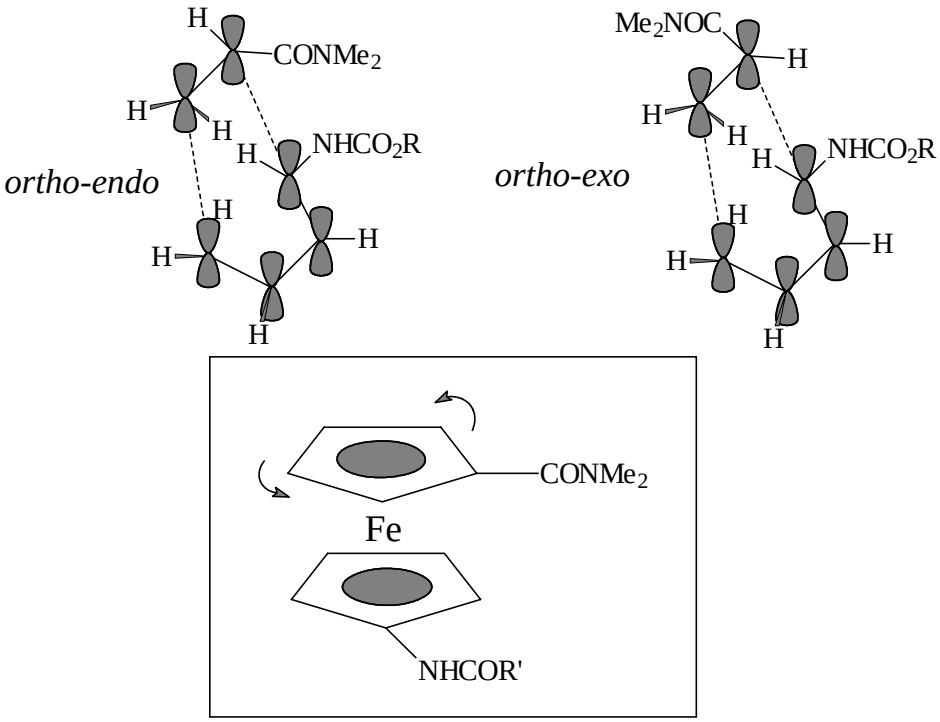
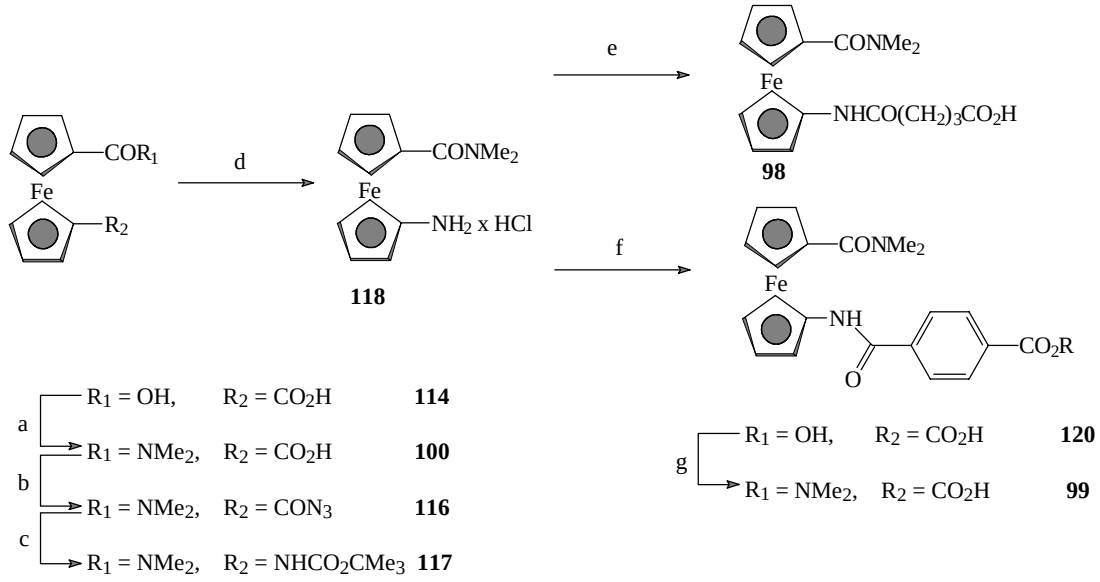


Figure 8. Transition-states of the *ortho-endo* and *ortho-exo* Diels–Alder reactions and the ferrocene mimic for these transition-states, R = $NHCH_2CO_2(C_6H_4)CO_2H$, R' = $(CH_2)_3CO_2H$ or 4-carboxyphenyl.

Hapten **98** was prepared in the following manner. Amide bond formation was accomplished between 1,1'-ferrocenedicarboxylic acid **114** and dimethylamine hydrochloride using BOPCl (Diago-Meseguer *et al.*, 1980) as coupling agent (7.0 equiv. of EDIA, $CHCl_3$, 0 °C, 1 h). Monoamide **100** was recovered along with the bisamide **115** in a moderate yield of 58%. The modified Curtius rearrangement (Shioiri *et al.*, 1972) of the acyl azide **116** prepared from **100**, with diphenylphosphoryl azide (2 equiv. of

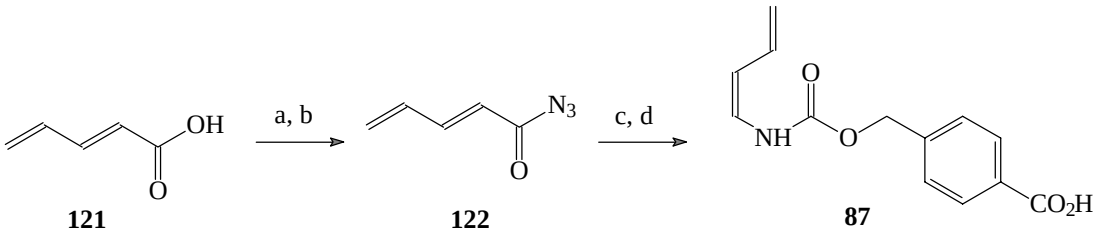
TEA, PhMe, rt, 25 min, 98%) gave the *tert*-butyl carbamate **117** in good yield (*t*-BuOH, PhH, 95 °C, 15 min, 83%). The use of 2 equiv. of TEA instead of 1 equiv. improved the yield of acyl azide **116** from 73 to 98% (Patel *et al*., 1993). Hapten **98** was obtained in 76% yield (2 steps) from **117** after removal of the *t*-Boc and attachment of glutaric anhydride to the amine hydrochloride **118** in the presence of EDIA in $CHCl_3$ (Scheme 33).



Scheme 33. (a) $Me_2NH{\cdot}HCl$, BOPCl, EDIA, $CHCl_3$, 0 °C, 1 h, 58%; (b) $(PhO)_2PON_3$, TEA, PhMe, rt, 25 min, 98%; (c) *t*-BuOH, PhH, Δ, 15 min, 83%; (d) 4 M HCl in 1,4-dioxane, rt, 20 min, 85 %; (e) glutaric anhydride, EDIA, $CHCl_3$, 0 °C, 13 h, 90%; (g) $LiOH{\cdot}H_2O$, $MeOH/H_2O$ (5:1), 0 °C, 25 min, 91%.

Hapten **99** was conveniently prepared by coupling ferrocenyl amine **118** and methyl 4-chloroformylbenzoate **119** (EDIA, $CHCl_3$, 0 °C, 13 h, 90%). The hydrolysis of methyl ester **120** provided hapten **99** in good yield ($LiOH{\cdot}H_2O$, $MeOH/H_2O$ 5:1, 0 °C, 25 min, 91%) (Scheme 33).

The diene substrate, 4-carboxybenzyl *trans*-1,3-butadiene-1-carbamate **87**, was prepared from *trans*-2,4-pentadeinoic acid **121** in four steps with minor modifications to the work reported by Weinstock and Overman groups (Weinstock, 1961, Overman *et al*., 1978). The formation of a mixed anhydride and acyl azide **122** was followed by Curtius rearrangement in the presence of methyl 4-(hydroxymethyl)benzoate **123** (Scheme 34). The hydrolysis of methyl ester carbamate afforded the diene substrate **87**, which was converted to its sodium salt.



121 **122** **87**

Scheme 34. (a) EtO_2CCl, EDIA, Me_2CO, 0 °C, 1 h; (b) NaN_3 in H_2O, 0 °C, 45 min; (c) **123**, PhMe, 100 °C, 40 min, 51%; (d) $LiOH·H_2O$, $MeOH/H_2O$ (20:1), 0 °C, 90 min, 87%.

4.2.2 Kinetic behaviour of the antibody catalysts

The most efficient *endo* catalyst 4D5 and *exo* catalyst 13G5 were kinetically characterised in detail. To determine the kinetics of these reactions, the differences in the initial rates between the catalysed and uncatalysed reactions were measured. Catalysis of the *endo* and *exo* Diels–Alder reactions by antibodies 4D5 and 13G5, respectively, were examined as random bireactant systems. Both antibodies showed multiple turnovers, and product inhibition was not observed. Lineweaver–Burk plots were constructed by holding one substrate at a fixed concentration while varying the concentration of the second. The kinetic parameters for the antibody-catalysed Diels–Alder reaction are presented in Table 10. The kinetic data are comparable to the results obtained in the previous study of antibody catalysis of the same Diels–Alder reaction (Gouverneur *et al*., 1993).

Table 10. Kinetic parameters for the antibody-catalysed [4π + 2π] cycloaddition between diene **87** and dienophile **88**.

Hapten	Antibody	K_m [M] Diene	K_m [M] Dienophile	k_{cat} [s^{-1}]	k_{uncat} [s^{-1}]	k_{cat}/k_{uncat} [M]
89	22C8 *exo*	7.0×10^{-4}	7.5×10^{-3}	5.28×10^{-5}	2.92×10^{-6}	18
91	7D4 *endo*	9.6×10^{-4}	1.7×10^{-3}	5.73×10^{-5}	1.19×10^{-5}	4.8
98	13G5 *exo*	2.7×10^{-3}	1.0×10^{-2}	2.00×10^{-5}	2.92×10^{-6}	6.9
99	4D5 *endo*	1.6×10^{-3}	5.9×10^{-3}	5.80×10^{-5}	1.19×10^{-5}	4.9

4.2.3 Enantioselectivity

The uncatalysed background reaction (10 mM PBS, pH 7.4, 37 °C) gave a diastereomeric *endo/exo* (**91**/**90**) mixture of *ortho* adducts in a ratio of 85:15 as well as the expected 50:50 ratio of each enantiomeric pair.[21]

The mAbs 13G5 and 4D5 catalysed the Diels–Alder reaction regio-, diastereo- and enantioselectively. They specifically catalysed the formation of either an enantiomer of the *ortho-exo* **90** or an enantiomer of the *ortho-endo* **91** adduct, respectively, with 95 ± 3% enantiomeric excess.

4.2.4 Affinity constants

As the inhibition constants (K_i) of the monoclonal antibody catalysts could not be obtained because of the hydrolysis of the ferrocenyl inhibitor, 1-[(acetyl)amino]-1'-[(dimethylamino)carbonyl]ferrocene **124**, under assay conditions, antibody-hapten affinity was independently measured by the quenching intrinsic antibody fluorescnece upon binding to a ligand. Ferrocene derivative **100** was selected as a simplified hapten mimic for both mAbs 4D5 and 13G5. Titration of **100** with mAbs 4D5 and 13G5 followed by Scatchard analysis (Scatchard, 1949) provided dissociation constants (K_d) of 209 and 48 μM, respectively. Both antibodies also displayed two binding sites.

4.2.5 Cross-reactivity and comparison of the antibodies elicited against the ferrocenyl and constrained bicyclo[2.2.2]octene haptens

The antibody catalysis of the Diels–Alder reaction has now been achieved with two structurally different sets of haptens, using either highly constrained bicyclo[2.2.2]octenes **89** and **92** (Gouverneur *et al*., 1993) or the conformationally flexible ferrocenes **98** and **99** as haptens (III). ELISA cross reactivity studies were

[21] Formation of the *meta* regioisomers was not detected in any of the mAb-catalysed or background reaction mixtures by either HPLC or ^{1}H NMR.

undertaken to probe the structural requirements of the combining sites of both sets of antibodies. The results are reported as the antibody dilution, in parentheses, which was required to give 50% maximum OD at 405 nm. A titer of 256+ was the maximum and hence sensitivity of the assay.

The *endo* catalyst 4D5 had a reasonably strong preference for the *endo* bicyclo[2.2.2]octene hapten **92** (128+) versus the *exo* hapten **89** (8 to 16). The *exo* catalyst 13G5 showed marginal affinity (8 to 16) for either the *exo* or *endo* haptens. In a second experiment, BSA–**98** and BSA–**99** were affixed to ELISA plates and allowed to react with anti-bicyclo[2.2.2]octene catalytic antibodies, 7D4 (*endo* specificity) and 22C8 (*exo* specificity). In this case both antibodies failed to bind the ferrocenyl haptens to any great extent.

These studies show that the anti-ferrocene antibody catalysts contain binding sites flexible enough to cross-react with the bicyclo[2.2.2]octene haptens, but not *vice versa*. This can be rationalised because of the disparity in the relative size of the two sets of haptens. The ferrocene haptens are much larger, having an inter-ring distance of approximately 3.3 Å, compared to the corresponding 1.55 Å C–C bond-lengths in the bicyclo[2.2.2]octenes (Gouverneur *et al*., 1993). Hence, it seems reasonable that the ferrocenyl haptens could not enter the binding pockets of the antibodies elicited against the smaller bicyclo[2.2.2]octene haptens. Conversely, the bicyclo[2.2.2]octene haptens could enter the larger binding pockets of the anti-ferrocenyl antibodies.

If the determined values of the Michaelis constant (K_m) are treated as representative of apparent dissociation constants for the antibody-substrate complex, then the bicyclo[2.2.2]octene haptens generated marginally tighter antibody-substrate binding complexes than were observed with the ferrocene haptens. The transition-state of this cycloaddition reaction has been shown to be asynchronous. The C–C bonds being formed have unequal lengths of either 2.05 Å and 2.35 Å for the *endo* transition-state or 2.03 and 2.38 Å for the *exo* transition-state (Gouverneur *et al*., 1993). For the bicyclo[2.2.2]octene haptens, the relevant C-C bond lengths are 0.50 to 0.85 Å shorter than the calculated transition-state. In the ferrocenyl haptens, the cyclopentadienyl ring separation is at least 0.90 Å greater than in the modelled transition-structure, strongly implicating a higher K_m for these antibodies.

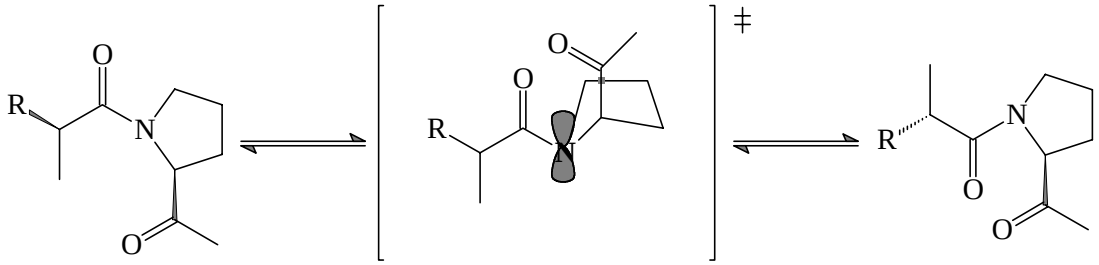
In the case of the *exo* catalyst 22C8, this increase in antibody substrate binding provides only a small increase in k_{cat} compared to that of the mAb 13G5. However, comparison of the two sets of *endo* catalysts, 7D4 and 4D5, shows that this increased antibody substrate interaction does not manifest any additional advantage in k_{cat}. The extra space imparted by the ferrocenyl haptens, while increasing the K_m, may also serve to increase turnover by allowing the product to diffuse away at a faster rate than it can from the more restricted binding site elicited by bicyclo[2.2.2]octene. Thus, it appears that the stereoelectronic features designed into the bicyclo[2.2.2]octene haptens **89** and **92** to model the transition-state do not provide any significant advantage over the orbital interactions modelled by the ferrocene haptens.

The immunisation with a single freely-rotating antigen was able to elicit antibodies with the Diels–Alderase activity. They were also capable of controlling the stereochemical outcome of the Diels-Alder reaction and providing an acceptable mimic of the stereoelectronic features important in the transition-state. Both concepts were realised during the study since Diels–Alderases capable of catalysing the formation of the *endo* or *exo* adducts were obtained from ferrocene haptens **98** and **99**.

This type of strategy is complementary to the conformationally-constrained hapten method and represents a new application of bio-organometallic chemistry to immunological recognition. Particularly noteworthy of this type of design is that (1) product inhibition does not occur (Hilvert *et al*., 1989, Suckling *et al*., 1992) and (2) enhancement of reaction rates, regio-, diastereo- and enantioselectivity are all comparable to those of antibodies elicited from more conformationally rigid haptens for this reaction.

4.3 The antibody-catalysed peptidyl-prolyl *cis-trans* isomerisation (IV)

As noted in chapter 1.4, the peptidyl-prolyl *cis-trans* isomerases comprise an abundant family of enzymes that efficiently catalyse the rotation about the P_1-proline amide bond (Scheme 35, Fischer, 1994). The cyclophilins and FK506 binding proteins (FKBPs) have been identified and characterised as two distinct classes implicated to play isomerase-dependent and -independent roles in protein folding and immunoregulation, respectively. Although the nature of catalysis remains to be completely elucidated, it appears that distortion is a major component in the mechanism of action (Harrison and Stein 1990b, 1992, Park *et al*., 1992, Stein, 1993).

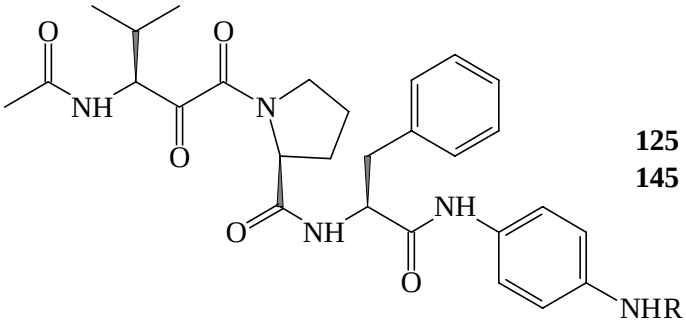


cis *trans*

Scheme 35.

4.3.1 Design of the hapten

Catalytic antibodies may be useful tools for the investigation of contributions to catalysis observed in enzymes. The design of appropriate hapten makes it possible to programme and study antibody active-sites that model a subset of features perhaps used by enzymes. The tripeptide dicarbonyl moiety containing hapten **125** was envisioned to generate antibody's binding sites that were hydrophobic and complementary to the α-keto amide functionality (Fig. 9). Since the hapten **125** places the linker distal to the α-keto amide moiety, presentation of the hapten **125** during the immune response should favour recognition of this region, including the hydrophobic valyl residue. An important factor might be — not the bulk dielectric constant of the binding site — but the interactions of specific amino acids and the side chains that provide a contact surface with the hapten and substrates (Mian *et al*., 1991).



125 R = $CO(CH_2)_3CO_2H$
145 R = CHO

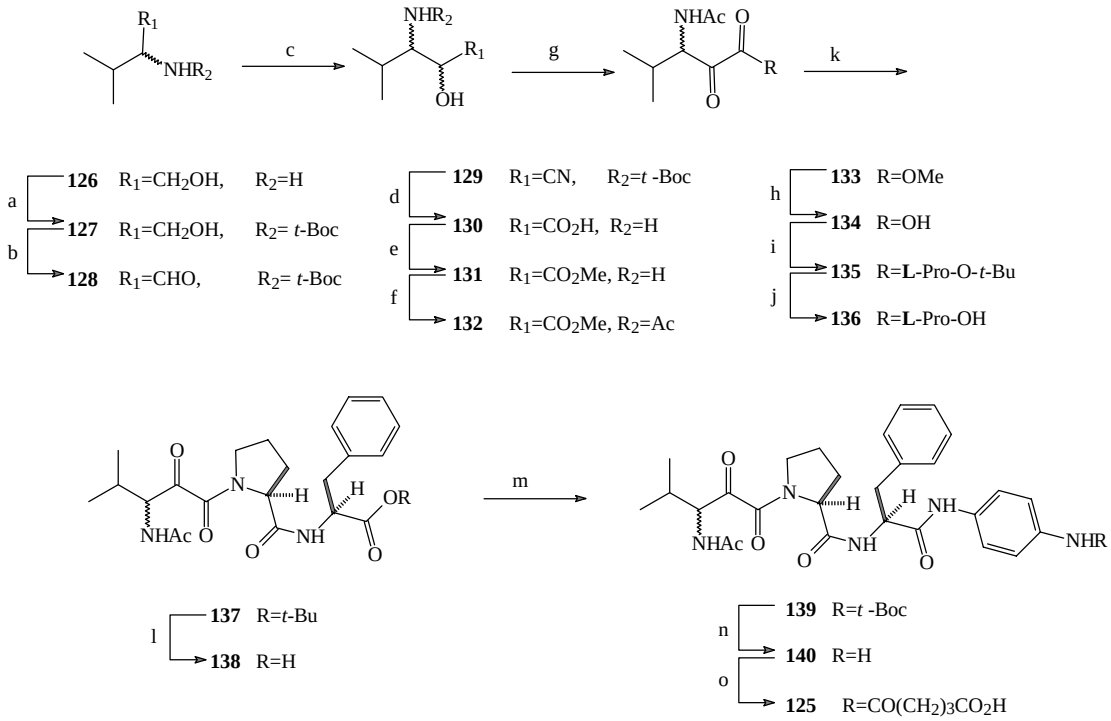
Figure 9. Structure of the hapten **125** for elicitation of the monoclonal antibodies with PPIase activity and structure of the corresponding α-keto amide inhibitor **145**.

The isomerisation of the proline amide bond is greatly facilitated in non-polar solvents (Eberhardt *et al*., 1992, Radzicka *et al*., 1992). Moreover, the α-keto amide moiety in FK506 **96**, rapamycin **97** and in less complex pyruvylamides is known to adopt an orthogonal conformation and possibly serve as a twisted amide mimic (Fischer *et al*., 1971, Albers *et al*., 1990, Liu *et al*., 1990, Rosen *et al*., 1990, Holt *et al*., 1993). Subsequently, it was hypothesised that desolvation and geometric effects might provide sufficient energetics to accelerate the rate of amide *cis-trans* isomerisation upon antibody binding of appropriate substrates (Scheme 35).

4.3.2 Synthesis of the hapten and substrates

The synthesis of tripeptidyl α-keto amide hapten **125** is presented in Scheme 36. Racemic 2-amino-3-methyl-1-butanol, (±)-valinol **126** was treated with di-*tert*-butyl dicarbonate to yield *N*-*t*-Boc protected valinol **127** (*t*-Boc_2O, $CHCl_3$, 4 °C, 2 h, quant.) which was subsequently oxidised using Swern conditions (oxalyl chloride, DMSO, TEA, CH_2Cl_2, −70 °C to rt, 1 h, 99%) to afford *N*-*t*-Boc valinal **128** in an excellent yield (Mancuso *et al*., 1978, Mancuso and Swern 1981). The aldehyde **128** was converted to the corresponding cyanohydrin ($NaHSO_3$, H_2O, NaCN, 0 °C, 2.5 h, quant.) **129** via a bisulfite addition compound (Corson *et al*., 1932). The cyanohydrin **129** was immediately hydrolysed to 3-amino-2-hydroxy-4-methylpropionic acid

hydrochloride **130** (35% HCl, 80 °C, 10 h, 92%). The Fischer esterification (MeOH, HCl (g), Δ, 1 h, 98%, **131**), *N*-acetylation (Ac_2O, $NaHCO_3$, MeCN, rt, 1.5 h, 88%, **132**), another Swern oxidation (oxalyl chloride, DMSO, TEA, CH_2Cl_2, –70 °C to rt, 1 h, 89%, **133**) and alkaline hydrolysis of the methyl ester **133** ($LiOH·H_2O$, $MeOH/H_2O$ 4:1, 0 °C, 10 min, 79%) gave 3-acetamido-4-methyl-2-oxopentanoic acid **134** in a good yield. The rest of the synthesis involves standard methodologies of peptide chemistry (**135** to **140**). BOPCl was used as a peptide coupling reagent, and all the *N*-*t*-Boc protective groups were cleaved using 4 M HCl in 1,4-dioxane when needed.



Scheme 36. (a) $(t\text{-BOC})_2O$, $CHCl_3$, 4 °C, 2 h, quant.; (b) $(ClCO)_2$, DMSO, TEA, CH_2Cl_2, –70 °C to rt, 1 h, 99%; (c) 1. $NaHSO_3$, H_2O, 0 °C, 30 min; 2. NaCN, H_2O, 0 °C, 2.5 h, quant.; (d) 37% HCl, 80 °C, 10 h, 92%; (e) MeOH, HCl (g), Δ, 1 h, 98%; (f) Ac_2O, $NaHCO_3$, MeCN, rt, 90 min, 88%; (g) $(ClCO)_2$, DMSO, TEA, CH_2Cl_2, –70 °C to rt, 1 h 89%; (h) $LiOH·H_2O$, $MeOH/H_2O$ (4:1), 0 °C, 10 min, 79%; (i) **L**-proline *tert*-butyl ester, BOPCl, TEA, CH_2Cl_2, 0 °C, 93%; (j) TFA, CH_2Cl_2, rt, 1 h, 91%; (k) **L**-phenylalanine *tert*-butyl ester, BOPCl, TEA, CH_2Cl_2, 0 °C, 2 h, 83%; (l) 1. 4 M HCl in 1,4-dioxane, rt, 3 h; 2. $NaHCO_3$, H_2O, rt, 96%; (m) mono-*t*-Boc protected 1,4-phenylenediamine, BOPCl, TEA, CH_2Cl_2, rt, 85%; (n) 4 M HCl in 1,4-dioxane, rt, 1 h, 99%; (o) glutaric anhydride, EDIA, CH_2Cl_2, rt, 68%.

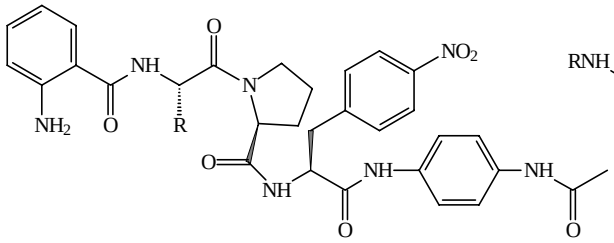
4.3.3 Kinetic characterisation of the antibody catalysts

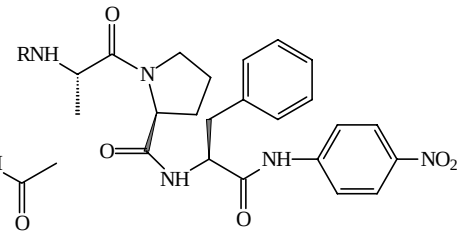
A panel of 28 monoclonal antibodies was raised against **125** coupled to the KLH carrier protein and screened preliminarily for catalytic activity employing both a spectrophotometric chymotrypsin-coupled and direct (uncoupled) fluorescence assay. The population of the *cis* conformers of the peptide substrates was increased by dissolving the substrates in 470 mM LiCl in 2,2,2-trifluoroethanol according to Rich and co-workers (Kofron *et al*., 1991, García-Echeverría *et al*., 1992).

Two mAbs, 1E3 and 35C8, were found to increase the rate of *cis* to *trans* isomerisation of the fluorophoric tripeptides **141** and **142** and the 4-nitroanilides **143** and **144**, respectively (Table 11). These antibodies did not exhibit cross-reactivity between the substrate sets. The *N*-formyl derivative of the hapten **145** was used in binding and inhibition studies. The dissociation constant K_d was found to be 3 μM and the K_i 10 μM, which were determined by the quenching of antibody fluorescence and the kinetic fluorescence assay, respectively. Taken together, the binding and competitive inhibition data suggest that catalysis occurred specifically at the antibody active site. Interestingly, product inhibition, identical to binding of the *trans* isomer, did not appear to be significant.

Using the fluorescence assay, the addition of 20 mol-% of peptide substrates equilibrated in buffer did not affect the rate. In addition, competition ELISA experiments using **142** and **144** versus the immobilised BSA conjugate of **125** showed that neither 35C8 nor 1E3 bound the *trans* isomer of these peptides (100 μM). In fact, only 8 of the 28 mAbs gave significant binding under these conditions where the *trans* isomer constituted 90% of the isomer population. Interestingly, the *cis* conformers bind preferentially in cyclophilin–substrate complexes (Kallen *et al*., 1991, Kallen and Walkinshaw, 1992, Ke *et al*., 1993).

Table 11. Kinetic constants for mAb 1E3 and 35C8 substrates.





141 R = CH_3
142 R = $(CH_3)_2CH$

143 R = Suc
144 R = Ac

Compound	k_{uncat} [s^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_m [M^{-1} s^{-1}]	k_{cat}/k_{uncat}
141	4.1×10^{-3}	0.11	1.1×10^{3}	27
142	3.0×10^{-3}	4.0×10^{-2}	4.0×10^{2}	13
143	4.5×10^{-3}	6.8×10^{-2}	6.8×10^{2}	15
144	4.9×10^{-3}	1.7×10^{-2}	1.7×10^{2}	3.4

4.3.4 Mechanistic considerations of the antibody-catalysed peptidyl-prolyl *cis-trans* isomerisation reaction

The data suggest a distortion-based mechanism wherein removal of the tripeptide from an aqueous environment into the non-polar, hydrophobic cavity of the antibody and subsequent stabilisation of a twisted intermediate promote prolyl-amide rotation. The reaction was not found to be sensitive to pH variation in the physiological range, and no D_2O solvent isotope effect was observed. This is similar to the enzymatic behaviour.

In general, antibody's binding sites are hydrophobic in nature, and the association of antibody and hapten-like molecules is facilitated by classical hydrophobic effects (Ben-Naim, 1983, Smithrud and Diederich, 1990). The rate enhancements produced here can be compared to the 46-fold acceleration for proline

model compounds in going from water to toluene (Radzicka *et al.*, 1992). Additionally, given that only two of 28 mAbs showed activity with unique substrate fidelities, it is likely that geometric influences induced by the twisted α-keto amide moiety are also necessary for antibody catalysis. This is further reflected in both the apparent preferential binding of the *cis* isomer and the less polar P_1-alanyl peptide **141**, which is better substrate than **142**. This indicates that elements other than simple hydrophobic interactions are involved. The function of the α-keto amide may be coupled, in a subtle way, to dielectric effects and other catalytic phenomena such as transition-state stabilisation and ground-state destabilisation. Indeed, the calculations implicate that these principles along with conformational constraints (autocatalysis) play a major role in FKBP catalysis (Fischer *et al.*, 1993, 1994).

Although k_{cat}/K_m and k_{cat} for the mAbs 1E3 and 35C8 are far less in relation to those of cyclophilin (10,000-fold), they are only a thousand times slower than those for FKBP using optimum tetrapeptides and within an order of magnitude of the enzyme's rates with its poorer substrates. The FKBP activity is much more influenced by hydrophobic factors than is that of cyclophilin which may invoke minor alterations in its mode of catalysis (Harrison and Stein, 1990b, Fischer *et al.*, 1993, Ke *et al.*, 1993). That the antibody models can begin to approach FKBP catalysis implicates perhaps a more primitive isomerase activity for FKBP compared to that for cyclophilin.

5 Conclusions and future perspectives

In the present investigation, five structurally new antigens were prepared to elicit monoclonal antibodies for the subsequent studies of their catalytic properties in (1) acyl-transfer reactions, (2) the Diels–Alder reaction, and (3) the *cis-trans* isomerisation of the prolyl amide bond. The new antigens were twisted α-keto amides for acyl-transfer and peptidyl-prolyl *cis-trans* isomerisation reactions and conformationally flexible ferrocenyl haptens for the Diels–Alder reaction.

The proposed concept of using twisted α-keto amides as stable ground-state analogues of the transition-state of the acyl-transfer reactions did not seem to produce catalytic antibodies for the hydrolysis of ester and amide substrates (I). However, recent studies of structurally similar twisted, non-hydrated α-keto piperazine amides as transition-state analogues have shown them to be viable haptens being capable of inducing antibodies that hydrolyse alkyl esters (M. Taylor, J. Yli-Kauhaluoma and K. D. Janda, unpublished results).

During the investigation of acyl-transfer hydrolyses, a new, synthetically useful alkylative decarbonylation and decarboxylation reaction of 3-phenyl-2-oxopropanoic acids was serendipitously discovered (II). It turned out to be a practical method for the preparation of isopropyl anisoles and veratroles, producing them in high yields. Moreover, the method developed was found to be devoid of unwanted regioisomers.

The use of conformationally unrestricted ferrocene haptens as a loose transition-state mimic is also a new concept developed during this study (III). The ferrocene antigens successfully elicitated catalytic antibodies with Diels–Alderase activities. Previously, it has been thought that hapten design, for reactions proceeding through a highly-ordered transition-state, must implicitly reflect this highly constrained structure. In this study, it was found that provided the hapten is able to achieve a conformer which mimics this highly-ordered transition-state, the immune system is able to elicit anti-bodies to sequester this structure and are liable to generate catalytic antibodies. The found and characterised Diels–Alderases displayed comparable accelerations of reaction rates, regio-, diastereo- and enantioselectivity with those antibodies raised against the more “classically-designed” haptens for this reaction.

Unique to the found antibodies with Diels–Alderase activities was that they were devoid of the product inhibiton which has been regarded as a potential problem for antibody-catalysed Diels–Alder reactions (III). Perhaps the most exciting feature of this new bio-organometallic method of using freely rotating η^5-cyclopentadienyl iron complex as haptenic group might be its potential applicability not only to the Diels–Alder cycloadditions but also to other reactions such as Claisen rearrangement, proceeding through highly-ordered, pericyclic transition-states. The only limitation being that the reactions should not face an energy barrier in excess of 20 kcal mol^{-1} that is widely accepted as being the maximum binding energy an antibody can deliver.

Despite the difficulties with the antibody generation for the acyl-transfer reactions, the antibodies raised against the α-keto amide tripeptide hapten were found to catalyse the *cis-trans* isomerisation of the prolyl peptide substrates (IV). During this study, the first monoclonal antibody catalysts with peptidyl-prolyl *cis-trans* isomerase activity were found and kinetically characterised. The investigation herein supports the hypothesis of other workers that the α-keto amide moiety in PPIase ligands, such as in macrolides FK506 and rapamycin, evolved to provide a contributing element in peptidyl-prolyl *cis-trans* isomerase activity (IV).

Although the present study showed that monoclonal antibodies elicited against α-keto amides or stable organometallic haptens were able to accelerate the rates of the peptidyl-prolyl *cis-trans* isomerisation and the Diels–Alder reactions, other questions remain to be answered. The catalytic mechanisms of antibodies with PPIase and Diels–Alderase activities are not yet known. Future studies with additional hapten modifications should enable the other features of catalysis to be examined. The antibodies derived from these new designs will then give more insight also into the unique mechanisms of both the PPIases and antibodies with PPIase activity. In the case of the Diels–Alder reaction, new haptens that could incorporate a Lewis acid mimic and/or co-factor in the ferrocene (or the respective osmocene or ruthenocene) antigens studied here are in preparation. It is hoped that the new antibodies elicited against them will be more effective catalysts which also retain the exquisite regio- and stereoselectivities observed in this study.

The field of catalytic antibodies is now eleven years old. Until today, approximately eighty different chemical reactions have been catalysed by antibodies.

The antibody catalysts studied have generally shown characteristics very similar to the enzymes: substrate specificity, stereospecificity, Michaelian saturation kinetics, competitive inhibition by the transition-state analogue and reversibility. Very interestingly, catalytic antibodies have also been used in guiding the course of the chemical reactions under kinetic control to thermodynamically disfavoured reaction pathways: The generally *endo*-selective Diels–Alder reaction produced *exo* diastereomers (Gouverneur *et al.*, 1993, III), the *syn*-elimination to *cis* olefin has previously not been regarded as a possible organic chemical transformation (Cravatt *et al.*, 1994), not to mention the highly disfavoured 6-*endo*-*tet* anti-Baldwin type ring closure of an epoxy alcohol to a tetrahydropyran (Janda *et al*., 1993).

Monoclonal antibody catalysts could also be useful in practical small-scale organic synthesis because they can be designed and produced almost to any chemical reaction whose transition-state is known or can be approximated by the methods of theoretical chemistry. There are already examples of multigramme-scale syntheses with catalytic antibodies (Shevlin *et al*., 1994, Reymond *et al*., 1994), and in one case catalytic antibody has already been used in the asymmetric synthesis of (–)-α-multistriatin (Sinha and Keinan, 1995). However, the turnovers (k_{cat}) of antibody mediated reactions are still very modest relative to the enzymes, the rate enhancements (k_{cat}/k_{uncat}) are typically lower than those of the enzymes, and problems with product inhibition do occur in some instances. These are problems which could be addressed, for example, by the rational hapten design and by exploiting the antibody gene libraries. Antibody production and accessibility should also be facilitated when the new bacterial or yeast expression systems or the production of antibodies in green plants are developed further (Schultz and Lerner, 1995).

Very interesting is the use of catalytic antibodies in medicinal and pharmacological applications, such as prodrug activation (Miyashita *et al.*, 1993, Campbell *et al*., 1994, Smiley and Benkovic, 1995), degradation of cocaine (Landry *et al.*, 1993) and antibody-directed prodrug therapy (Wentworth *et al*., 1996).

Ultimately, catalytic antibodies with improved turnovers, rate enhancements, chemical stabilities and antibodies that are devoid of product inhibition can be produced economically in large amounts to be used as highly efficient, selective and specific biocatalysts complementary to enzymes, maybe bringing hope to the future.

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