Catalytic Antibodies for
Amide Cleavage and Prodrug Activation

by

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ABSTRACT

The first part of the thesis describes a novel approach to generate amide cleaving antibodies. Two haptons, O1 and O2, were designed and synthesized to create a hydrophobic pocket for an auxiliary nucleophile as well as elicit complementary basic/acidic side chain residues in the antibody combining binding site. The recently established heterologous immunization protocol was utilized to induce multiple catalytic side chain residues in antibodies. Three antibodies were isolated capable of catalyzing the hydrolysis of propionyl-p-nitroanilide in the presence of phenol, as the nucleophilic cofactor. Further studies with substrate and nucleophile analogs clearly showed that the nucleophilic phenolic hydroxyl group is essential for catalysis, much like amide hydrolysis catalyzed by serine proteases.

The second part of the thesis describes the generation of catalytic antibodies capable of removing a protection group for the simultaneous activation of multiple prodrugs. To this end, a protective group was designed which could be attached to any kind of cancer drug, in order to convert a cancer drug into a prodrug. The protective group relies on a β-sulfone elimination process to release the active drug. The corresponding hapten design includes a non-specific element to allow broad substrate tolerance with regard to the drug portion. Two haptons, R1 and R2, both structurally similar, but with different functionalities were designed and synthesized. Both haptons include a perfectly placed ammonium residue for α-proton abstraction. The second hapten design, R1, further sought to induce an acidic residue for carbamate leaving group stabilization. A comparison of both haptons showed that leaving group stabilization did not improve antibody performance.

Another subject crucial to the prodrug activation project concerns the detection of catalytic activity in hybridoma supernatants. A new method called "Capture"-CatELISA was developed, which allowed the efficient screening for catalysis of hundreds of hybridoma supernatants in a very short period of time. To this end, a substrate C1 was developed, which upon activation covalently traps catalytic antibodies, and therefore immobilizes them on a solid support. A conventional ELISA-assay was then applied to identify the catalytic clones.

Thesis Supervisor: Professor Satoru Masamune

Title: Arthur C. Cope Professor of Chemistry
This thesis is dedicated to my parents, who always supported my extravagant ideas, and unusual life path with their prayers and spiritual guidance.

This thesis is also dedicated to my girlfriend Elena, who patiently carried me through the hardest times, and shared with me the saddest and happiest moments.
"The great tragedy of science -
the slaying of a beautiful hypothesis by an ugly fact."

_Aldous Huxley_

"Science can purify religion from error and superstition.
Religion can purify science from idolatry and false absolutes."

_Pope John Paul II_
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# Table of Contents

Chapter 1: Introduction .................................................................................................................. 10
   1.1. A semi-philosophical review of antibody catalysis ......................................................... 10
   1.2. About this thesis .............................................................................................................. 14

Chapter 2: Acyl-Transfer Processes Catalyzed by Antibodies: A Review .............................. 15
   2.1. Introduction .................................................................................................................... 15
   2.2. Phosphonate based haptens .......................................................................................... 15
      2.2.1. Regio- and enantioselective ester hydrolysis ............................................................ 16
      2.2.2. The first example of amide hydrolysis .................................................................... 17
      2.2.3. The first antibody catalyzed multistep reaction catalyzes the rearrangement of a peptidic bond ...................................................................................... 18
   2.3. The "bait and switch" strategy ......................................................................................... 18
      2.3.1. Pyridinium hapten induces esterolytic antibodies .................................................... 19
      2.3.2. Esterolytic antibodies induced to haptens with a 1,2-amino alcohol ......................... 19
   2.4. Heterologous immunization ............................................................................................ 20
   2.5. Reactive immunization .................................................................................................. 21
   2.6. Joint hybridoma and combinatorial antibody library approach .................................... 22

Chapter 3: Antibody Catalyzed Cleavage of an Amide Bond Using an External Nucleophile ............................................................ 25
   3.1. Introduction .................................................................................................................... 25
   3.2. Serine Proteases ........................................................................................................... 25
   3.3. Introduction of an auxiliary nucleophile ........................................................................ 26
   3.4. Hapten design .............................................................................................................. 27
   3.5. Results .......................................................................................................................... 28
      3.5.1. Hapten synthesis .................................................................................................... 28
      3.5.2. Kinetic results and discussion ................................................................................. 30

Chapter 4: Antibody Catalyzed Prodrug Activation ................................................................. 35
   4.1. Introduction .................................................................................................................... 35
   4.2. Antibody Directed Enzyme Prodrug Therapy (ADEPT) and Antibody Directed Antibody Prodrug Therapy (ADAPT) .............................................................. 36
      4.2.1. ADEPT .................................................................................................................. 36
      4.2.2. ADAPT ................................................................................................................. 37
   4.3. A brief synopsis of prodrug activating antibodies ......................................................... 38
4.3.1. Ester protected prodrugs .........................................................38
4.3.2. Intracellular prodrug activation .................................................40
4.3.3. Carbamate prodrug activation .....................................................41

Chapter 5: Catalytic Antibodies for Prodrug Activation: Initial Studies .................................................42
  5.1. Prodrug Design ........................................................................42
  5.2. Hapten design ........................................................................42
  5.3. Results ....................................................................................43
    5.3.1. Substrate synthesis ..............................................................43
    5.3.2. Growth inhibition studies ....................................................44
    5.3.3. Kinetic stability of prodrug ....................................................45

Chapter 6: Simultaneous Activation of Multiple Prodrugs by Catalytic Antibodies .................................................47
  6.1. Introduction ............................................................................47
  6.2. Protection Group Design ..........................................................47
  6.3. Hapten design ........................................................................49
  6.4. Results ....................................................................................50
    6.4.1. Hapten and substrate syntheses ..........................................50
    6.4.2. Antibody generation and purification ....................................54
      6.4.2.1. Antibody generation and selection ..................................54
      6.4.2.2. Antibody production and purification ...............................55
  6.4.3. Kinetic results and discussion ...............................................55
  6.5. Outlook ..................................................................................58
    6.5.1. Improvement of antibody performance ..................................58
    6.5.2. Generation of bispecific antibodies ......................................59
    6.5.3. In vitro experiments ............................................................60

Chapter 7: "Capture" CatELISA .......................................................................61
  7.1. Introduction ............................................................................61
  7.2. A brief review of CatELISA ......................................................62
  7.3. The new screening method .......................................................64
  7.4. Results ....................................................................................66
    7.4.1. Synthesis of "Capture" CatELISA substrate C1 ...................66
    7.4.2. Assay results ....................................................................68

Chapter 8: Experimental ..........................................................70
  8.1. Syntheses of haptens and substrates .........................................70
8.1.1. General methods ................................................................. 70
8.1.2. Synthesis of hapten O2 ......................................................... 70
8.1.3. Synthesis of hapten R1 ....................................................... 76
8.1.4. Synthesis of "Capture CatELISA" substrate C1 ...................... 82
8.1.5. Synthesis of substrate S1 ..................................................... 88
8.1.6. Synthesis of prodrug analog 25 .......................................... 89

8.2. Biological methods ............................................................... 91
  8.2.1. Preparation of carrier protein-hapten conjugates .................... 91
  8.2.2. Immunizations .................................................................. 91
  8.2.3. Hybridoma generation ....................................................... 92
    8.2.3.1. Fusion ......................................................................... 92
    8.2.3.2. Hybridoma selection ................................................... 95
  8.2.4. Ascites production from monoclonal hybridomas .................. 97
  8.2.5. Purification of monoclonal antibodies ................................... 97
  8.2.6. Cytotoxicity assays ........................................................... 98
  8.2.7. Assays for catalysis .......................................................... 99

References .................................................................................. 101

Appendices ................................................................................ 108

Biographical Note ....................................................................... 138
Chapter 1
Introduction

1.1. A semi-philosophical review of antibody catalysis

The science of antibody catalysis takes as its goal the production of enzymes in real time. One may ask why one would wish to build new enzymes since some 4000 or so enzymes already exist in nature. A simple answer might be that even this large number of enzymes cannot encompass all the chemical transformations that one wishes to catalyze. Thus, one can conceive of many important reactions for which there are no enzymes, and, even when enzymes exist, their substrate restrictions in some cases, or promiscuity in other cases, may preclude their use. But there is a more compelling reason that cannot be put in better words than those of the late Richard Feynman, who wrote, "What I cannot create I do not understand". This is very important in the study of the evolution of enzymes, where contributors to rate acceleration act in concert so that each is perturbed by the other, making it very difficult to discern individual components of catalysis. However, as one makes an enzyme in real time, it becomes possible to learn much about the physical organic chemistry of catalysis, and ultimately about the ability of protein binding pockets to do chemistry. Implied in these ideas are issues of how proteins impart strain, overcome entropic barriers, deal with highly reactive intermediates, control stereoelectronic parameters, and use their hydrophobic interiors to accomplish chemistry that would be impossible in solution.

So how do we make an antibody catalyst? Much of what is important was explained by Linus Pauling in 1948 at the end of his Silliman lecture. When Pauling turned to antibodies and enzymes, he said that they differ in that antibodies bind to ground states whereas enzymes bind to transition states, thereby lowering the energy barrier and increasing the rate of the reaction. This essentially summarizes all the necessary elements for antibody catalysis. First, he presented a fundamental theory of catalysis that suggests what kind of compounds should induce antibodies capable of catalyzing a reaction. Second, he pointed out that a protein diversity system exists that efficiently binds anything that can be synthesized.

So what kind of chemical machine is the antibody molecule? It is in every sense of the word the product of combinatorial chemistry, starting with its synthesis which will not be further elaborated here. It is naive, which is a feature often overlooked but extremely important to systems that must evolve binding in real time. This system achieves the all-important linkage of recognition and replication that is, of course, the main feature of many biological systems including sperm and egg interaction, development and differentiation, and antibody induction. The linkage of recognition and replication in immune cells allows for the special process of somatic mutation to yield antibodies with ever higher affinity for the inducing antigen. Thus, the immune system is a biological system that depends on the presence of the inducer for replication while at the same time
producing a product (the antibody molecule) that removes the inducer. If this feature is combined with somatic mutation and the linkage of recognition and replication, then one has a system that, by its nature, drives toward the highest binding energy.

However, there is a fundamental problem in harnessing this system for catalysis in that the system selects for tight binding, whereas in catalysis, the exact way in which binding occurs is the key. The immune system recruits a wonderful array of functionalities around the antigen which are even more powerful since they are in a hydrophobic pocket. However, to achieve catalysis the experimenter must cause these functionalities to be arranged in highly specific ways around the transition state of a reaction. This is done by giving a chemical education to the protein via the nature of the inducer (or hapten) that the chemist makes. Often, the hapten is a stable analog of the transition state of the reaction. Antibody catalysis is therefore a chemical science where the art is to give detailed chemical instructions to the induction system to achieve catalytic perfection. Thus, the science is largely instructional and the better the instructions, the better the catalyst. On the other hand, an approach called combinatorial antibody libraries which is mainly done in bacterial phages rather than in vivo, takes the absolute opposite approach. It uses no (or very little) instructions from the experimenter and the system runs to perfection by random mutation, selection, and amplification. These combined approaches are currently emerging as very powerful tools in the quest for ever more efficient catalysts.

The ability of the antibody molecule to take instructions pertinent to catalysis can be seen in the first X-ray structure of a catalytic antibody for the Claissen reaction of compound 1 to form compound 2, solved by Hilvert et al. (Figure 1). The Claissen rearrangement is formally a 3,3 sigmatropic rearrangement in which the enol pyruvate must move from its extended conformation (4) to lie over the cyclohexadienal system in the transition state (5). It was already known that the oxabicyclic system 3, designed to mimic the transition state, was an inhibitor of the natural enzyme. When Schultz and Hilvert independently made antibodies to this transition state analog 3, they were able to obtain highly efficient antibodies. The crystal structure of one of the antibodies complexed to the hapten illustrates how well the antibody binding pocket adapted to the instructions.

The antibody binding pocket deeply buries the oxabicyclic hapten 3 and presumably also buries the substrate, shielding the reaction from water. This may not be so important for this concerted transformation, but clearly helps the entropic contribution to the binding energy. The shape of the pocket will not allow binding of the substrate in its extended conformation 4. Thus, it should force the enol pyruvate side chain over the cyclohexadienal system which entropically favors the transformation. Finally, the asymmetry of the binding pocket explains the stereochemical outcome of the reaction in that only one antipode of the substrate is accepted by the antibody.
Thus, the antibody faithfully accepts the instructions of the chemist and in doing so becomes a catalyst. This does not necessarily mean that the instructions were perfect, since there may be considerable enolate character to the transition state of this reaction, and thus introduction of some charge into the hapten might be desirable. The general ideas discussed above have led to the induction of many catalysts which are discussed in some 650 publications.

Figure 1

Since its inception, the process of immunization has dealt with inert antigens. The process of reactive immunization is just the opposite of this. It attempts to immunize with a very reactive compound so as to push forward a chemical reaction in the antibody binding pocket during the process of induction. Then, this same chemical reaction is used later as part of the catalytic mechanism. In a sense one immunizes with a chemical reaction rather than with a chemical. In the ordinary process of immunization, the process of somatic mutaion leads to a series of improved interactions with the antigen that, as with enzymes, leads to tighter binding. However, in reactive immunization, the antigen makes a covalent bond with the antibody binding site, giving this particular clone a competitive advantage over all others. Thus, the antibody that makes a covalent bond obviously has a better binding energy than any antibody using non covalent interactions. When the functionality that leads to the covalent bond appears early in the process of antibody evolution, further refinement is aborted, and that clone becomes dominant in the immune process.

Lerner et al. applied this concept to the construction of antibody aldolases that are both very efficient and broad in scope. The aldol addition is one of the most important carbon-carbon bond forming processes in chemistry and biology. In nature, this reaction is accomplished by enzymes
which are highly efficient but narrow in scope. In chemistry, most catalysis used are stochiometric and require protecting groups, and preformed enolates. One of the two classes of natural enzymes, the class II aldolases, uses a highly reactive lysine residue to form a carbon nucleophile donor that adds to a second carbonyl carbon via an enamine mechanism (Figure 2).

![Figure 2](image)

The centerpiece of this chemical mechanism is the formation of the enamine. Lerner tried to mimic the formation of the enamine by immunizing with the reactive immunogen 6 (Figure 3). The hapten 6 features a 1,3 dicarbonyl functionality that was chosen to select for a reactive lysine residue during the immunization process. When this lysine appears during the process of somatic refinement, it can attack one of the carbonyl carbons to form a Schiff base (7). The Schiff base can further tautomerize to an enamine which, because of the second carbonyl, forms a stable vinylogous amide (8). The vinylogous amide exhibits an absorption maximum at 316 nm and therefore, antibodies that make this interaction can be detected.

![Figure 3](image)

The crystal structure of one of two catalytic aldolase-like antibodies was solved and it showed, as expected, a lysine residue deeply buried within the antibody binding pocket. Like in most enzymes, this lysine residue has highly perturbed pK\(_a\) of 6.5. The antibody is only a hundred-fold less efficient than the natural aldolase, which is the centerpiece of sugar metabolism. But most remarkably, the antibody accepts a wide range of substrates in its binding pocket and accomplishes over 100 different aldol additions and condensations with almost equal efficiency.

In the generation of enzymes which are the result of the process of natural selection, the driving force is improved chemical function. Thus, they are selected for their ability to accomplish chemistry. The immune system also works on evolutionary principles albeit, in real time, and it
selects for simple binding. The reactive immunization concept therefore combines the two features of selection for catalysis and evolution in real time to yield a very efficient catalyst.

Biological systems and chemical systems each have unique powers. Biology has evolution, diversity, and replication. Chemistry has the power to understand reaction mechanisms, synthesize molecules of any kind and know their structure. As shown above, the exploration of a combination of the unique powers of each system can generate new functions such as enzymes catalyzing new reactions or accepting different substrates. The research at the interface of biology and chemistry is as yet at its infancy and will lead to more powerful systems with hitherto unimagined properties.

1.2. About this thesis

The field of antibody catalysis has come a long way since its inception in 1986. Antibodies capable of catalyzing more than 70 reactions not found in nature have been isolated thus far, and the number is growing almost every week. We learn to understand how the antibody recruits amino acid side chains for catalysis and how the immune system replicates the evolution process of enzymes in real time. However, despite the huge efforts spent on antibody catalysis there are still some major areas left to explore. The present thesis deals with two of the most important goals of antibody catalysis, namely the sequence specific hydrolysis of peptides and the activation of prodrugs.

In chapters two and three, a new approach for the cleavage of amide bonds is presented. While we still have a long way to go before we can isolate tailor made and specific proteases, an encouraging new approach is presented where an external nucleophile aids the cleavage of an activated amide bond.

In chapters four, five, and six, instead of trying another concept of hapten design for the generation of more efficient catalytic antibodies, a possible use of catalytic antibodies for medical purposes is explored, namely the antibody mediated activation of prodrugs. To date, there is no successful drug delivery system which could activate prodrugs in the vicinity of tumors, or other lesions without affecting healthy cells. The ability to create a unique catalyst for which there is no natural enzyme counterpart would open a new field for the treatment of hitherto untreatable diseases.

Chapter seven deals with the development of a new methodology, called "Capture CatELISA", for the rapid screening of hybridoma supernatants for catalysis. This improved methodology, based on the established CatELISA methodology, allowed us to tremendously shorten the time and effort spent on identifying and isolating catalytic antibodies for the prodrug activation project.
Chapter 2

Acyl-Transfer Processes Catalyzed by Antibodies: A Review

2.1. Introduction

Acyl-transfer processes are ubiquitous in both biochemical metabolism and organic chemistry and, as such, have remained a major focus of antibody catalysis since the first reports of these programmable proteins appeared in the mid-1980's. In the initial demonstrations of antibody catalysis, simple reactions such as ester and amide hydrolysis were chosen because the reaction mechanisms were well understood. Also, these reactions exhibited a large difference in shape and charge between the transition state and the substrates. However, while catalysis of ester hydrolysis seemed easy to accomplish, the more challenging amide hydrolysis met only limited success thus far.

Amide hydrolysis is of unique importance in enzymology. Therefore, it has been a prime target of antibody technology to generate catalytic antibodies which are capable of hydrolyzing peptides in a sequence specific manner. However, the hydrolysis of an amide bond is much more challenging than the hydrolysis of an ester bond since the latter reaction is kinetically disfavored. In order to hydrolyze an amide bond, it is not only necessary to stabilize the tetrahedral intermediate, but also to stabilize (and protonate) the amine leaving group. It is seemingly very difficult to program all these features into one hapten design.

The numerous antigens used for the elicitation of catalytic acyl-transfer antibodies can be broadly categorized in five different groups:

1. Phosphonate based transition state analog (TSA) haptens
2. Bait and Switch haptens
3. Heterologous Immunization
4. Reactive Immunization
5. Joint Hybridoma and Combinatorial Antibody Library Approach

Another approach which was developed in the Masamune Group uses programmed nucleophilic catalysis to accomplish amide cleavage. The author was part of these efforts and the successful outcome of this approach will be discussed in chapter 3.

2.2. Phosphonate based haptens

During hydrolysis, a planar and uncharged ester or amide proceeds along the reaction coordinate toward the charged tetrahedral intermediate, which collapses to the acid and alcohol/amine products. The phosphonate molecule is an analog of this high-energy transition state
because it is tetrahedral and negatively charged, and the phosphonate-oxygen bond is approximately 20% longer than the carbon-oxygen bond. The phosphonate thereby mimics the stretching of the latter bond in the transition state of the reaction after attack of water or hydroxide ion at the electrophilic carbonyl carbon. Such phosphonates were, and continue to be, very successful in inducing esterolytic (and to some extend amidolytic) antibodies. As the crystal structures of esterolytic catalytic antibodies continue to emerge, we are learning how faithfully the antibody molecule accepts the instructions of the chemist to build a pocket that accepts the tetrahedral shape and neutralizes the developing negative charge. A brief summary of the milestones in the development of phosphonate based haptens is discussed below.

2.2.1. Regio- and enantioselective ester hydrolysis

Antibodies have unique three dimensional binding sites that can be programmed by the appropriate hapten to achieve regio-and enantioselective catalysis of a substrate. This obviates the need for the protection or chemical differentiation of similarly reactive sites in the molecule.

As a first example of enantioselective catalysis, Napper and coworker\(^9\) raised antibodies against racemic hapten 9 (Figure 4). The assumption was that only one of two enantiomeric haptens will elicit a particular monoclonal antibody. Therefore, it should be possible to induce antibodies with both stereospecificities by immunizing with a racemic mixture.

![Figure 4](image)

One antibody, 24B11, did indeed catalyze the cyclization of substrate 10 to lactone 11 with an enantiomeric excess (ee) of 66%. The low ee is not due to poor selectivity, but rather to the small ratio of \(k_{\text{cat}}/k_{\text{uncat}} = 167\) (\(k_{\text{cat}} = 0.5\ \text{min}^{-1}\)). This translates into an ee of 94% for the antibody-catalyzed reaction. Later on, this concept was applied to generate antibodies useful for natural product synthesis and kinetic resolution of esters.\(^10\)

Fujii and coworkers extended this concept and isolated esterolytic antibodies which were capable of deprotecting an acylated carbohydrate in a stereo- and regioselective manner (Figure 5).\(^11\) Antibody 17E11 raised against hapten 12 specifically hydrolyzed the 4'-acetyl group of substrate 13a with a \(k_{\text{cat}} = 0.182\ \text{min}^{-1}\), especially when high concentrations (30 μM) of catalyst were used. Very little hydrolysis of the 3'-acetyl group was observed under these conditions.
Also, enantioselectivity was established since the galactopyranoside 13b was not subject to catalysis. Antibody 17E11 did not discriminate between the α and the β configuration of the anomic center and accepted both enantiomers as substrates. Therefore, this antibody may be suitable for the regioselective hydrolysis of nonreducing termini in a variety of complex oligosaccharides.

2.2.2. The first example of amide hydrolysis

As a first step towards the goal of amide hydrolysis, Lerner, et al. reported the generation of an antibody (43C9), raised to phosphonamidate hapten 14, capable of hydrolyzing an activated nitroanilide 15 (Figure 6).\(^{8a}\)

The 250,000-fold rate acceleration (at pH 9) accomplished by this antibody remains the highest ever accomplished for an amide substrate. Further mechanistic investigations revealed that the catalytic mechanism involves the fortuitous formation of a covalent acyl-antibody intermediate as a consequence of complementary side chain residues at the antibody site.\(^{12}\) The catalytic mechanism involves two key residues: His-L91, which acts as a nucleophile to form the acyl-antibody intermediate, and Arg-L96, which stabilizes the anionic tetrahedral moieties. It was suggested that the introduction of general acid-base catalysis, by either improved hapten design or site directed mutagenesis, may improve the catalytic performance of antibody 43C9. However, none of these approaches have shown success thus far.
In 1994, Martin et al. isolated an antibody that was capable of hydrolyzing an unactivated primary amide substrate using a phosphonate based hapten, although the accomplished rate acceleration was far more modest (132 fold). 

2.2.3. The first antibody catalyzed multistep reaction catalyzes the rearrangement of a peptidic bond

An alternative approach to the direct hydrolysis of the peptidic bond is the catalysis of the well-documented β-aspartyl shift, the natural mechanism of protein aging. This may eventually provide a powerful method for the deactivation specific proteins. Benkovic et al. reported the generation of antibodies raised against the bi-functional hapten 16, which not only catalyzed the conversion of asparginyl-glycyl(N-phenethyl)amide 17 to succinimide 16, but also the subsequent hydrolysis to the products 19 and 20 (Figure 7).

Figure 7

![Chemical Structures]

Investigations of the mechanistic characteristics of two such antibodies, 23C7 and 2E4, revealed two tetrahedral binding sites of unequal effectiveness, which were induced within the antibody combining site in response to the tetrahedral mimics (phosphinate and secondary hydroxyl). Antibody 2E4 was characterized kinetically and showed a of $k_{cat} = 0.0072 \text{ min}^{-1}$ (at pH 9.0) which corresponds to a rate acceleration of about 70 when compared to the water catalyzed reaction.
2.3. The "bait and switch" strategy

Tetrahedral transition state analogs (TSA), like phosphonates, presumably work by transition state stabilization, and by stabilization of the developing oxyanion. However, most proteolytic enzymes (such as aspartate and serine proteases) also feature functional groups in their active sites, which act as a general acid-base pair. These groups are usually charged at physiological pH. It was recognized early on that such functional groups may be inducible in the antibody combining site by simple charge complementarity within the hapten. The charged hapten serves as the bait for attracting catalytic functions within the antibody; it is then switched for the substrate.

2.3.1. Pyridinium hapten induces esterolytic antibodies

Janda et al. were the first researchers to apply the hypothesis of charge complementarity to acyl transfer reactions after a seminal report showed that this concept worked well for elimination reactions (see chapter 6).\textsuperscript{14} He synthesized pyridinium hapten 21 in the hope of inducing a negatively charged residue within the antibody, which is supposed to aid the deprotonation of an incoming water molecule (Figure 8). In addition, the secondary alcohol was supposed to mimic the tetrahedral geometry of the transition state. Hapten 22 is nearly identical to 21, but lacks the positive charge at physiological pH and served as a control.

![Figure 8](image)

Antibody 30C6 (raised against hapten 21) was screened against benzoate ester 23 and displayed Michaelis-Menten kinetics with a $k_{\text{cat}}$ of $5 \times 10^{-3}$ min$^{-1}$ and $K_m = 1.12$ mM. The pH-rate profile implicated the involvement of a basic catalytic residue in the antibody binding site with an apparent $pK_a$ of 6.3. This confirms the success of the charge-complementarity approach to the generation of esterolytic antibodies. As expected, only hapten 21 could induce catalytic antibodies while hapten 22 failed to do so.

2.3.2. Esterolytic antibodies induced to haptenes with a 1,2-amino alcohol functionality

Masamune et al. extended Janda's approach by making the bait and switch strategy more general through the use of a 1,2-amino alcohol functionality.\textsuperscript{15} This approach does not carry the
limitation of Janda's hapten which was only applicable to benzoate esters. The idea was to generate aspartyl-protease like antibody by using the same bait and switch mechanism. Three different hapten structures were synthesized: a 1,2-amino-alcohol 24, a 1,2-trimethylammonium alcohol 25, and a 1,2-trimethylammonium phosphate 26 (Figure 9).

Figure 9

![Chemical structures](image)

24

25

26

27a  \(X = O\)

27b  \(X = NH\)

It was intended that these groups would induce a functional side chain in the antibody that would act as a general base, facilitating the deprotonation of an incoming water molecule as well as the reprotonation of the departing alcohol or amine product. Also, the haptens were supposed to elicit a neutral/acidic group that would stabilize the oxyanion generated in the transition state. These are the roles performed by the two aspartyl side chains in aspartyl proteases such as renin. Interestingly, all three haptens generated esterolytic antibodies that hydrolized ester 27a with roughly a 3000-fold rate acceleration and similar specificity constants. None of the antibodies catalyzed the hydrolysis of the amide substrate 27b.\(^1\)\(^6\) Apparently hapten 26 failed to induce an acidic residue within the antibody close to the reaction site. Therefore, it can be assumed that all three haptens induced antibodies with one functional side chain residue by virtue of the charged ammonium residues.

2.4. **Heterologous immunization**

The conclusion from the bait and switch strategy is that charge complementarity alone can induce rate accelerations in the order of \(10^3\)-fold in acyl-transfer reactions. Since transition state and oxyanion stabilization (using phosphonate haptens) yield similar rate accelerations, the coupling of these two features might produce more efficient antibodies. This might be accomplished by using zwitterionic haptens featuring a phosphonate and an ammonium moiety in
very close proximity. However, it is often very difficult to synthesize such a molecule and in many cases they are not stable enough to be used for immunizations.

Masamune et al. envisioned that the phosphonate and ammonium groups of a zwitterionic hapten could be bisected into two haptens. These could then be used to immunize mice in succession and generate antibodies that would be crossreactive to both of the haptens, and display enhanced catalytic efficiency. It was anticipated that a subset of the host's memory B-cell population which was induced by the first hapten could get stimulated by a second, structurally similar but differently functionalized hapten. This reasoning was inspired by earlier immunological studies of antibody cross reactivity to two distinct but structurally similar antigens that was termed "original antigenic sin".

With the aim of generating amidase antibodies that catalyze the hydrolysis of amide 27b, Masamune et al. immunized mice according to two different protocols. In the homologous immunization protocol, mice were immunized three times in two week intervals with either 14 or 24. In the heterologous immunization protocol, mice were immunized twice with the same hapten (14 or 24) followed by a final boost with the complementary hapten (Figure 10).

Figure 10

As it could be expected from previous studies, the homologous immunization protocol yielded antibodies that catalyzed the hydrolysis of ester 27a with rate accelerations of about one- to ten-thousandfold. However, the best catalyst obtained from heterologous immunization, H5H2-42, accelerated the ester hydrolysis more than $10^5$-fold above the background reaction at pH 6.6 and displayed more than 500 turnovers. The antibody was shown to be competitively inhibited by both haptens, 14 and 24, while no product inhibition was seen with either $p$-nitrophenol or the acid. The pH dependence studies pointed to the existence of two ionizable antibody residues that participated in catalysis with apparent pKa's of 5.6 and 8.7; thus confirming the generation of bifunctional antibody residues with the heterologous immunization strategy. Furthermore, the percentage of catalysts that did not suffer from severe product inhibition among all hapten binding antibodies was significantly higher with the heterologous immunization protocol (ca. 38%) than with either one of the homologous immunization protocols (ca. 15%). Unfortunately, neither antibody catalyzed the hydrolysis of amide 27b.
2.5. Reactive immunization

An intriguing alternative to the conventional approach of immunization with stable, unreactive haptns, is to use compounds that can react covalently with amino acid side chains. "Reactive Immunization" provides a strategy for inducing this functionality in the antibody combining site and bringing covalent catalysis into the realm of design rather than serendipity (see also chapter 1).4 An effective demonstration of this concept was recently reported by Janda and Lerner, using the electrophilic phosphonate aryl esters 28 and 29 (Figure 11).4a Although the intact phosphonate diester is not a transition state analog, displacement of one of the phenolic esters by an active site nucleophile affords a mimic of the tetrahedral intermediate in the acylation step of an acylation/deacylation mechanism for ester hydrolysis.

The antibodies isolated after immunization with a conjugate mixture of 28 and 29 show an interesting spectrum of activity, comprising not only the ability to hydrolyze the carboxylate ester 30 but also the diaryl phosphonate 29 (R = Me). Cleavage of the phosphonate, however, inactivates the catalyst. One of the antibodies, 49H4, catalyzes the hydrolysis of ester 30 with a specificity constant ($k_{cat}/K_m$) of about $1 \times 10^5$ M$^{-1}$ min$^{-1}$ which puts it among the best hydrolytic antibodies generated thus far. The hydrolysis appears to proceed via a covalent intermediate, since an acyl-antibody intermediate can be trapped at low pH. In the meantime, Janda successfully applied this approach to the kinetic resolution of a racemic mixture of $p$-methysulfonylphenyl esters of naproxen.19
2.6. Joint hybridoma and combinatorial antibody library approach

Traditional hybridoma technology samples only less than 10% of the immune system during the practical process involved. This insufficient screening of the immune library is a major reason for moving to alternative methods of monoclonal antibody generation, and phage display is a step forward in this respect. Janda et al. tried a combined approach where the spleen of a mouse which was previously immunized with hapten 32 (Figure 12) was used to construct a phage Fab library with the ultimate goal of catalyzing the hydrolysis of the primary amide in 31a.88

Figure 12

\[
\begin{align*}
31a: & \quad R = \text{NH}_2 \\
31b: & \quad R = \text{OMe} \\
31c: & \quad R = \text{OH}
\end{align*}
\]

The choice of a trigonal boronic hapten drastically departs from the traditional approaches, namely phosphonate haptens. However, it was reasoned that given the pKₐ of 8.0 for the hydration of boronic acids, there will be a high proportion of this form available to the immune system (assuming a plasma pH of 7.4). The tetrahedral boronic hydrate may also serve as an excellent mimic of the transition state for the catalysis of water to a carbonyl center (Figure 13).  

Figure 13

\[
\begin{align*}
\text{B(OH)}_3 & \quad \text{pK}_{a} \sim 8.0 \\
\text{B(OH)}_3 & \quad \text{B(OH)}_3
\end{align*}
\]

It was also hoped that the Lewis acidic nature of hapten 32 could select for a complementary Lewis base in the antibody combining site, therefore inducing nucleophilic catalysis (see chapter 3.2.). This hypothesis was supported from boronic acid inhibitors of serine proteases which are known to bind by coordination of the active-site serine or histidine to form a tetrahedral anionic mimic of the transition state for peptide bond cleavage.20

The panning procedure was performed at pH 7.5 using BSA-32. Under these conditions the effective concentration of the trigonal and tetrahedral form of hapten 32 was expected to be equivalent. After four rounds of panning, Fab-BL25 was isolated and further characterized. Fab-BL25 exclusively hydrolyzed the L-isomer of 31a to the acid 31c with a rate acceleration of about
$4 \times 10^4$ at pH 9. Fab-BL25 exhibited an excellent substrate specificity, accepting neither S-31a nor the methylester 31b as substrates. A pH-dependent competitive ELISA study revealed that Fab-BL25 most likely binds to the tetrahedral form of 32, suggesting that catalysis occurs by transition state stabilization rather than by nucleophilic catalysis. In a control study, boronic hapten 32 was used to immunize mice and 25 monoclonal antibodies were isolated by standard hybridoma technology. None of these antibodies showed catalysis.
Chapter 3
Antibody Catalyzed Cleavage of an Amide Bond Using an External Nucleophilic Co-Factor

3.1. Introduction

Recent investigations of X-ray crystal structures and computer modeling of a series of antibodies suggest that combining sites created by similar haptens converge to a single motif. Five hydrolytic antibodies that were raised against different haptens contain an apolar pocket surrounded by aromatic sidechains (to bind the hapten's aromatic rings) and charged sidechains (arginine, lysine or histidine) to help neutralize the negatively charged phosphonyl oxygens.\textsuperscript{7d} A convergence of motifs also occurs in monoclonal antibodies raised against a single hapten.\textsuperscript{21} This suggests that the immune system recruits the same motifs for certain kind of haptens (in these cases phosphonate haptens), and that catalysis occurred mainly through transition state stabilization.

The maximum flux for transition state stabilization catalysis is restricted to be on the order of $10^4$ to $10^5$. This value is calculated by using the equation for determining the flux ratio for catalysis, $(\nu_{\text{cat}}/\nu_{\text{uncat}})_{\text{max}} = [\text{Ab}]/K_{\text{TS}}$, an antibody concentration of $10^{-5}$ M and a general value $K_{\text{TS}} (= K_{d, \text{hapten}})$ of $10^{-10}$ M.\textsuperscript{22} The conversion of binding energy into turnover is generally accomplished by overcoming the translational and rotational modes of freedom of the substrate by confinement within the antibody's combining site and by specific interactions through non covalent bond formation between the substrate and sidechains of the amino acids. Greater catalytic rates could potentially occur for abzymes that perform covalent catalysis. However, there has been only one example where nucleophilic catalysis was involved in antibody catalysis beyond a reasonable doubt (see chapter 2.2.2). Unfortunately, this nucleophile was introduced in the antibody binding pocket solely by serendipity and it would be desirable to reliably program such a nucleophile into the hapten design.

3.2. Serine proteases

In our previous efforts to generate amidolytic antibodies (see chapter 2.4.) we successfully introduced two catalytic side chain residues into the antibody by using our heterologous immunization protocol. This approach was modeled after aspartatic proteases where two complementary acidic and basic side chain residues facilitate catalysis. While we generated very efficient esterolytic antibodies, we did not succeed in isolating amidolytic antibodies.

Serine proteases are another class of proteolytic enzymes which utilize an optimally placed internal serine residue in their active site to achieve the nucleophilic attack at the amide bond (Figure 14).\textsuperscript{23} The nucleophilic attack is aided by general base catalysis through a histidine which is in turn activated by an aspartate, leading to the formation of the acyl-enzyme tetrahedral
intermediate. The subsequent expulsion of the amine is aided by its protonation by histidine, generating the acyl-enzyme species. Then the process is repeated in reverse for the hydrolysis of the acyl intermediate liberating the acid portion of the substrate.

Figure 14

Our previous studies showed that we can introduce complementary acidic/basic sidechain residues into the antibody. However, it became apparent that we also need to introduce nucleophilic catalysis if we wanted to improve the rate acceleration beyond the hundred thousand-fold suggested by theoretical considerations (vide supra) or catalyze the hydrolysis of the kinetically more stable amide bond.

3.3. Introduction of an auxiliary nucleophile

Ideally, the nucleophile of the hydrolytic reaction is programmed into the antibody binding site in the form of an amino acid side chain residue. In practice, it is not a simple task to generate such a precisely placed residue even with prior knowledge of the binding site geometry.24 On the other hand, an auxiliary nucleophile can be tightly bound in an appropriately created pocket of the antibody binding site, and it should prove equally effective compared with an internal nucleophilic residue. In this present study, phenol was chosen as the auxiliary nucleophile since it is a readily water soluble compound and yet incorporates a phenyl ring that can take advantage of hydrophobic binding interactions to optimally place it in the antibody binding pocket. Furthermore, it is a good nucleophile that would generate a water-labile phenyl ester through the reaction with the model
amide, substrate T1. The phenol assisted cleavage of propionyl p-nitroanilide T1 is schematically shown in Figure 15.

Figure 15

![Chemical structure](image)

We also designed a unimolecular substrate T2 which aided the initial screening process for antibody catalysts. This avoided the difficulty of testing more than a hundred potential candidates in an initial bi-molecular screen which is very labor and time intensive. Once antibodies were identified that catalyzed the lactonization of substrate T2, those were then screened for catalysis in the bimolecular version (i.e. with amide T1 and phenol).

3.4. Hapten design

Complementary haptons O1 and O2 were designed by sorting out the functional groups of a hypothetical hapten O3, which is supposed to generate acidic and/or basic residues in the antibody combining sites (Figure 16).

Figure 16

![Chemical structure](image)

Hapten O2, bearing a pyridinium salt, was intended to induce a basic antibody residue to accept the proton from the attacking phenolic hydroxyl group. Furthermore, this protonated basic
residue could thereafter deliver its proton to the amine functionality of the tetrahedral intermediate, facilitating its departure. The tertiary hydroxyl group in O2 serves as an isosteric replacement for the tetrahedral intermediate.\textsuperscript{24} Phosphonamidate hapten O1 was designed to generate in the catalytic site an acidic residue that would stabilize the developing oxy-anion of the transition state. The arrangement of the phenol and the phosphonamidate in a six membered cyclic structure is intended to ensure the correct orientation of the hydroxyl group in close proximity to the amide carbonyl group during the process of catalysis.

3.5. Results

3.5.1. Hapten synthesis\textsuperscript{26}

The synthesis of hapten O2 was accomplished in thirteen steps (10.9\% overall yield) as shown in Figure 17. Alkylation of the phosphonate 34 with 4-nitrobenzyl bromide\textsuperscript{27} followed by a Horner-Emmons reaction with formaldehyde\textsuperscript{28} afforded the unsaturated ester 35.

Figure 17
The DIBAL-H reduction of ester 35 at -78°C, followed by triethylsilyl protection of the resulting primary alcohol 36 and subsequent epoxidation of the double bond with m-CPBA provided 38 in good yield (86%, 3 steps). The nitro group in 38 impeded the nucleophilic opening of the epoxide with 2-picoyllithium even when high order cyano cuprates were used. Therefore, it was decided to reduce it to an amine and protect it. Hydrogenation of the nitro group with Pd/C immediately followed by protection of the resulting amino moiety with trityl chloride proceeded almost quantitatively. The opening of the resulting epoxide 39 with 2-picoyllithium then proceeded smoothly to give 40 in 69% yield. Deprotection of the TES group with TBAF gave the diol 41 quantitatively. Activation of the primary hydroxyl group with methanesulfonyl chloride (MsCl) and subsequent cyclization in refluxing acetone afforded the quinolizinium salt 42. Finally, the trityl-group of 42 was removed under biphasic conditions by refluxing it in a mixture of water/ethyl acetate, followed by condensation of obtained 43 with glutaric anhydride to yield hapten O2 as an amorphous solid.

The synthesis of hapten O1 and substrates T1 and T2 was carried out by Dr. Oguz Ersoy (Figure 18).

Figure 18
Figure 18 (continued)

Both haptens were individually coupled to carrier proteins Keyhole Limpet Hemocyanine (KLH) and Bovine Serum Albumin (BSA) via the reaction of their activated N-hydroxysuccinimide esters with the surface lysine residues of these proteins. Successful conjugation was confirmed by UV spectrophotometry. The KLH conjugates were used in the immunizations as this protein is known to induce strong immune responses. BSA conjugates were used in the subsequent Enzyme Linked Immuno Sorbent Assays (ELISA's) of the generated hybridomas.

3.5.2. Kinetic results and discussion32

Details of immunization protocols, antibody generation, cloning and purification were already reported in Dr. Ersoy's thesis. In brief, Balb/c mice were immunized according to two different protocols. In the homologous immunization protocol, mice were immunized three times in two week intervals with either O1 or O2 conjugated to KLH. In the heterologous immunization protocol, mice were immunized twice with the same hapten (O1 or O2) followed by a final boost with the complementary hapten. Three days after hyperimmunization, mice were sacrificed and monoclonal antibodies were prepared. The amide hydrolysis of 1 was carried out with or without antibodies at 25 °C and at the appropriate pH's (three component buffer: 12.5 mM Mes, 12.5 mM Hepes, 25 mM Diethanolamine). The reaction was followed by HPLC, monitoring p-nitroaniline release at 382 nm.

Two out of 39 monoclonal antibodies generated against hapten O1 and one out of 20 monoclonal antibodies generated by hapten O2 were found to be a catalyst. Three out of 15 antibodies generated by the heterologous immunization protocol by immunizing twice with O1 followed by a boost with O2 exhibited catalytic activity (the reversed order, O2 followed by O1, did not yield crossreactive antibodies).33 The best antibody from each protocol was characterized further and the Michaelis-Menten parameters are summarized in Table 1.
Table 1

<table>
<thead>
<tr>
<th>Catalytic Antibody</th>
<th>Haptens</th>
<th>Immunization</th>
<th>$k_{cat}$ (10^{-3} min^{-1})</th>
<th>$K_m$ (µM)</th>
<th>$K_i$ - O1$^*$ (µM)</th>
<th>$K_i$ - O2 (µM)</th>
<th>$k_{Substr. T2}$ (10^{-7} min^{-1})</th>
<th>pH optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-17</td>
<td>O1 / O1 / O1</td>
<td>Homo</td>
<td>2.7</td>
<td>980</td>
<td>36</td>
<td>N.D.</td>
<td>4.2</td>
<td>8</td>
</tr>
<tr>
<td>3-49</td>
<td>O2 / O2 / O2</td>
<td>Homo</td>
<td>4.2</td>
<td>770</td>
<td>N.D.</td>
<td>380</td>
<td>5.7</td>
<td>9</td>
</tr>
<tr>
<td>14-10</td>
<td>O1 / O1 / O2</td>
<td>Hetero</td>
<td>15.0</td>
<td>1270</td>
<td>51</td>
<td>790</td>
<td>4.2</td>
<td>8</td>
</tr>
</tbody>
</table>

* Due to the instability of hapten O1 at higher pH's, Ki's for O1 were determined at pH 7 and not at the pH optimum.

The background rate of hydrolysis of amide T2 at pH 6.8 was measured to be $2.7 \times 10^{-7}$ min$^{-1}$, and that of substrate 51 (Figure 19) lacking the phenolic hydroxyl of substrate T2 was found to be almost identical ($k$ for 51 = $2.8 \times 10^{-7}$ min$^{-1}$ at pH 6.8). These data show that if the phenol participates in the hydrolysis of substrate amide T2, the rate of this lactonization reaction should not exceed the $k$ observed for the hydrolysis of substrate amide T1. This result sets the upper limit for the uncatalyzed lactonization.

The evidence for the intramolecular participation of the phenol of T2 emerged from the studies with substrate 51. While this substrate that lacks the phenolic hydroxyl group was shown to have an identical background rate of hydrolysis as compared to T2, none of the three antibody catalysts (6-17, 3-49, and 14-10) accepted 51 as a substrate.

The three antibody catalysts were then screened for the acceleration of the cleavage of propionyl p-nitroanilide T1 at varying concentrations of phenol at their pH optimum. The Michaelis-Menten parameters were determined for the three antibody catalysts by varying either the phenol or propionyl p-nitroanilide T1 concentrations. These values are listed in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Catalytic Antibody</th>
<th>Haptens</th>
<th>Immunization</th>
<th>$k_{cat}$ (10^{-5} min^{-1})</th>
<th>$K_m$ phenol (µM)</th>
<th>$K_m$ substr. T1 (µM)</th>
<th>pH screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-17</td>
<td>O1 / O1 / O1</td>
<td>Homo</td>
<td>2.1</td>
<td>78</td>
<td>310</td>
<td>8</td>
</tr>
<tr>
<td>3-49</td>
<td>O2 / O2 / O2</td>
<td>Homo</td>
<td>1.3</td>
<td>14</td>
<td>77</td>
<td>8</td>
</tr>
<tr>
<td>14-10</td>
<td>O1 / O1 / O2</td>
<td>Hetero</td>
<td>13.3</td>
<td>136</td>
<td>370</td>
<td>8</td>
</tr>
</tbody>
</table>
The participation of phenol in antibody catalysis was clearly established with a number of control experiments. First and foremost, in the absence of phenol, none of the three antibodies showed any rate acceleration of the cleavage of amide T1. Furthermore, neither 2-naphthol 53 nor 4-t-butyl phenol 54 were accepted as nucleophilic co-factors for catalysis by any of the three antibodies.

Figure 19

These results indicate the existence of a specific binding pocket for the phenol auxiliary nucleophile. Similarly, these catalysts were also specific for anilide T1. t-Butylacetyl p-nitroanilide 52 was not accepted as a substrate, indicating that a specific binding pocket also exists for T1. Together with the observation that substrate 51 lacking the nucleophilic hydroxyl group was not subject to antibody catalysis, these results confirm that the participation of phenol is essential for the antibody catalyzed cleavage of amide T1. The product of the reaction, phenyl propionate 50, was shown to not inhibit the antibody catalyzed cleavage of T1, even at relatively high concentrations (5 mM 50 and 0.5 mM T1). Furthermore, none of the three antibodies displayed any acceleration of the rate of hydrolysis of 50. Thus, it can be stated that the product 50 readily diffuses out of the antibody binding pocket upon its formation. Finally, the comparatively fast uncatalyzed rate of hydrolysis of product 50 regenerates the phenol co-factor thus completing the catalytic cycle.

The background rate of the hydrolysis of amide T1 was found to be $2.1 \times 10^{-7}$ min$^{-1}$ at pH 8.0. Interestingly, this rate does not vary with a change in the concentration of phenol in the aqueous solution, indicating that phenol does not participate in the uncatalyzed hydrolysis of amide T1. Therefore, the $k_{cat}$ values in Table 2 can not be directly compared with this background hydrolysis rate. Rather, the antibody catalyzed reaction appears to proceed by a pathway that is "disfavored" in aqueous solution. Mechanistically, it is possible that phenol can attack the amide bond in the presence of competing water (even though phenol is a rather poor nucleophile). However, the breakdown of the formed tetrahedral intermediate would most likely favor the
expulsion of phenol due to the huge $pK_a$ difference (greater than 6 units) between the phenol and nitroaniline. The antibodies are capable of rerouting this process in favor of nitroaniline expulsion.

The $K_m$ values for substrate T2 are consistently three to four times higher than those for substrate T1. One possible explanation may be that substrate T2 exists in aqueous solution in a predominately transoid conformation. The difference in $K_m$ values would then reflect the binding energy required to rotate substrate T2 into a cisoid (and therefore more hapten-like) conformation. It is also possible that the antibody cannot use the binding energy for conformational changes. In this case, the antibody can only bind to the cisoid conformation which means that the effective substrate concentration is much lower. In either case, one would expect a higher $K_m$ value which is not necessarily reflective of the “real” $K_m$. A further analysis of $K_m$ values shows that the $K_m$ phenols of all three antibodies are smaller than the respective $K_m$ substrates (Table 1). This is readily explained by consideration of the hapten designs. In both cases, the part of the hapten that corresponds to phenol is positioned furthest from the linker site of the haptons. It is therefore likely, that phenol is bound in a deep, hydrophobic pocket in the antibody binding sites, while the binding pocket for amide T1 is more solvent accessible.

The antibody catalyzed bimolecular reaction between T1 and phenol is about two orders of magnitude slower than the antibody catalyzed lactonization of T2. This difference seems rather small considering the potential steric repulsion between the ortho-hydrogen of phenol and the $\beta$-hydrogens of propionamide T2 (the hapten structure represents this as a carbon-carbon bond; see Figure 20).

Figure 20

As a result, phenol and amide T1 might not assume an optimal orientation within the antibody in terms of the phenol attack angle, phenol deprotonation, and anilide protonation. However, it seems that antibodies do provide some flexibility in accommodating different substrates.

The product phenyl ester 50 appears to freely diffuse out of the binding pocket (i.e. no product inhibition is observed), and its rate of hydrolysis in the buffer employed ($k = 2.0 \times 10^{-4}$ min$^{-1}$ at pH 8.0) is three order of magnitudes faster than the buffer-catalyzed rate of hydrolysis of amide T1 at this pH. Therefore, it can be said that all three antibodies have, in fact, achieved the hydrolysis of the amide bond in substrate T1. This represents a formal, two step hydrolysis of an amide bond where the initial transfer of the amide group to phenol is followed by the relatively
facile hydrolysis of the formed phenol ester 50. These findings indeed demonstrate the power of using an external nucleophile to effect the cleavage of amide bonds by antibody catalysts.
Chapter 4
Antibody Catalyzed Prodrug Activation

4.1. Introduction

Numerous catalytic antibodies have been prepared for a wide variety of reactions ranging from esterolysis to pericyclic reactions. Advances in both catalytic efficiency and specificity have been impressive in some cases, and the generation of tailor-made enzymes for applications in medicine, seems attainable. The activation of prodrugs for cancer therapy could be a possible use of catalytic antibodies in medicine. To date, there is no successful drug delivery system which could activate cancer prodrugs at the tumor site without affecting healthy cells. The ability to create a unique catalyst for which there is no natural enzyme counterpart would allow an additional degree of specificity in the process of prodrug design and delivery.

Considerable effort has focused on the development of prodrugs that are activated by antibody-enzyme conjugates (Antibody Directed Prodrug Therapy, ADEPT). However, several clinical trials using this approach were unsuccessful due to severe side effects and minimal efficacy of these conjugates (see chapter 4.2.). Many of these problems may be overcome by replacing the antibody-enzyme conjugate with a bispecific antibody, derived from a tumor specific antibody and a catalytic antibody. To this end, several groups have reported the generation of catalytic antibodies capable of converting prodrugs into cytotoxic compounds (see chapter 4.3.). However, none of these systems would be suitable for in vivo applications since the kinetic stability of the prodrugs was very low and/or the antibody catalyzed rates were too slow.

In the early phase of this project (see chapter 5) we designed an amide based p-phenylenediamine prodrug which was expected to display a greatly reduced toxicity compared to the parent drug. However, cytotoxicity studies comparing this prodrug with the parent drug, p-phenylenediamine, revealed that the prodrug was almost as toxic as the parent drug. We reasoned that the prodrug was either not kinetically stable enough, and/or it was a substrate for nonspecific enzymes. It was also possible that the protecting group would not reduce the toxicity of the parental drug by the expected extend. Furthermore, kinetic experiments showed that the kinetic lability of the prodrug was the main reason for high toxicity of the prodrug.

In the next phase of the project (chapter 6) we designed a carbamate based prodrug which according to preliminary experiments could be expected to be more stable in serum. The improved prodrug design also addressed another problem. One major limitation of all the prodrug activating antibodies generated thus far is that they are limited to only one cancer drug since the features of the cancer drug were included in the hapten design. This presents a severe limitation to cancer chemotherapy since most current therapies involve multiple drug combinations. Consequently, despite the considerable number of prodrug activating antibodies in literature, the ultimate goal of
developing a clinically useful bispecific catalytic antibody has not been realized thus far. Therefore, it would be desirable to generate a catalytic antibody that will be capable of simultaneously activating several different prodrugs.\textsuperscript{45} To this end, two appropriate haptens were designed which were expected to generate an antibody combining site that specifically recognizes the protective group, but allows broad substrate tolerance with regard to the drug portion.\textsuperscript{46} This approach will open the possibility of a multi drug combination therapy using a single bispecific catalytic antibody, thus greatly broadening the applicability of bispecific catalytic antibodies in cancer therapy.

4.2. Antibody Directed Enzyme Prodrug Therapy (ADEPT) and Antibody Directed Antibody Prodrug Therapy (ADAPT)

4.2.1. ADEPT

A number of attempts have been made to exploit tumor-specific antibodies in the treatment of cancer with limited success (i.e., Herceptin from Genentech, recently approved by the FDA). The one approach that is most closely related to this project is the use of an antibody-enzyme conjugate to activate a prodrug at tumor sites, known as Antibody-Directed Enzyme Prodrug Therapy (ADEPT). ADEPT has been developed to reduce unwanted non-specific toxicity associated with cytotoxic anticancer drugs (Figure 21).\textsuperscript{40} It consists of two components: a tumorspecific antibody-enzyme conjugate and a stable prodrug (in this example p-phenylenediamine mustard protected with a tripeptide) of lower toxicity.

Figure 21

\begin{center}
\includegraphics[width=\textwidth]{figure21.png}
\end{center}

In a typical treatment scheme, the conjugate is administered first and allowed to accumulate at the tumor site through antibody binding to a tumor-associated antigen. Once excess conjugate has been cleared from the plasma, the prodrug is administered. Subsequent cleavage of the prodrug to generate the active cytotoxic agent by the enzyme component of the conjugate occurs selectively at the tumor site and therefore leads to both enhanced efficacy of the anticancer agent and reduced
Peripheral cytotoxicity. Since its inception, this concept has been applied to many different enzyme-prodrug pairs over the years. However, several problems have been found to be associated with ADEPT which have caused severe side effects in patients and have limited its efficacy. (1) In order to reduce non-specific prodrug activation by human enzymes, the enzyme component commonly selected has been of bacterial origin, which is immunogenic. Also, most of the antibodies used in this context were of murine origin and therefore highly immunogenic as well. (2) The high catalytic efficiency of these enzymes causes residual conjugates in the bloodstream to activate prodrugs at undesired sites distant from the tumor. It has been suggested that higher selectivity and less peripheral toxicity might be accomplished by using less efficient catalysts. Similarly, widespread distribution of relatively non-selective host enzymes capable of prematurely activating a prodrug also caused undesirable side effects. (3) As ADEPT has been employed to target drugs for tumor cells, there is a size limitation for these conjugates as they have to penetrate tissues to reach tumor cells. Recent experiments suggest that optimal tumor infiltration may be accomplished with proteins between 60 and 80 kD in size. However, the ADEPT conjugates usually have a molecular mass of 200 kD and larger. For these reasons, it is not surprising that several clinical trials using the ADEPT approach were unsuccessful.

4.2.2. ADAPT

One solution to overcome the immunogenicity of the enzyme would be to replace the enzyme in the antibody-enzyme conjugate with a catalytic human antibody, since “humanized” antibodies have been shown to be well tolerated by humans without causing serious immune responses. In order to avoid the non-specific activation of prodrugs by endogenous enzymes, one can create a unique antibody catalyst for which there is no natural enzyme counterpart. This idea has been put forward by Bagshawe, who has proposed that humanized catalytic antibodies could replace the enzyme component of ADEPT in an improved targeted therapy (Figure 22). This concept, coined ADAPT (for Antibody Directed Antibody Prodrug Therapy) may prove superior to the ADEPT approach.

The use of a catalytic antibody should make possible both the activation of the prodrug, via a reaction not catalyzed by endogenous enzymes, and also eliminate the need for an immunogenic enzyme. As a bacterial enzyme is not involved, the immunogenicity observed for the antibody-enzyme conjugate should be greatly reduced. Furthermore, “humanization” of all the antibody fragments involved will diminish or eliminate the inherent immunogenicity of bispecific catalytic antibodies.
Catalytic antibodies usually exhibit catalytic rates lower than their natural enzyme counterparts, therefore reducing the chances of peripheral toxicity by activation of the prodrug through residual immunoconjugates at sites distant from the tumor. Recent calculations\textsuperscript{43d} suggested that a rate of 1 min\textsuperscript{-1} to 1 s\textsuperscript{-1} is fast enough for efficient prodrug activation, a rate that is definitely within the realm of catalytic antibody technology. In addition, modern antibody technology allows the modulation of the size of bispecific antibody constructs (e.g. minibodies,\textsuperscript{50} diabodies,\textsuperscript{53} etc.). Actually, Figure 22 does not imply any particular kind of bispecific antibody construct as different fragments may have different advantages and deficiencies. The different kind of bispecific antibody fragments will be discussed in chapter 6.5.2.

The flexibility of the ADAPT system offers many solutions for the shortcomings of the ADEPT-strategy.

4.3. A brief synopsis of prodrug activating antibodies

4.3.1. Ester protected prodrugs

In 1993, Fujii and coworkers isolated the first catalytic antibody which could activate a prodrug of Chloramphenicol 57 (Figure 23).\textsuperscript{43a} They raised antibodies to the phosphonate 59 and 60 to cleave a phenyl acetate 55 and liberate the antibiotic chloramphenicol 57. As the phenyl acetate exists in a mixture of two regioisomers 55 and 56, product inhibition was expected to be low. None of the 23 antibodies raised to hapten 59 were found to be catalytic, while 6 out of the
12 to hapten 60 were. The best antibody, 6D9, displayed a $k_{\text{cat}}$ of 0.133 min$^{-1}$ which corresponds to a rate acceleration of about $2 \times 10^3$. It displayed multiple turnovers, as well as growth inhibition in a bacterial assay. In a paper-disc agar diffusion assay, the combination of prodrug and antibody 6D9 inhibited the growth of bacteria in a circular zone of 12 mm. While this was an encouraging first effort, the author noted that more efficient catalysts in terms of rate enhancement as well as turnover will be needed for further applications. However, they also commented that this protocol could be used as a selection tool during the phagemid expression of antibodies in bacteria.

Figure 23

In the next study, Schultz et al. used the 5'-D-Val ester 61 of the antitumor agent 5-fluorodeoxyuridine 62 (Figure 24). They reasoned that unspecific esterases, in general, do not hydrolyze β-branched D-Val esters to any appreciable degree. Antibodies against transition state analog 63 were raised and one antibody, 49.AG.659.12, was found to catalyze the hydrolysis of ester 61. The Michaelis Menten parameters were determined and the antibody exhibited a $k_{\text{cat}}$ of 0.03 min$^{-1}$ and a $K_m$ of 218 μM, which corresponds to a one thousand-fold rate acceleration. In a bacterial assay they could show that growth inhibition could be accomplished by exposing *E. coli* to prodrug 61 and antibody, while prodrug alone did not inhibit the growth to any appreciable extend.
4.3.2. Intracellular prodrug activation

Benkovic et al. reported the generation of an antibody which could catalyze the decarboxylation of the prodrug 5-fluoroorotate 64 to the anti-metabolite 5-floururacil 65 (Figure 25).43b

Initially, his group attempted to create antibodies capable of decarboxylating orotate, an intermediate in the de novo pyrimidine synthetic pathway.54 An auxotrophic E. coli strain lacking OMP decarboxylase (orotidine-5’-monophosphate carboxy-lyase) could synthesize uracil monophosphate (UMP) if provided with a replacement enzyme. For example, a catalytic antibody with orotate decarboxylase activity could function like such an enzyme, since the product, uracil, could get converted to the nucleotide UMP by the salvage pathway enzyme uracil phosphoribosyltransferase. In a combined immunization/combinatorial library approach, hapten 66 was used to immunize mice. Then, the spleen was extracted, and antibody cDNA was isolated. The cDNA was utilized to assemble a combinatorial heavy- and light-chain phagemid library. The phage library was then used to infect the above pyrimidine auxotrophic E. coli strain which was grown on pyrimidine free medium. Six antibody encoding plasmids were isolated which conferred a growth advantage to this strain on pyrimidine free medium. These six plasmids were then converted into single chain antibodies and E. coli bacteria were infected with these plasmids. Benkovic could show that one plasmid, pSC-8, conferred high drug sensitivity to a strain of E. coli which is resistant to 5-fluoroortate 64, by converting this prodrug into 5-flourouracil 65.
4.3.3. Carbamate prodrug activation

In the most promising approach yet, Blackburn et al. targeted the activation of a nitrogen mustard prodrug by antibodies.\textsuperscript{43d} Antibody EA11-D7, elicited to the phosphonamidate 70, catalyzes the hydrolysis of carbamate prodrug 67 to release the phenolic drug 68 (Figure 26). Significant inhibition of human colonic tumor cell growth was observed in \textit{in vitro} assays comprising EA11-D7 and 67. It is interesting to note that despite the substantial dissimilarity between hapten and substrate, efficient catalytic antibodies were isolated. Complementary kinetic investigations of a series of similar aryl carbamate substrates (71a - 71e) using another antibody, DF8-D5, elicited to 70 clearly established a mechanistic switch from the favored E1cB (first order elimination via the conjugate base) process for aryl carbamate ester hydrolysis to a highly disfavored BAc2 (second order base catalyzed acyl-bond cleavage) mechanism.\textsuperscript{55} The highest rate acceleration was accomplished with substrate 71e, where $k_{\text{cat}}$ was 4.9 min\textsuperscript{-1}, which corresponds to a rate acceleration of over 10\textsuperscript{6}-fold.

Figure 26
5.1. Prodrug design

In the initial design, we sought to generate a trifluoroacetamide prodrug 72 of $p$-phenylenediamine mustard 73. This target was chosen because the mustard drug has been well studied over the years, especially in conjunction with the ADEPT system (see chapter 4.2.), and its derivatization is straightforward (Figure 27). $p$-Phenylenediamine mustard 73 is a DNA alkylation agent which has been shown to confer cell death by crosslinking DNA strands.

We chose the trifluoroacetamide group as the protection group for the following reasons. We envisioned that the electron withdrawing effect of the trifluoroacetamide group would greatly diminish the electron density of the mustard nitrogen and therefore reduce the DNA-alkylating property of this molecule. Studies with $p$-nitrophenyl mustard 74 have shown that this analog is up to seven thousand-fold less toxic than $p$-phenylenediamine mustard. Secondly, nobody has yet isolated an antibody which is capable of efficiently hydrolyzing the kinetically very stable amide bond. We reasoned that the trifluoro group would activate the amide bond to such an extent that the kinetic stability would be reduced to the stability of a phenyl ester, therefore increasing the chances of finding an antibody catalyst. Thirdly, the trifluoroacetamide group is very small and provides very few recognition elements for unspecific hydrolytic enzymes.

5.2. Hapten design

The transition state of the trifluoroacetamide hydrolysis is shown in Figure 28.
Most hapten designs for the hydrolysis of esters and amides feature a phosphonate group as a transition state mimic. However, these designs could, in general, only provide rate accelerations of about $10^3$ to $10^4$-fold (see discussions in chapter 2). Drawing from our experience with heterologous immunization and bifunctional hapten, we designed three hapten for the hydrolytic cleavage of the trifluoroacetamide group (Figure 29).

**Figure 29**

**Bifunctional Hapten:**

![Bifunctional Hapten](image)

**Heterologous Immunization:**

![Heterologous Immunization](image)

Bifunctional hapten 75 features a phosphonamidate group, which is supposed to generate in the catalytic site an acidic residue that would stabilize the developing oxy-anion of the transition state and mimic the tetrahedral transition state. It also bears a pyridinium salt which is intended to induce a basic antibody residue to accept the proton from the attacking phenolic hydroxyl group. Furthermore, this protonated basic residue could thereafter deliver its proton to the amine functionality of the tetrahedral intermediate, facilitating its departure. Haptens 76 and 77 were conceived as alternatives if it turned out that hapten 75 is not stable. These two hapten were designed by sorting out the different functionalities of hapten 75, and were intended to be used in a heterologous immunization protocol.59

### 5.3. Results

#### 5.3.1. Substrate synthesis

$p$-Phenylenediamine mustard 73 was prepared according to literature procedures and used as the stable hydrochloride salt.56 Trifluoroacetamide prodrug 72 was obtained by reacting mustard 72 with trifluoroacetic acid (TFAA) in the presence of triethylamine (Figure 30).
Compound 79, an analog of 72, which was used for kinetic assays (see chapter 5.3.3.), was prepared very efficiently in a three step procedure which did not require extensive workup between steps (Figure 31). Thus, the known precursor 78\(^{58a}\) was methylated with methyl iodide in the presence of potassium hydride as a base. The resulting dimethyl ether was hydrogenated over palladium on carbon and the resulting amine was immediately reacted with trifluoroacetic anhydride in the presence of triethylamine to afford 79 in good yield.

**5.3.2. Growth inhibition studies\(^{60}\)**

LS174T human colon carcinoma cells (5 \(\times\) 10\(^3\)) were plated in Dulbecco's modified Earl's medium (DMEM + 10\% fetal bovine serum) in 96 well plates and incubated for 24h. Prodrug and drug were incubated at twelve different concentrations (between 1 \(\times\) 10\(^{-4}\) M and 5 \(\times\) 10\(^{-8}\) M) with these cells for 1h. The cells were then washed (three times) with medium to remove any remaining compound, and reincubated with drug free medium for a further six days. At the end of this period, the cells were fixed and stained. The concentration of cellular protein from the surviving cells was quantified by a sulforhodamine B assay.\(^{61}\) The optical density of each well was plotted against the drug/prodrug concentration and the results are shown in Graph 1.

The IC\(_{50}\) value for the parent drug \(p\)-phenylene diamine 73 is about 1.8 \(\times\) 10\(^{-6}\) M which is in good agreement with literature values (2 \(\times\) 10\(^{-6}\) M).\(^{57}\) And the IC\(_{50}\) value for the prodrug was estimated to be 3 \(\times\) 10\(^{-5}\) M, which indicates that the prodrug is only about 17-fold less toxic than the parent drug. This means that this prodrug/drug pair is not very well suited for the antibody mediated prodrug activation since a differential toxicity of at least a eighty to a hundred-fold would be desirable in order to make this approach clinically useful.
5.3.3. Kinetic stability of prodrug

Since the drug/prodrug pair 72/73 did not yield the expected differential toxicity, we tried to understand whether the considerably high toxicity of the prodrug was either due to unspecific hydrolysis of the trifluoroacetamide group, or due to insufficient deactivation of the nitrogen mustard by the trifluoroacetamide group.

The kinetic stability of the trifluoroacetamide group in 72 in serum could not be measured directly since the mustard group also contributes to the decomposition of 72. In order to get a good estimate for the hydrolysis rate of the trifluoroacetamide group in 73, compound 79 was prepared (Figure 32). The methylmustard analog 79 cannot decompose by activation of the mustard group and therefore, its rate of hydrolysis provides a good estimate for the kinetic stability of the trifluoroacetamide group in 72.

Accordingly, we incubated prodrug analog 79 with fetal bovine serum and monitored the appearance of compound 80 by HPLC. The half life of 79, and therewith of prodrug 72, was estimated to be 14h ($t_{1/2} = \ln 0.5/k_{obs}$). This means that after a drug incubation period of 1h,
about 5% of the prodrug was hydrolyzed to the parental drug. Accordingly, at the prodrug IC$_{50}$ concentration of 3 $\times$ 10$^{-5}$ M, there was a parental drug 73 concentration of about 1.5 $\times$ 10$^{-6}$ M present. This corresponds roughly with the IC$_{50}$ value of the parent drug 73. It is therefore very likely that the low differential toxicity between drug and prodrug is entirely due to the kinetic instability of the trifluoroacetamide group, and not due to insufficient deactivation of the nitrogen mustard by the trifluoroacetamide group.

Figure 32

This particular prodrug and hapten design was later discontinued and the improved system is discussed in the next chapter.
Chapter 6
Simultaneous Activation of Multiple Prodrugs by Catalytic Antibodies

6.1. Introduction

As outlined earlier (see chapter 4.1.), part of the improved prodrug design was a protecting group which could be attached to any type of common drug. One catalytic antibody would therefore be capable of activating a range of different prodrugs, provided that they all feature the same protecting group. This way, we could circumvent the limitation of previous approaches where a different antibody would be needed for each particular prodrug.

Figure 33 shows a random selection of cancer drugs currently used in chemotherapy. It becomes apparent that if the protective group was equally suitable for hydroxyl and amino functionalities, it would cover a wide range of cytotoxic agents. The arrows indicate the site of protective group attachment, which would render these drugs less or non toxic based on their mode of action.

Figure 33

5-Fluoro-2'-desoxyuridine (Anti-metabolite)
p-Phenylenediamine mustard (DNA-crosslinking agent)
Camptothecin (Topoisomerase inhibitor)
Duocarmycin C2 (DNA-alkylating agent)

Etoposide (DNA-intercalating agent)
Doxorubicin (DNA-Intercalating agent)
Aminopterin (Dihydrofolate Reductase Inhibitor)

R = site of protection group attachment

6.2. Protection group design

The reactions which have been used to deprotect prodrugs by catalytic antibodies are mainly the hydrolysis of esters and carbamates (see chapter 4.3.). Due to (i) the widespread distribution and substrate promiscuity of esterases in vivo, and (ii) their inherent kinetic
instability (a lesson we learned from the previous design), esters do not seem to be an appropriate choice for the protective group design. Carbamates and (non peptidic) amides seem to be a better choice since they are reasonably stable in serum, and are not good substrates for hydrolytic enzymes.\textsuperscript{63} However, the hydrolysis of amides and carbamates (especially non activated alkyl amides and carbamates) by catalytic antibodies was shown to be notoriously difficult (see chapters 2 and 3).\textsuperscript{64} The only other reaction besides the hydrolysis of esters that catalytic antibodies can reliably catalyze are eliminations.\textsuperscript{65} We therefore decided to include in the protective group design a trigger mechanism based on an elimination process, in order to hydrolytically fragment a carbamate bond which ultimately releases free drug. We designed a 2-arylsulfonylethoxycarbonyl group which serves as the trigger mechanism which upon deprotonation eliminates carbamic acid.\textsuperscript{66} The carbamic acid is unstable and ultimately decomposes into carbon dioxide and free amine.

The 2-arylsulfonylethoxycarbonyl group $\mathbf{P1}$ - $\mathbf{P4}$ (Figure 34) was chosen for several reasons. To our knowledge, there is no endogenous enzyme that would catalyze the sulfone elimination. Modulation of the acidity of the $\alpha$-proton by selecting an appropriate R group (e.g. $R$ = Me, H, Cl, NO$_2$) in the phenyl ring allows fine tuning of the reactivity of the prodrug.\textsuperscript{67}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure34.png}
\caption{Figure 34}
\end{figure}

Also, the phenyl group provides a good 'handle' for antibody recognition through hydrophobic and $\pi$-bonding interactions. Inserting a methylene bridge between the carbamate and hydroxyl group ($\mathbf{P5}$ - $\mathbf{P8}$) allows the application of this group to be extended to alcohol functionalities (Figure 35).\textsuperscript{68}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure35.png}
\caption{Figure 35}
\end{figure}
Substrates S1 through S4 (Figure 36) were designed as preliminary screening substrates for the determination of the kinetic stability of the protection group and the identification of antibody catalysts. The fluorescent dansyl group allowed the screening of the hydrolytic stability of all four compounds in serum in an easy TLC-format. These substrates also proved very useful for the HPLC screening of antibody catalysts.

Figure 36

6.3. Hapten design

The ethyl phenylsulfone β-elimination reaction proceeds either via an E2 or E1cB mechanism depending on the electronic properties of the 4-phenyl substituent (Figure 37). Most hapten designs for elimination reactions include a positively charged ammonium group to induce a negatively charged carboxylate residue in the antibody combining site for easier proton abstraction. However, Uno et al. accomplished the catalysis of a dehydratation reaction by stabilizing the protonated form of the OH group, converting it to H₂O, a much better leaving group. It was suggested that a catalytic dyad which facilitates proton abstraction as well as leaving group stabilization may induce more efficient catalytic antibodies.

Figure 37

Transition State:
To test this hypothesis, hapten \textbf{R1} (Figure 38) was designed. \textbf{R1} features a positively charged (at physiological pH) ammonium functionality positioned to abstract the corresponding proton of bound substrate. It also features a negatively charged phosphonate which is expected to stabilize the incipient charge on the carbamate moiety. The phosphinate group is an isosteric replacement for the sulfone, and it should also induce an acidic residue to increase the acidity of the α-sulfone hydrogen in the substrate. In order to measure the effectiveness of leaving group stabilization, hapten \textbf{R2} was designed in which the phosphonate group of \textbf{R1} is replaced by an amide group. Comparison of the two haptens will yield valuable mechanistic insights into elimination processes catalyzed by antibodies.

![Figure 38](image)

Furthermore, the piperidine functionality is supposed to link a non-specific element to the hapten design to induce a broad substrate tolerance.\textsuperscript{46} The 3,4-dimethylphenyl moiety creates a pocket space that can accommodate different substituents in the aromatic rings of \textbf{P1} and \textbf{P2}.\textsuperscript{65\textsuperscript{e}} In addition, dissimilarity of hapten and product, both in shape and charge, minimizes possible problems due to product inhibition.

6.4. Results

6.4.1. Hapten and substrate syntheses

The synthesis of hapten \textbf{R1} was carried according to Figure 39.

![Figure 39](image)
A solution of methyl phosphinate 82 in methyl orthoformate was prepared according to the method of Fitch. Methylphosphinate 82 was reacted with 4-iodo-o-xylene under palladium catalysis to form phosphinate 83. 4-Hydroxypipendine 84 was protected with 9-fluorenylmethyl chloroformate to yield 85 almost quantitatively. Only very diprotected product (-OH and -NH) was observed. 3-Mercaptopropionic acid 86 was protected with tritylchloride to yield 87 in quantitative yield. Phosphonate ester 88 was hydrolyzed with bromotrimethylsilane and then immediately reacted with oxalylchloride to yield dichlorophosphonate 89. Without workup, the dichlorophosphonate was first reacted with alcohol 85 under 1-H-tetrazole catalysis, and then quenched with methanol to yield mixed phosphonate 90 in moderate yield. The double bond in 90 was then ozonized to afford aldehyde 91. Aldehyde 91 was treated with tritylamine to form its Schiff base, which was then reacted with phosphinate 83 in refluxing toluene to yield phosphinate 92. After deprotection of the FMOC-group with piperidine, 92 was condensed with acid 87 to yield the fully protected hapten 93. Deprotection of
the hapten was accomplished in two steps. First, 93 was treated with bromotrimethylsilane to hydrolyze the phosphinate and phosphonate esters. Treatment of the resulting compound with trifluoroacetic acid to remove the N-trityl and S-trityl groups yielded hapten R1. Triethylsilane was added to the reaction mixture to quench the intermittently formed and highly reactive trityl carbocations.

The synthesis of hapten R2 was carried out by Dr. Maria Blanco-Pillado according to Figure 40.

Figure 40

The synthesis of substrates S2, S3, and S4 was carried out by Dr. Maria Blanco-Pillado according to Figure 41.
The synthesis of substrate S6 was carried according to Figure 42. Thus, 2-(4-nitrophenylthio)ethanol 109 was treated with diphosgene to form its chloroformate and then reacted with amine 105 to form carbamate 110. The carbamate 110 was reacted with m-CPBA, which not only oxidized the thioether to the desired sulphone, but also the tertiary amine of the dansyl group to the amine oxide. Reduction of the amine oxide with sodium sulfite yielded substrate S6 in excellent yield.
6.4.2. Antibody generation and purification

6.4.2.1. Antibody generation and selection

**Immunizations**

Haptens R1 and R2 were individually coupled to maleimide activated Keyhole Limpet Hemocyanin (KLH) and Bovine Serum Albumin (BSA) via their thiol residues. Successful conjugation was confirmed with UV spectrophotometry. The KLH conjugates were used in the immunizations as this protein is known to induce strong immune responses; BSA conjugates were used in the subsequent Enzyme Linked Immuno Sorbent Assays (ELISA's) of the generated hybridomas.

Hapten conjugates KLH-R1 and KLH-R2 were emulsified in RIBI adjuvant and four mice for each hapten were injected subcutaneously with 100 µg of protein per mouse. A booster injection was given after two weeks using the same protocol. Ten days after the second injection, mice were bled and the serum was titrated using ELISA-methods. All animals displayed a strong immune response to their respective BSA-conjugates (titer > 25,600).

**Fusion**

All fusions were performed using a methylcellulose based semi-solid media for hybridoma cloning soon after the fusion. This technique offers several advantages over traditional fusion procedures. First, it virtually eliminates the need for subcloning and serial dilutions of hybridomas resulting in a significant time and material savings. Second, faster growing hybridoma clones do not overgrow slower growing clones. Third, the number of clones to be screened for secretion of specific antibodies is minimized, since identical daughter cells are not produced prior to the direct cloning.

Thus, four weeks after the second injection, the mouse with the highest titer received a final boost intraperitoneally with the respective KLH-conjugate. Three days later, the mouse was sacrificed and its spleen extracted. A single cell suspension from each spleen was fused to SP2/0 myeloma cells using polyethylene glycol (diluted 1/1 with serum free medium) as the fusing agent. The fusion mixture was suspended in a semi solid methylcellulose medium (which is supplemented with a HAT-selection mixture) and plated into ten 100 mm petri dishes. Ten to fourteen days later, individually growing colonies were transferred into 96-well plates with a pipettor. This method yields about 500 colonies per fusion. About four days later, the cells were confluent and screened for the presence of hapten binding antibodies using ELISA. Out of about 900 colonies, 67 colonies specific for R1 and 63 colonies specific for R2 were identified and transferred into 24 well plates. Once these colonies were confluent, the hybridoma supernatants were screened for catalysis using
the newly developed "Capture"-CatELISA assay (see chapter 7). Based on this assay, four antibodies specific for R1 and five antibodies specific for R2 selected for large scale production.

6.4.2.2. Antibody production and purification

Antibody production

The selected cell lines were grown to 10 ml scale and high viability (>95%) was accomplished by continuously diluting the cells to a concentration of 0.3 - 0.5 × 10^6 cells per ml. Healthy hybridoma cells (1.5 × 10^6) were suspended in PBS (0.5 ml) and injected into the intraperitoneal cavity of pristane primed Balb/C mice. After seven to ten days the mice produced large amounts of antibody enriched ascites fluid, which was collected via one to three taps with a hypodermic needle. The ascites fluid was immediately frozen after it was collected.

Antibody Purification

Antibodies were purified in a protocol that uses the least number of manipulations. Thus, frozen ascites fluid was pooled, diluted with a neutral phosphate buffer (1/1), centrifuged, and filtered through a 0.22 μm syringe filter. The solution was then applied to a HiTrap Protein G column (Pharmacia), and antibody was eluded with an acidic glycine buffer. Protein G was selected because it binds very well to all subclasses of mouse antibodies with high specificity. The UV active (λ = 280 nm) fractions were pooled and quantified.

6.4.3. Kinetic results and discussion

The half life of substrates S1, S2, S3, and S4 was measured in serum at 37 °C. Thus, the substrates were incubated with serum at a concentration of 0.1 mM and the release of 105 was monitored by TLC methods using fluorescent light for detection. As expected, S1 (R = NO2) was the most reactive substrate with a half life of less than 3h. It was followed by substrate S2 (R = Cl) with a half life of 72h, while S3 (R = H) and S4 (R = Me) displayed half lives of longer than 150h. It therefore became clear that while P1 was too reactive to be used as a protecting group, P2, P3, and P4 would be suitable choices based on their stability. The half life of S2 (and therefore of the corresponding protecting group P2) is about five times longer than the half life of the trifluoroacetamide group of prodrug 72 (see chapter four). Considering that the trifluoroacetamide protecting group provided a differential toxicity of 16 times that of the parental drug, one can expect to see a minimum differential toxicity of 80 times using P2 as a protecting group for any given cytotoxic compound. The differential toxicity of P3 and P4 can be expected to be even greater. These are reasonable values which make P2 through P4 good choices for protecting group design.
Nine purified antibodies were assayed for their ability to catalyze the elimination process using S2 through S4 as substrates. The reactions were carried out in Tris-buffer (50 mM, pH 7.0, 100 mM NaCl) at 37 °C and the release of compound 105 was monitored by HPLC at 248 nm. While there was little catalysis observed with substrates S3 and S4, all nine antibodies displayed catalysis with the more reactive substrate S2.

The best catalyst from each immunization were characterized further and their Michaelis-Menten constants were determined. The best antibody, FD2, generated to hapten R1, showed a $k_{\text{cat}}$ of 0.015 min$^{-1}$ and a $K_m$ of 890 μM. Antibody BH4, generated to hapten R2, displayed a $k_{\text{cat}}$ of 0.010 min$^{-1}$ and a $K_m$ of 497 μM. The antibody catalyzed reaction was inhibited by the addition of free hapten and a value of 530 μM for $K_i$ was measured for antibody FD2 (using R1 as the inhibitor). The two antibodies showed multiple turnovers and no product inhibition as evidenced by the fact that even after 24h there was no decrease of catalysis observed.

The background rate (zero buffer rate) for the elimination of substrate S2 was measured to be $1.5 \times 10^{-5}$ min$^{-1}$. This translates into a rate acceleration ($k_{\text{cat}}/k_{\text{uncat}}$) of exactly one thousand-fold for antibody FD2 while antibody BH4 performed slightly worse ($k_{\text{cat}}/k_{\text{uncat}} = 670$). However, if the catalytic efficiencies (as defined by $k_{\text{cat}}/K_m$) of the two antibodies are compared they both perform almost equally well (FD2: 16.85 min$^{-1}$M$^{-1}$; BH4: 20.12 min$^{-1}$M$^{-1}$). This suggests that if the phosphonate in hapten R1 induced an acidic residue for leaving group stabilization, it would not provide an impact on the catalytic rate. Therefore, it can be assumed that the rate determining step is the proton abstraction rather than the departure of the leaving group. Consequently, the mechanism for the elimination should proceed more like a E1cb process than a E2 process. The antibody catalyzed, as well as the uncatalyzed reaction, yielded the expected elimination product, the vinylphenylsulphone, while no alcohol formation could be observed (Figure 43). As expected, the carbamate group was perfectly stable under the assay conditions. Also, the vinylphenylsulphone did not react with antibody or buffer molecules ruling out the possibility that the vinylphenylsulfone could react as a mechanism based inhibitor.

Figure 43
The rather high $K_m$ values of both antibodies suggest that the phosphinate groups in \textbf{R1} and \textbf{R2} may not be a great mimic for the sulphone group. However, an attempt was made to synthesize a hapten \textbf{R3} which features an $\alpha$-amino sulphone instead of an $\alpha$-amino phosphinate (Figure 44). Unfortunately, it turned out that this molecule is not stable and the design was not pursued further.\textsuperscript{85}

Figure 44

![Hapten R3](image)

Similarly, the fact that absolutely no product inhibition was observed vindicated the design of the protection group, which upon activation, fragments into three different molecules. The fragmentation products bear little resemblance to the hapten and can therefore easily diffuse out of the antibody binding pocket. This should be an important feature of future protecting group designs.

The $pK_a$ of phenylmethylsulphones is approximately 27. Considering the activating effect of the $p$-chloro substituent in substrate \textbf{S2}, it is fair to assume that the $pK_a$ of the $\alpha$-proton in \textbf{S2} is around 24. This compares with a $pK_a$ of less than 19 for the $\alpha$-keto proton of a substrate \textbf{111}. Antibody 43D4-3D12, generated to hapten \textbf{112}, was previously shown to affect the elimination of HF using compound \textbf{111} as a substrate (Figure 45).\textsuperscript{65c} The $k_{cat}$ of this antibody is only about tenfold higher than the $k_{cat}$ accomplished by antibody FD2 with substrate \textbf{S2}. Considering the $pK_a$-difference of five orders of magnitude, it seems very surprising that antibody FD2 and BH4 could accomplish the catalysis of such a difficult transformation with quite a remarkable rate.

Figure 45

![111 and 112](image)
6.5. Outlook

6.5.1. Improvement of antibody performance

While the best catalytic antibody that was isolated (FD2) missed the target rate of 1 min⁻¹ by two orders of magnitude, there are many approaches to improve its performance. Joint hybridoma and combinatorial antibody library approaches have shown that these methods could boost the performance of antibodies by two orders of magnitude using the same hapten. Using this method, one would immunize mice with R₁ or R₂, extract the spleen and isolate the antibody cDNA. This cDNA could then be utilized to assemble a hapten biased combinatorial heavy- and light-chain phagemid library. However, instead of basing the selection process on mere binding, one could conceive a selection process that is based on actual catalysis.

It has been shown that gene III protein (gIIIp) is critical for phage infectivity. Phages that are not expressed together with gIIIp as a fusion protein are not infective. A general strategy for selecting antibody catalysts could then rely on linking the infectivity of gIIIp with the catalytic event. This can be accomplished by using a bifunctional reagent, in which one end selects for chemistry at the antibody combining site and the other couples to an infection-promoting N1-N2 construct of gIIIp (Figure 46).

![Figure 46](image)

Applying this concept to the prodrug activation system of chapter 6, one could couple substrate C₁ (Figure 47) to the maleimide activated N-terminal domain of phage gIIIp. Phages that could activate the substrate would get covalently trapped and thus automatically linked to the infectivity-conferring gIIIp protein (refer to chapter 7 for a detailed discussion of the covalent trapping mechanism of substrate C₁).
Hence, only catalytic phages could restore infectivity and undergo amplification. By making the reaction conditions increasingly more stringent (i.e. lower pH, shorter reaction times) several rounds of panning could lead to the isolation of more efficient antibody phages.

### 6.5.2. Generation of bispecific antibodies

Once an efficient catalytic antibody is isolated, the next step would be the generation of bispecific antibody fragments. A number of bispecific antibody conjugates with varying sizes have been reported in literature (Figure 48):\(^{89}\) they include (i) a dimer of two antibodies, (ii) bispecific F(ab')\(_2\) fragments, (iii) minibodies and (iv) diabodies. Each of the four types of bispecific catalytic antibodies have potential advantages and disadvantages. The antibody dimer's large size may help reduce its rate of clearance, thus prolonging its half life in serum. Thus, if tumor penetration is an essential requirement for treatment, this antibody conjugate will perform very poorly. In comparison, all the other fragments are much smaller in size and therefore display improved tumor penetration, however, at the expense of an increased system clearance rate.
Also, bispecific F(ab')₂ fragments, diabodies, and minibodies may not be the best choices for in vivo experiments since these antibody constructs can provide only one valency for target binding. It is very possible that by sacrificing the bivalency of a tumor recognizing antibody, both affinity and specificity of the antibody for the given antigen may decrease. Therefore it might be advantageous to covalently link two small antibody fragments to accomplish both improved tumor penetration, and bivalent target binding. A further advantage of using the covalent linkage is the additional translational and rotational freedom of the catalytic moiety. Further studies will be required to determine the pharmacokinetically optimal protein size.

6.5.3. In vitro experiments

With a bispecific antibody in hand, easy in vitro experiments would verify its efficacy. For this model study, one could use LS174T and T380 colon carcinoma cell lines which are known to overexpress the human tumor-associated antigens CEA (carcinoembryonic antigen), TAG-72 (tumor-associated-glycoprotein-72) and KS1/4 (membrane glycoprotein). The corresponding antibodies directed against these proteins are CEM231, CC-49, and 007B, respectively. As the model prodrug system, doxorubicin protected with P1 (112) and etoposide protected with P6 (113) should be ideal candidates for prodrug design (Figure 49). Both drugs are currently in clinical use but exhibit dose limiting toxicity which makes them obvious candidates for prodrug design. There is literature precedent for their deactivation upon protection of the functionalities indicated and both molecules are easy to derivatize.

Figure 49

![Figure 49](image_url)
Chapter 7
"Capture" CatELISA

7.1. Introduction

Catalytic antibodies are usually selected by binding to the hapten or transition state analog (TSA). One fusion can yield up to several hundred clones which bind the TSA with a broad range of different affinities. Usually the best binders are selected, produced in large quantities, and then screened for catalysis. However, this method is very inefficient as the percentage of catalysts among hapten binding clones is usually only between 1% and 10%. Especially, if large antibody or phage libraries are to be screened, this method is very labor intensive and inefficient.

Recent studies have also shown that high affinity for the transition state does not necessarily translate into good catalysis. One reason may be our limited ability to mimic the transition state with a stable molecule. Another reason might be that the antibody may recognize certain structural features common to both, hapten and substrate, a selective destabilization of the substrate will then be unlikely. Therefore, the conventional selection strategy based on binding may lead to the isolation of good binders that are not necessarily good catalysts, while at the same time discarding antibodies that might have serendipitously developed catalytic activity not programmed by the structure of the hapten.

A solution to these problems is the direct screening for catalysis rather than binding of hybridoma supernatants. The most common methods of assaying catalytic activity rely on the release of a chromophore as the reaction proceeds. However, these efforts were hampered by several factors such as high background reaction in culture medium, generally low efficiency of antibodies, and contaminating enzymes which catalyze the same reaction. A potentially more successful approach, dubbed CatELISA, was reported by Tawfik et al. in which product is detected by specific antibodies as in a regular enzyme-linked immunosorbent assay (ELISA). However, product specific antibodies are either not always accessible or are time consuming to generate. Aside from this, the product specific antibody has to be able to discriminate between product and substrate. This is a difficulty which often requires careful screening of particular useful clones and often prohibits the use of polyclonal antibodies.

We therefore developed a more general approach called "Capture CatELISA" which circumvents the shortcomings of the traditional CatELISA approach by eliminating the need for a product specific antibody. In this assay the catalytic antibody gets covalently trapped by a reactive substrate and becomes immobilized. These antibodies are then detected by traditional ELISA-methods.
7.2. A brief review of CatELISA

In 1993, Tawfik et al. reported the development of an immunoassay method for screening large numbers of potential antibody catalysts by modifying the conventional enzyme-linked immunosorbent assay (ELISA) approach (Figure 50).

In their method, the potential substrate for the reaction is immobilized in the wells of a microtiter plate which is then exposed to the antibody library. This results in the cleavage of the substrate in the wells containing catalytically active antibodies. The supernatant is then removed from the well and an enzyme-labeled polyclonal antibody that specifically binds the product is then added. Using this method, Tawfik et al. were able to screen 1570 clones for esterolytic cleavage of a p-nitrobenzylester 114 using hapten 115 for immunization (Figure 51). They isolated a very efficient esterolytic antibody with $k_{cat} = 7.35 \text{ min}^{-1}$ and $K_m = 0.28 \text{ mM}$, which corresponds to a rate acceleration of $2.6 \times 10^5$ over the background reaction.

MacBeath and Hilvert applied this method to a reaction involving bond formation rather than cleavage, in this case a Diels-alder reaction (Figure 52). Thus, mice were immunized with
Hapten 121 and hybridoma supernatants were screened for their ability to catalyze the Diels Alder reaction between tetrachlorothiophen dioxide 117 and maleimide 118. The initial product of the reaction 119 is spontaneously air-oxidized to tetrachlorophtalimide 120. However, Hilvert et al. unsuccessfully used polyclonal antibodies for the detection of the reaction product 120. Only after they used monoclonal detection antibodies, did they successfully identify several catalytic clones.

Figure 52

Hilvert studied this method further using his reaction system and found that the success of CatELISA strongly depended on the quality of the detection antibody. The detection antibody must be able to efficiently discriminate between the substrate and product structures, which is especially difficult in cases where substrate and product share strongly antigenic elements. In those instances, polyclonal antibodies would not work for CatELISA assays. Furthermore, he found that in order to produce a highly sensitive system, care must be taken with both the concentration of detection antibody relative to antigen, and the relative dissociation constants for the binding of the detection antibody to the substrate and products. If the system is carefully optimized Hilvert estimates that the assay can detect catalyst concentrations as low as 10 nM.

Benedetti et al. found that in some cases the highly hydrophobic environment surrounding the immobilized substrates interferes with the reaction to be monitored. They reported the development of a very similar assay based on a competitive ELISA assay. In this assay, the detection antibody is immobilized on microtiterplates via a secondary antibody. The reaction is then carried out in solution rather on the solid phase. The reaction solution is incubated in the wells together with an enzyme conjugate of the product. Upon catalysis, the free product from the catalyzed reaction competes with the product-enzyme conjugate for binding to the immobilized anti-product antibody, resulting in a decrease of the absorbance signal obtained when a chromogenic enzyme substrate is added.
7.3. The new screening method

As outlined in the introduction (chapter 7.1.), the traditional CatELISA approach suffers from the difficulty of obtaining a product specific antibody. We therefore developed an improved method, dubbed "Capture"-CatELISA. In this method, the need for a product specific antibody is eliminated, which greatly broadens the scope and generality of the traditional CatELISA approach.

The reasoning was that if the catalytic event could lead to covalent binding, and therefore immobilization of the catalytic antibody onto the solid support, the need for a product specific antibody would be eliminated. We therefore designed a reaction that, if accomplished, leads to a covalent interaction between antibody and the insoluble matrix.

In literature, there is a great wealth of data on mechanism based inhibitors, which upon incubation with an enzyme covalently modify it, based on the enzyme's catalytic mechanism. A report by Halazy et al. which showed that both ortho- and para-(difluoromethyl)aryl-β-D-glucosides (122 and 123) are mechanism based inhibitors of β-glucosidases (Figure 53). Upon enzymatic cleavage, the difluoromethylphenol moiety 124 generates a reactive chalcone methide species, 125, at or near the active site, thereby alkylating any nucleophile (126).

Figure 53

Based on this mechanism, Janda et al. developed a chemical selection process for phage libraries where antibody particles with galactosidase activity were trapped by a substrate immobilized onto a solid support by a disulfide bond. The trapped phage particle was released by disulfide bond cleavage, allowing for the recovery of the abzymes gene and its subsequent antibody expression. The best antibody identified by this screening method catalyzed the
hydrolysis of p-nitrophenyl-β-galactopyranoside with a rate enhancement of $7 \times 10^4$ fold over the background reaction.

On this basis, we designed the new system, "Capture"-CatELISA, in which a catalytic antibody is covalently trapped by a modified "mechanism based inhibitor" substrate. Substrate C1 (Figure 54) consists of a head-group containing the protective group recognition unit (P4, R = Me), a latent quinone methide species and a linker group which allows the coupling of this substrate to maleimide activated BSA.

![Figure 54](image)

We chose the most stable protection group (R = Me) as the head-group for two reasons. First, we wanted to rule out unspecific cleavage and deactivation of the screening substrate during the coupling process to BSA or during incubation with cell culture supernatants. Second, we wanted to make the selection process as stringent as possible, in the hope that antibodies capable of hydrolyzing C1 may show even greater rate enhancement with a more activated substrate (i.e. C2).

The "Capture"-CatELISA consisted of a two step selection mechanism. First, hybridoma supernatants were screened for hapten binding using a conventional ELISA assay. This conventional ELISA step was necessary as we wanted to rule out surface catalysis by antibody clones unspecific for the hapten. This has been shown to be a not so uncommon phenomena and elimination reactions seem sensitive to surface catalysis in particular. The positive clones were taken into the second step, where C1-BSA coated microtiter plates were incubated with the cell culture supernatants from the previous screen. Nonbinders and antibodies which do not bind covalently are then removed by washing with a neutral and an acidic buffer, respectively. Upon activation by a catalytic antibody, the difluoromethylaniline moiety generated a highly reactive chinone methide intermediate close to the antibody combining site. The chinone methide could then react with nucleophilic side chain residues, thus immobilizing the antibody to the microtiter plate. A conventional ELISA assay was then used to detect the catalytic antibody. Figure 55 illustrates this process. Using this method, we were able to rapidly screen 130 hapten binding clones, (67 clones
binding R1 and 63 clones binding R2) allowing us to identify nine catalytic antibodies with different kinetic constants (see previous chapter).

Figure 55

![Chemical diagram showing the catalytic antibody reaction process.]

7.4. Results

7.4.1. Synthesis of "Capture CatELISA" substrate C1

The synthesis of "Capture CatELISA" substrate C1 was efficiently carried out in twelve steps as shown in Figure 56. Thus, 3-nitro-4-methylbenzoic acid 127 was reacted with dimethylformamide dimethylacetal (DMFDMA) in refluxing DMF to yield enamine 128. DMFDMA not only formed the enamine but also methylated the carboxyl group in 127. Interestingly, the benzylester of 127 was subjected to the same reaction conditions and again, 128 was isolated as the main product. Apparently, a benzylester is not stable under these reaction conditions. Without workup, 128 was treated with sodium periodate to yield aldehyde 129.
which was then protected with 1,2-ethanedithiol under Lewis acid catalysis to afford dithiane 130 in good yield.\textsuperscript{105} Since the removal of methyl esters requires alkaline conditions (conditions under which the carbamate in C1 might not survive), it was decided to replace the methylester with a benzylester which can later be removed by hydrogenolysis under neutral conditions. Thus, 130 was hydrolyzed with lithium hydroxide to yield the free acid, which was then condensed with benzylalcohol to yield benzylester 131.

Figure 56

\[
\begin{align*}
&\text{127} \xrightarrow{\text{DMFDMA, DMF, } \Delta T} \text{128} \xrightarrow{\text{NaO}_4} \text{129} \\
&\text{127} \xrightarrow{\text{BF}_3 \cdot 2\text{AcOH, Ethanedithiol}} \text{130} \xrightarrow{1. \text{LiOH}} \text{130} \xrightarrow{2. \text{BnOH, DCC}} \text{131} \\
&\text{130} \xrightarrow{\text{TiCl}_3} \text{132} \\
&\text{133} \xrightarrow{1. \text{Diphosene}} \text{134} \xrightarrow{\text{HF/pyridine, DBH}} \\
&\text{135a: } R = \text{CHF}_2^+ \xrightarrow{\text{DAST}} \\
&\text{135b: } R = \text{CHO}
\end{align*}
\]
The reduction of the nitrogroup to aniline 132 was accomplished by using titanium(III)chloride which proceeded without interference from the dithiane group.\textsuperscript{106}

Commercial 2-(toluenesulphonyl)ethanol 133 was treated with diphosgene to form its chloroformate and then reacted with aniline 132 to afford the carbamate 134.\textsuperscript{107} The dithiane group in 134 was then converted to the gem-difluorogroup by exposing 134 to 1,3-dibromo-5,5-dimethylhydantoin (DBH) and pyridinium poly(hydrogen fluoride) in methylenechloride.\textsuperscript{108} This reaction yielded the gem-difluorocompound 135a in 36\% yield, while 43\% was recovered as the aldehyde 135b. The aldehyde 135b can easily be converted into 135a upon treatment with N,N-diethylaminosulfur trifluoride (DAST).\textsuperscript{109} Hydrogenolysis of the benzylester in 135a yielded the free acid which was then condensed with 2-pyridylthioethyl amine\textsuperscript{110} to afford compound 136. After 136 was treated with DTT and catalytic amounts of triethylamine, C1 was isolated in excellent yield.

7.4.2. Assay results

Substrate C1 was coupled to maleimide activated BSA (PIERCE) via its thiol residue. Successful conjugation was confirmed by UV-spectrophotometry. Eight hi-affinity protein binding plates (ELISA plate) were coated with C1-BSA and then blocked using a concentrated solution of BSA in PBS (2 mg/ml, pH 7.2). Hybridoma supernatants, which previously showed affinity for their respective hapten (by conventional ELISA-assay), were added to the ELISA plates (in quadruplicates) and the plates were incubated for either 1, 2, 8 or 48 hours in a cell culture incubator at 37 °C. After the incubation period, one set of four plates was washed with a neutral buffer only, while another set of four plates was additionally washed with an acidic glycine buffer (0.1 M, pH 2.7). The wells were treated with an anti-mouse IgG-HRP conjugate and then developed with \(\alpha\)-phenylenediamine and hydrogenperoxide as the substrates.

As described above, 67 hapten R1 binding clones and 63 hapten R2 binding clones were screened. After only two hours of incubation, 25 clones developed a strong signal. The signal intensified only marginally when plates were incubated with hybridoma supernatants for longer periods of time. Of these 25 clones, nine clones showed a positive signal on the plates which underwent the acidic washing protocol. These nine clones were grown on large scale and purified from ascites fluid. At each step of the purification, the antibodies were tested using the "Capture"-
Cat-ELISA assay to insure that no activity had been lost. All of the nine clones turned out to be catalysts and their further characterization has been described in the previous chapter.

The results show that in this particular system, a incubation period of two hours was sufficient to observe a strong positive signal. The fact that longer incubation periods only marginally intensified the signal shows that the signal is limited by the C1 substrate concentration on the BSA-C1 conjugate. However, the "Capture"-CatELISA will not yield any quantitative results since the concentrations of antibody varies greatly from clone to clone. A less efficient antibody may yield a strong signal if present at a high concentrations, while a highly active antibody may yield a weaker signal if present at very low concentrations. Unfortunately, an exact estimation of total antibody concentration is notoriously difficult since the heterogeneity of the newly generated antibodies can yield inaccurate results.

At one point, hybridoma supernatants were taken from clones whose medium was replaced 24h before the assay. In this experiment, some clones showed a much weaker signal, which indicated that the antibody concentration is an important factor in this assay. It is therefore recommendable to only take hybridoma supernatants from clones whose supernatants were not replaced for 72h. However, waiting even longer may have the opposite effect, since increasing cell death due to starvation may release unspecific proteases which could destroy the antibody.

The successful outcome of this new methodology should prove valuable for the screening of large antibody and phage libraries. The flexibility of this assay allows the researcher to screen their library against many different substrates, provided that a synthetic scheme can be developed. One can envision that the repeated screening and amplification of a library with increasingly difficult (i.e. kinetically stable) substrates and reduced reaction times could yield a new methodology for the evolution of catalysts in a very brief period of time.
8.1. Syntheses of haptens and substrates

8.1.1 General methods

All reactions were carried out in oven-dried glassware under an argon atmosphere unless otherwise noted. Aqueous reactions were run under ambient atmosphere. Column chromatography was performed using 230-400 mesh silica gel (Merck). Proton (1H NMR) magnetic resonance spectra were recorded at 300 MHz on a Varian XL-300 instrument. The chemical shifts of 1H NMR spectra were referenced to CDCl$_3$ (7.24 ppm), DMSO (2.49 ppm), CD$_2$Cl$_2$ (5.32 ppm), or CD$_3$OD (3.30 ppm). 1H NMR peak multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintuplet), m (multiplet), br (broad). Coupling constants (J) are reported in Hz. Proton-decoupled-carbon (13C-NMR), phosphorus (31P-NMR), and fluorine (19F-NMR) magnetic resonance spectra were recorded at 75.44 MHz, 121.44 MHz, and 290 MHz respectively on a Varian XL-300 instrument. The chemical shifts of 13C NMR spectra were referenced to CDCl$_3$ (77.0 ppm), DMSO (39.5 ppm), CD$_2$Cl$_2$ (53.8 ppm), or CD$_3$OD (49.0 ppm). 31P-NMR and 19F-NMR spectra were referenced to 85% H$_3$PO$_4$ and CHF$_3$ respectively as an external standard. Infrared (IR) spectra were recorded on a Perkin-Elmer 283B spectrometer. High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT 8200 spectrometer. Reagents were used as supplied by the vendors, except in moisture sensitive reactions where reagents were dried: CH$_2$Cl$_2$ (over CaH$_2$), THF (over Na), Pyridine (over CaH$_2$) and toluene (over Na).

8.1.2. Synthesis of hapten O2

\begin{center}
\begin{align*}
\text{EtO} & \text{P} & \text{CO}_2\text{Et} \\
\text{EtO} & \text{O} & \\
\end{align*}
\end{center}

1. NaH, $\rho$-NO$_2$-BnBr  \\
2. CH$_2$O aq., K$_2$CO$_3$

\begin{center}
\begin{align*}
\text{CO}_2\text{Et} & \\
\end{align*}
\end{center}

A solution of triethyl phosphono-acetate 34 (900 µL, 4.5 mmol) in THF (5 ml) was added dropwise to a suspension of sodium hydride (200 mg, 5 mmol, 80%) in THF (5 ml) at 0 °C. The resulting mixture was allowed to stir at rt for 15 min (potassium salt precipitated as white solid)
followed by the addition of a solution of 4-nitrobenzyl bromide (970 mg, 4.5 mmol) in THF (5 ml). The resulting solution was stirred for 1 h at rt (the precipitate was completely dissolved). The mixture obtained was partitioned between hydrochloric acid (10 ml, 5% aq.) and methylenechloride (10 ml). The combined organic layers were dried, filtered over MgSO₄, and evaporated. Without further purification, the alkylated phosphono acetate was suspended in formaldehyde solution (1.8 ml, 18 mmol, 35% aq.) and a solution of potassium carbonate (1.4 g, 10 mmol) in H₂O (2 ml) was added dropwise at rt. The reaction mixture was stirred at 80 °C for 7 h. After cooling, the reaction mixture was partitioned between ammonium chloride (sat., 5 ml) and diethyl ether (6 ml). The combined organic layers were washed with brine, dried over MgSO₄ and concentrated. Purification by flash chromatography (gradient of 50% to 90% EtOAc in hexane) afforded 443 mg (42%) of 35.

\[ ^1H \text{ NMR (CDCl}_3\): } \delta 8.13 (d, J = 7.8 \text{ Hz}, 2H), 7.35 (d, J = 7.8 \text{ Hz}, 2H), 6.29 (s, 1H), 5.55 (s, 1H), 4.15 (q, J = 6.8 \text{ Hz}, 2H), 3.71 (s, 1H), 1.23 (t, J = 7.7 \text{ Hz}, 3H). \]

\[ ^13C \text{ NMR (CDCl}_3\): } \delta 166.4, 147.0, 139.2, 129.9, 127.2, 123.8, 61.1, 38.3, 14.3. \]

[Diagram of chemical structures]

DIBAL-H (8.5 ml, 8.5 mmol, 1.0 M in hexane) was added dropwise to a cooled (-78 °C) solution of acrylester 35 (940 mg, 4 mmol) in THF (35 ml). After 1h, the solution was allowed to warm up to rt. The reaction mixture was partitioned between hydrochloric acid (30 ml, 10% aq.) and diethylether (40 ml). The combined organic layers were washed with sodium bicarbonate (sat.), brine, dried over MgSO₄, filtered and concentrated. The residue was filtered through a plug of silica gel (hexane - EtOAc 3/1) to yield 760 mg (90%) of the allylic alcohol 36.

\[ ^1H \text{ NMR (CDCl}_3\): } \delta 8.13 (d, J = 8.4 \text{ Hz}, 2H), 7.35 (d, J = 8.4 \text{ Hz}, 2H), 5.17 (s, 1H), 4.87 (s, 1H), 4.02 (s, 2H), 3.49 (s, 2H), 1.68 (s, 1H). \]

\[ ^13C \text{ NMR (CDCl}_3\): } \delta 147.2, 147.0, 130.0, 123 8, 113.1, 65.4, 39.6. \]
Triethylamine (2.78 ml, 20 mmol), chlorotriethylsilane (20 ml, 20 mmol, 1.0 M in THF) and DMAP (15 mg, 0.12 mmol) were added to a stirred solution of alcohol 36 (4.22 g, 20 mmol) in THF (150 ml) at r.t. After 3h the solution was filtered and evaporated. The residue was purified by flash chromatography (hexane - EtOAc 4/1) to give 6.5 g (100%) of 37.

$^1$H NMR (CDCl$_3$): δ 8.13 (d, $J = 8.7$ Hz, 2H), 7.34 (d, $J = 8.7$ Hz, 2H), 5.17 (s, 1H), 4.81 (s, 1H), 4.02 (s, 2H), 3.45 (s, 2H), 0.91 (t, $J = 8.1$ Hz, 9H), 0.57 (q, $J = 8.1$ Hz, 6H).

$^{13}$C NMR (CDCl$_3$): δ 147.4, 146.7, 129.8, 123.6, 112.8, 65.1, 39.4, 6.8, 4.5.

To a solution of silyl ether 37 (6.5 g, 20 mmol) in methylene chloride (200 ml) was added $m$-CPBA (9.65 g, 40 mmol, 80%). The solution was refluxed for 3h and then stirred at r.t. for another 3h. The solution was washed with sodium bicarbonate (sat.), brine, dried over MgSO$_4$, filtered and concentrated. The residue was purified by flash chromatography (gradient of 5% to 20% EtOAc - hexane) to yield 6.5 g (95%) of 38.

$^1$H NMR (CDCl$_3$): δ 8.14 (d, $J = 8.5$ Hz, 2H), 7.40 (d, $J = 8.5$ Hz, 2H), 3.54 (s, 2H), 3.09 (s, 2H), 2.69 (d, $J = 4.6$ Hz, 1H), 2.53 (d, $J = 4.6$ Hz, 1H), 0.93 (t, $J = 8.0$ Hz, 9H), 0.57 (q, $J = 8.0$ Hz, 6H).

$^{13}$C NMR (CDCl$_3$): δ 147.3, 144.7, 130.9, 123.6, 65.1, 59.6, 50.4, 37.7, 6.9, 4.6.
Pd/C (cat. 10%) was added to a solution of 38 (4.5 g, 13.25 mmol) in deoxygenated ethyl acetate (200 ml). The resulting solution was stirred under hydrogen (1 atm) at r.t. After the hydrogen consumption stopped (4 h) the solution was filtered through celite and evaporated. The residue was immediately dissolved in methylenechloride (135 ml), cooled at 0°C and triethylamine (1.86 ml, 13.25 mmol) and tritylchloride (3.72 g, 13.25 mmol) were added. After 1h, the solution was washed with sodium bicarbonate (sat.), brine, dried over MgSO₄, filtered and concentrated. The residue was purified by flash chromatography (gradient of 5% to 20% EtOAc in hexane) to afford 6.9 g (96%) of 39.

¹H NMR (CD₂Cl₂): δ 7.67-7.45 (m, 15 H), 7.14 (d, J = 8.1 Hz, 2H), 6.58 (d, J = 8.0 Hz, 2H), 3.82 (d, J = 11.7 Hz, 1H), 3.23 (d, J = 11.9 Hz, 1H), 3.09 (d, J = 13.8 Hz, 1H), 2.88 (d, J = 13.9 Hz, 1H), 2.87 (d, J = 5.0 Hz, 1H), 2.73 (d, J = 4.8 Hz, 1H), 1.19 (t, J = 8.4 Hz, 9H), 0.85 (q, J = 8.4 Hz, 6H). ¹³C NMR (CD₂Cl₂): δ 146.1, 145.5, 130.0, 129.5, 128.5, 127.1, 126.1, 116.5, 72.0, 60.6, 50.3, 37.3, 7.2, 4.9.

To a solution of phenyllithium (21.5 ml, 38.6 mmol, 1.8 M in cyclohexane-ether) in diethyl ether (100 ml) was added dropwise (20 min) a solution of 2-picoline (3.81 ml, 38.6 mmol) in diethyl ether (40 ml) The solution turned dark red. After 1 h of stirring at r.t. the solution was cooled to 0°C and a solution of epoxide 39 (6.9 g, 12.85 mmol) in diethyl ether (40 ml) was added over a period of 30 min (with a syringe pump). After 3h, the reaction mixture was partitioned between ammonium chloride buffer (pH 8, sat., 150 ml) and diethyl ether (200 ml). The combined organic layers were dried over potassium carbonate, filtered and concentrated. The residue was
purified by flash chromatography (gradient of 30% to 50% EtOAc-hexane) to afford 5.64 g (69%) of 40.

$^1$H NMR (CD$_2$Cl$_2$): δ 8.44 (d, J = 4.2 Hz, 1H), 7.58 (dt, J = 7.8 Hz, 1H), 7.18-7.39 (m, 15H), 7.09-7.13 (m, 2H), 6.80 (d, J = 8.6 Hz, 2H), 6.30 (d, J = 8.5 Hz, 2H), 3.34 (d, J = 9.6 Hz, 1H), 3.28 (d, J = 9.6 Hz, 1H), 2.87 (m, 2H), 2.64 (d, J = 13.2 Hz, 1H), 2.56 (d, J = 13.4 Hz, 1H), 1.82 (m, 2H), 0.94 (t, J = 7.8 Hz, 9H), 0.59 (q, J = 7.9 Hz, 6H).

$^{13}$C NMR (CD$_2$Cl$_2$): δ 161.1, 149.0, 146.1, 145.4, 137.2, 130.6, 129.7, 128.4, 127.1, 126.8, 123.7, 121.6, 116.7, 72.0, 67.3, 43.0, 35.6, 32.4, 7.0, 4.6.

TBAF (2.22 ml, 2.22 mmol, 1.0 M in THF) was added at 0 °C to a solution of 40 (1.4 g, 2.22 mmol) in THF (20 ml). After 10 min, the solvent was evaporated and the residue was purified by flash chromatography (gradient of 0% to 10% acetone - EtOAc) to yield 1.03 g (90%) of 41.

$^1$H NMR (CD$_2$Cl$_2$): δ 8.43 (d, J = 4.8 Hz, 1H), 7.62 (dt, J = 7.7 Hz, 1H), 7.13-7.40 (m, 17H), 6.78 (d, J = 8.1 Hz, 2H), 6.32 (d, J = 8.7 Hz, 2H), 3.32 (d, J = 9.5 Hz, 1H), 3.28 (d, J = 9.5 Hz, 1H), 2.92 (m, 2H), 2.60 (s, 2H), 1.82 (m, 2H).

$^{13}$C NMR (CD$_2$Cl$_2$): δ 161.4, 149.1, 146.3, 145.5, 137.4, 130.9, 129.8, 128.5, 127.4, 126.9, 123.8, 121.8, 116.8, 72.2, 67.7, 43.1, 35.4, 32.2.
Triethylamine (279 μL, 2.0 mmol) and methanesulfonyl chloride (155 μL, 2.0 mmol) were added to a precooled (0°C) solution of diol 41 in THF (20 ml). After 2h, the solution was filtered, evaporated and the residue was purified by flash chromatography (5% MeOH in methylenechloride). The resulting mesylate turned out to be not very stable and was therefore immediately submitted to the next step. The crude product was dissolved in acetone (100 ml) and the solution was refluxed overnight. The suspension obtained was cooled to 0 °C, the product was filtered off and dried under vacuum to yield 820 mg (70%) of 42.

\(^1\)H NMR (CD\(_3\)OD): \(\delta\) 8.53 (d, \(J = 6.3\) Hz, 1H), 8.28 (t, \(J = 4.4\) Hz, 1H), 7.83 (d, \(J = 8.4\) Hz, 1H), 7.75 (t, \(J = 6.8\) Hz, 1H), 7.08-7.32 (m, 15H), 6.74 (d, \(J = 8.8\) Hz, 2H), 6.37 (d, \(J = 8.3\) Hz, 2H), 4.40 (d, \(J = 13.5\) Hz, 1H), 4.27 (d, \(J = 13.5\) Hz, 1H), 3.30 (m, 2H), 2.72 (s, 2H), 2.62 (s, 3H), 1.96 (m, 1H), 1.82 (m, 1H).

\(^1\)C NMR (CD\(_3\)OD): \(\delta\) 158.0, 147.2, 146.5, 145.8, 132.5, 131.4, 130.6, 130.0, 128.9, 128.2, 127.8, 126.3, 117.8, 72.7, 70.4, 65.6, 46.6, 39.7, 27.4.

\[\text{42} \xrightarrow{\text{H}_2\text{O} / \text{EtOAc, reflux}} \text{43}\]

A suspension of 42 (670 mg, 1.13 mmol) in water/ethylacetate (2/1, 35 ml), was vigorously stirred and refluxed until both phases cleared up (2h). The aqueous phase was washed with ethyl acetate (20 ml) and the water was azeotropically removed (coevaporation with 1:1 benzene - acetonitrile) to yield 396 mg (100%) of 43.

\(^1\)H NMR (CD\(_3\)OD): \(\delta\) 8.54 (d, \(J = 5.7\) Hz, 1H), 8.29 (t, \(J = 6.9\) Hz, 1H), 7.82 (d, \(J = 7.5\) Hz, 1H), 7.74 (t, \(J = 6.3\) Hz, 1H), 6.99 (d, \(J = 8.4\) Hz, 2H), 6.63 (d, \(J = 8.4\) Hz, 2H), 4.47 (d, \(J = 14.1\) Hz, 1H), 4.33 (d, \(J = 13.2\) Hz, 1H), 3.31 (m, 2H), 2.83 (s, 2H), 2.62 (s, 3H), 2.01 (m, 1H), 1.87 (m, 1H).

\(^1\)C NMR (CD\(_3\)OD): \(\delta\) 157.8, 147.9, 146.3, 145.7, 132.4, 129.4, 126.4, 125.8, 116.6, 70.3, 65.4, 65.3, 46.4, 39.6, 27.3.
To a solution of the aniline quinolizinium salt 43 (396 mg, 1.13 mmol) in acetonitrile (15 ml) was added glutaric anhydride (193 mg, 1.64 mmol) at r.t. After 36h, the solvent was evaporated and the residue redissolved in water. The aqueous solution was washed with ethyl acetate and then azeotropically evaporated (coevaporation with 1:1 benzene - acetonitrile) to yield 460 mg (88%) of hapten O2.

$^1$H NMR (CD$_3$OD): δ 8.54 (d, $J = 6.3$ Hz, 1H), 8.29 (t, $J = 7.7$ Hz, 1H), 7.83 (d, $J = 8.1$ Hz, 1H), 7.73 (t, $J = 6.5$ Hz, 1H), 7.43 (d, $J = 8.4$ Hz, 2H), 7.18 (d, $J = 8.4$ Hz, 2H), 4.48 (d, $J = 14.1$ Hz, 1H), 4.29 (d, $J = 14.1$ Hz, 1H), 3.31 (m, 2H), 2.90 (s, 2H), 2.58 (s, 3H), 2.28 (m, 4H), 2.02 (m, 1H), 1.86 (m, 2H), 1.78 (m, 1H).

$^{13}$C NMR (D$_2$O): δ 180.7, 177.5, 158.5, 147.4, 147.2, 138.5, 134.8, 134.0, 131.2, 127.8, 124.7, 72.1, 67.0, 66.0, 47.2, 41.1, 38.1, 35.7, 31.5, 28.3.

8.1.3. Synthesis of hapten R1

A solution of methyl phosphinate in methyl orthoformate was prepared according to the method of Finch. Anhydrous crystalline phosphinic acid 81, prepared by rotary evaporation of 50% aqueous H$_3$PO$_2$ (1.25 g, 19 mmol), was allowed to react with methyl orthoformate (12.2 ml, 110 mmol) at r.t. for 1h. This methyl phosphinate solution (82) was added via cannula to a solution of 4-iodo-o-xylene (1.05 ml, 7.2 mmol), propylene oxide (504 μl, 7.2 mmol), palladium(II) acetate (82 mg, 0.36 mmol), and triphenylphosphine (378 mg, 1.44 mmol) in acetonitrile (35 ml). The yellow solution was refluxed for 1h (exact!) and the solvent removed under reduced pressure. The brown residue was triturated with ethyl acetate, filtered, and the
solvent removed at reduced pressure. The resulting yellow oil was purified twice by flash chromatography (1% MeOH in Methylenechloride) to yield 807 mg (60.6%) of compound 83 as a slightly yellow oil.

$^1$H-NMR (CDCl$_3$): $\delta$ 2.33 (s, 6H), 3.78 (d, $J = 12.1$ Hz, 3H), 7.28 (m, 1H), 7.52 (m, 2H), 7.53 (d, $J = 563.4$ Hz, 1H).
$^{13}$C-NMR (CDCl$_3$): $\delta$ 19.0, 20.1, 52.4 (d, $J = 7.1$ Hz), 127.9 (d, $J = 13.4$ Hz), 130.4 (d, $J = 14.2$ Hz), 132.1 (d, $J = 12.0$ Hz), 137.6, 142.8.
$^{31}$P-NMR (CDCl$_3$): $\delta$ 32.26.

To a solution of 4-hydroxypiperidine (202 mg, 2 mmol) and diisopropylethylamine (700μl, 4 mmol) in methylenechloride (15 ml) was added 9-fluorenymethyl chloroformate (429 mg, 1.9 mmol) at 0 °C. The solution was stirred for 3h at 0 °C and then washed twice with citric acid (5% solution in water) and brine. The organic phase was dried over MgSO$_4$ and evaporated to yield 526 mg (81%) of compound 85 as a white foam. The product was used without further purification.

$^1$H-NMR (CDCl$_3$): $\delta$ 1.43 (m, 2H), 1.81 (m, 2H), 3.11 (m, 2H), 3.83 (m, 3H), 4.25 (t, $J = 6.9$ Hz, 1H), 4.41 (d $J = 6.9$ Hz, 2H), 7.30 (dd, $J = 7.2$ Hz, 2H), 7.39 (dd, $J = 7.2$ Hz, 2H), 7.57 (d, $J = 7.2$ Hz, 2H), 7.75 (d, $J = 7.2$ Hz, 2H).
$^{13}$C-NMR (CDCl$_3$): $\delta$ 155.3, 144.1, 141.4, 127.8, 127.2, 125.1, 120.1, 67.4, 67.3, 47.4, 41.5, 34.1.
A stream of nitrogen was bubbled through a solution of 3-mercaptopropionic acid 86 (174 µl, 2 mmol) and tritylchloride (558 mg, 2 mmol) in methylenechloride. After 3h, the solution was evaporated and the resulting residue was recrystallized from ethyl acetate to give 582 mg (83%) of 87 as white crystals.

$^1\text{H-NMR (MeOH-}d_4\text{): } \delta 2.22 \text{ (t, } J = 6.9 \text{ Hz, 2H), 2.44 (t, } J = 6.9 \text{ Hz, 2H), 7.18 - 7.44 (m, 15H).}\\
^1\text{C-NMR (MeOH-}d_4\text{): } \delta 25.3, 32.7, 126.0, 127.2, 128.9, 144.1, 173.8.$

![Chemical Reaction]

A solution 4-(diethylphosphono)-1-butene 88 (313mg, 1.63 mmol) in methylenechloride was treated with bromotrimethylsilane (645 µl, 4.89 mmol) at r.t. for 2h. The solution was thoroughly evaporated under reduced pressure and the residue redissolved in methylenechloride (10 ml). To the solution was added oxalyl chloride (427 µl, 4.89 mmol) and DMF (10µl) which was stirred for an additional 30 min until the evolution of gases subsided. The solution was again thoroughly evaporated and the residue (89) was redissolved in benzene (5ml). This solution was added via cannula to a solution of alcohol 85 (526 mg, 1.63 mmol), diisopropylethylamine (1.13 ml, 6.52 mmol) and 1$^H$-tetrazole (cat. amounts) in benzene (10 ml) at 0 °C. After stirring overnight, methanol (5 ml) was added, and after 3h, the reaction mixture was evaporated. Flash chromatography (gradient of 1% to 3% MeOH in methylenechloride) afforded 525 mg (70%) of the mixed diester 90 as an oil (some alcohol could not be separated from the product as it is visible in the NMR-spectrum; however the alcohol was easy to separate from the product in the next step).

$^1\text{H-NMR (CDCl}_3\text{): } \delta 1.64 \text{ (br, 2H), 1.82 (m, 4H), 2.34 (m, 2H), 3.27 (br, 2H), 3.63 (m, 2H), 3.70 (d, } J = 11.1 \text{ Hz, 3H), 4.20 (t, } J = 6.9 \text{ Hz, 1H), 4.41 (d } J = 6.9 \text{ Hz, 2H), 4.59 (m, 1H), 5.05 (dd, } J = 21.3, 9.9 \text{ Hz, 2H), 5.81 (m, 1H), 7.26 (dd, } J = 7.2 \text{ Hz, 2H), 7.37 (dd, } J = 7.2 \text{ Hz, 2H), 7.53 (d, } J = 7.2 \text{ Hz, 2H), 7.73 (d, } J = 7.2 \text{ Hz, 2H).}\\
^1\text{C-NMR (CDCl}_3\text{): } \delta 32.4, 40.7, 47.5, 53.5 \text{ (d, } J = 11.0 \text{ Hz), 67.4, 77.4, 115.1, 120.1, 125.0, 127.2, 127.9, 137.2, 141.5, 144.0, 155.3.}\\
^3\text{P-NMR (CDCl}_3\text{): } \delta 34.09.$
Through a solution of mixed ester 90 (525 mg, 1.14 mmol) in methylenechloride (15 ml) and methanol (1 ml) was bubbled ozone at -78 °C until the solution turned dark blue (10 min). A stream of nitrogen was passed through the solution for 30 min after which triphenylphosphine (450 mg, 1.72 mmol) was added. The solution was slowly warmed up to r.t., evaporated and chromatographically purified (gradient of 1% to 4% MeOH in methylenechloride) to yield 320 mg (61%) of aldehyde 91 as an oil.

$^1$H-NMR (CDCl$_3$): δ 1.64 (br, 2H), 1.82 (m, 2H), 2.05 (m, 2H), 2.76 (m, 2H), 3.27 (br, 2H), 3.63 (m, 2H), 3.70 (d, $J = 11.1$ Hz, 3H), 4.20 (t, $J = 6.9$ Hz, 1H), 4.41 (d, $J = 6.9$ Hz, 2H), 4.59 (m, 1H), 7.26 (dd, $J = 7.2$ Hz, 2H), 7.37 (dd, $J = 7.2$ Hz, 2H), 7.53 (d, $J = 7.2$ Hz, 2H), 7.73 (d, $J = 7.2$ Hz, 2H), 9.76 (s, 1H).

$^{13}$C-NMR (CDCl$_3$): δ 32.5, 37.0, 40.9, 47.5, 52.5 (d, $J = 11.3$ Hz), 67.4, 72.3, 120.1, 125.0, 127.2, 127.8, 141.5, 144.1, 155.2, 198.5.

$^{31}$P-NMR (CDCl$_3$): δ 32.34.

A solution of aldehyde 91 in toluene (6 ml) was cooled to 0 °C and treated with tritylamine (105 mg, 0.41 mmol). The mixture was warmed up to r.t. and anhydrous Na$_2$SO$_4$ (500 mg, flame dried under oil pump vacuum) was added. The mixture was stirred for 2h after which an additional 500 mg of Na$_2$SO$_4$ was added and stirring was continued for another 2h. The mixture was then filtered and concentrated to afford the crude imine. The imine was dissolved in toluene (7 ml) and phosphinate 83 (75 mg, 0.41 mmol) was added. After refluxing the solution for 3h an additional 35 mg (0.2 mmol) of phosphinate 83 was added and refluxing was continued for another 3h. The
solution was evaporated and chromatographed (gradient of 1% to 5% MeOH in methylene chloride) to afford 160 mg (46%) of compound 92 as an oil.

\(^{1}\)H-NMR (CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 1.3 - 1.9 (br, 8H), 2.34 (s, 3H), 2.37 (s, 3H), 3.01 (br, 1H), 3.34 (br, 2H), 3.51 (d, \(J = 10.8\) Hz, 3H), 3.60 (d, \(J = 10.8\) Hz, 3H), 3.5-3.7 (br, 2H), 4.26 (t, \(J = 6.9\) Hz, 1H), 4.40 (d \(J = 6.9\) Hz, 2H), 4.59 (m, 1H), 7.0 - 7.5 (m, 19H), 7.60 (d, \(J = 7.2\) Hz, 2H), 7.78 (d, \(J = 7.2\) Hz, 2H).

\(^{13}\)C-NMR (CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 20.3, 23.1, 32.8, 41.1, 44.5, 47.9, 51.6, 67.6, 71.9, 120.5, 125.5, 127.1, 127.3, 127.5, 128.1, 128.3, 128.4, 129.2, 129.4, 130.2, 141.8, 144.7, 146.8, 155.4.

\(^{31}\)P-NMR (CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 30.19, 43.55.

A solution of compound 92 (125 mg, 0.14 mmol) in DMF (4 ml) was treated with 400 µl of piperidine and stirred for 2h at r.t. The solution was evaporated and the residue was dried at 50 °C under oil pump vacuum for 3h to afford the crude amine. A solution of S-trityl-3-mercaptopropionic acid 87 in DMF (4 ml) was cooled to 0 °C and treated with HOBT (32 mg, 0.225 mmol) and EDC (50 mg, 0.25 mmol). After 3h, the solution was added via cannula to a solution of the crude amine in DMF (1 ml) at 0 °C. The solution was slowly warmed up to r.t. and stirring was continued overnight. The reaction mixture was evaporated and chromatographed (gradient of 1% to 5% MeOH in Methylene chloride) to afford 70 mg (51%) of compound 93 as an oil.

\(^{1}\)H-NMR (CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 1.1 - 1.3 (br, 2H), 1.3 - 1.5 (br, 2H), 1.6 - 1.8 (br, 4H), 2.11 (t, \(J = 7.2\) Hz, 2H), 2.32 (s, 3H), 2.36 (s, 3H), 2.47 (t, \(J = 7.2\) Hz, 2H), 3.00 (br, 1H), 3.26 (br, 2H), 3.51 (d, \(J = 10.8\) Hz, 2H), 3.57 (d, \(J = 10.2\) Hz, 3H), 3.5-3.7 (br, 2H), 4.39 (m, 1H), 7.1 - 7.7 (m, 33H).

\(^{13}\)C-NMR (CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 20.1, 20.4, 27.9, 33.0, 33.6, 38.8, 42.5, 51.8, 67.3, 71.9, 127.2, 128.5, 129.3, 129.4, 130.2, 145.5, 146.9, 169.6.

\(^{31}\)P-NMR (CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 29.28, 42.72.
A solution of protected hapten 93 (36 mg, 37.4 µmol) in methylenechloride (4 ml) was treated with bromotrimethylsilane (29 µl, 220 µmol) for 2h at r.t. The solution was evaporated and the residue redissolved in methylenechloride (4 ml). Triethylsilane (25 µl, 156 µmol) was dissolved in this solution and trifluoroacetic acid (1 ml) was added dropwise (a yellow color, indicating the presence of trityl cations briefly appears). After 1h, the solution was evaporated, the residue dissolved in water (10 ml), and the aqueous solution was extracted with diethyl ether and methylenechloride. After concentration of the aqueous solution under reduced pressure, the residue was dissolved in a little amount of water (1 ml), transferred into another flask, and evaporated to give 16 mg (89%) of hapten R1.

$^1$H-NMR (D$_2$O): δ 1.5 - 1.9 (br, 8H), 2.26 (s, 6H), 2.72 (s, 4H), 3.15 (m, 1H), 3.42 (m, 4H), 4.37 (m, 1H), 7.31 (m, 1H), 7.45 (m, 2H).

$^{13}$C-NMR (D$_2$O): δ 20.2, 20.5, 23.6, 32.7, 33.1, 33.8, 35.8, 37.6, 38.3, 40.1, 43.9, 45.9, 70.9, 130.8, 131.2, 134.0, 138.5, 142.3, 166.3, 173.3.

$^{31}$P-NMR (CD$_2$Cl$_2$): δ 24.42, 24.83.
8.1.4. Synthesis of "Capture"-CatELISA substrate C1

A solution of 3-nitro-4-methylbenzoic acid 127 (905 mg, 5 mmol) and \(N,N\)-dimethylformamide dimethyl acetal (2 ml, 15 mmol) in DMF (5 ml) was refluxed for 6h. The volatiles were removed under oil vacuum pressure and the residue (128) was redissolved in 50% aqueous THF. Sodium periodate (3.23 g, 15 mmol) was added and stirring was continued for 2h at r.t. The reaction mixture was filtered and the insolubles were washed with ethyl acetate. The combined filtrates were washed with water and saturated sodium bicarbonate solution and dried over MgSO₄. After evaporation of the solvents, the residue was chromatographically purified (gradient of 10% to 30% EtOAc in Hexane) to yield 465 mg (44.5%) of the desired aldehyde 129 as a yellow solid and 131 mg (13.4%) of the methyl ester 127.

\(^1\)H-NMR (CDCl₃): \(\delta\) 4.00 (s, 3H), 8.15 (d, \(J = 8.4\) Hz, 1H), 8.39 (dd, \(J = 8.4\) Hz, 2.1 Hz, 1H), 8.58 (d, \(J = 1.8\) Hz, 1H), 10.40 (s, 1H).

\(^13\)C-NMR (CDCl₃): \(\delta\) 53.3, 124.8, 131.1, 134.7, 135.3, 151.7, 164.2, 187.0.

Methyl 3-formyl-4-nitrobenzoate 129 (396 mg, 1.9 mmol) was dissolved in a mixture of 1,2-ethanediethiol (0.41 ml, 4.28 mmol) and boron trifluoride-acetic acid complex (0.33 ml, 2.14 mmol). The suspension was vigorously stirred for 2h at r.t., after which it was diluted with diethyl ether (10 ml). The ethereal solution was extracted with saturated sodium bicarbonate solution and brine and dried over MgSO₄. After removal of the solvent, the residue was chromatographically
purified (gradient of 10% to 30% EtOAc in Hexane) to yield 468 mg (86%) of the dithiane 130 as a yellow solid.

\[ ^1H\text{-NMR (CDCl}_3\]): \delta 3.37 - 3.49 (m, 4H), 3.95 (s, 3H), 6.09 (s, 1H), 7.84 (d, \( J = 8.7 \text{ Hz}, 1H \)), 7.94 (dd, \( J = 8.4 \text{ Hz}, 1.8 \text{ Hz}, 1H \)), 8.66 (d, \( J = 2.1 \text{ Hz}, 1H \)).
\]

\[ ^{13}\text{C-NMR (CDCl}_3\)): \delta 40.3, 50.2, 53.0, 124.6, 129.5, 131.9, 134.2, 137.6, 150.7, 164.9.\]

Methyl ester 130 (550 mg, 1.93 mmol) was dissolved in THF/water (3/1, 20 ml) containing lithium hydroxide (100 mg, 4.4 mmol) and stirred for 1h at r.t. The solution was acidified with 1N HCl to pH 1 and extracted with diethyl ether. The combined organic extracts were washed twice with brine and water, dried over MgSO\(_4\) and evaporated to yield 515 mg (100%) of crude acid. Without further purification the crude acid was suspended in methylenechloride (30 ml), and DCC (600 mg, 2.9 mmol), and benzylalcohol (300 μl, 2.9 mmol) were added at 0 °C. The solution was warmed up to r.t. and stirring was continued for 4h. The reaction mixture was filtered, evaporated, and chromatographically purified (10% EtOAc in Hexane) to afford 457 mg (66%, two steps) of the benzylester 131.

\[ ^1H\text{-NMR (CDCl}_3\]): \delta 3.37 - 3.45 (m, 4H), 5.40 (s, 2H), 6.11 (s, 1H), 7.40 (m, 5H), 7.85 (d, \( J = 8.4 \text{ Hz}, 1H \)), 8.04 (dd, \( J = 8.4 \text{ Hz}, 1.8 \text{ Hz}, 1H \)), 8.74 (d, \( J = 1.8 \text{ Hz}, 1H \)).
\]

\[ ^{13}\text{C-NMR (CDCl}_3\)): \delta 40.6, 50.6, 68.0, 125.0, 128.8, 129.2, 129.3, 129.9, 132.6, 134.7, 135.6, 138.2, 151.3, 164.8.\]
To a solution of nitrocompound 131 (31 mg, 81 µmol) in acetic acid (2 ml) was added an aqueous titan(III)chloride solution (0.6 ml of a 16% solution, 0.6 mmol). After stirring for 15 min at r.t. the reaction mixture was diluted with water (6 ml) and methylenechloride/methanol (9/1, 6 ml). The suspension was basified with saturated sodium bicarbonate solution (ca. 60 ml) and salted out by adding sodium chloride. The mixture was extracted with methylenechloride, and the combined extracts were washed with brine, and dried over MgSO₄. The solvent was evaporated and the residue was chromatographically purified (gradient of 10% to 30% EtOAc in Hexane) to afford 27 mg (94%) of the aniline 132 as a yellowish solid.

$^1$H-NMR (CDCl₃): δ 3.35 (m, 2H), 3.47 (m, 2H), 4.85 (br, 2H), 5.30 (s, 2H), 5.78 (s, 1H), 6.72 (d, $J = 8.4$ Hz, 1H), 7.33 (m, 5H), 7.83 (d, $J = 8.4$ Hz, 1H), 8.07 (s, 1H).

$^{13}$C-NMR (CDCl₃): δ 40.0, 55.4, 66.4, 117.0, 120.1, 120.3, 128.3, 128.7, 131.5, 132.5, 136.6, 148.7, 166.3.

Pyridine (162 µl, 2 mmol) was added to a solution of diphosgene (150 µl, 1.2 mmol) and 2-(p-toluenesulphonyl)ethanol 133 (400 mg, 2 mmol) in THF (2 ml) at 0 °C. The solution was warmed up to r.t. and stirred for an additional 1h. The reaction mixture was diluted with methylenechloride (10 ml) and washed with saturated ammonium chloride solution and brine. The organic phase was dried over MgSO₄ and evaporated to yield 425 mg (81%) of the crude chloroformate. Aniline 132 (121 mg, 370 µmol) was dissolved in THF (5 ml) and cooled to 0 °C. Pyridine (33 µl, 407 µmol) and above crude chloroformate (168 mg, 640 µmol) were added at 0 °C.
°C and stirring was continued for 2h while the solution was allowed to slowly warm up. The reaction mixture was diluted with methylenechloride (20 ml) and washed with saturated ammonium chloride solution and brine. The organic phase was dried over MgSO₄, evaporated, and chromatographically purified (gradient of 30% to 50% EtOAc in Hexane) to yield 137 mg (66%) of the carbamate 134 as an oil.

\[
\text{H-NMR (CDCl₃): } \delta 2.34 (s, 3H), 3.37 (m, 2H), 3.51 (t, J = 5.7 Hz, 2H), 3.53 (m, 2H), 4.49 (t, J = 6 Hz, 2H), 5.33 (s, 2H), 5.74 (s, 1H), 7.31 - 7.43 (m, 5H), 7.38 (d, J = 8.4 Hz, 2H), 7.79 (d, J = 8.4 Hz, 2H), 7.92 (d, J = 8.4 Hz, 1H), 8.00 (d, J = 8.7 Hz, 1H), 8.03 (s, 1H).
\]

\[
\text{C-NMR (CDCl₃): } \delta 21.8, 40.4, 55.4, 55.5, 58.9, 66.9, 121.6, 125.3, 128.2, 128.3, 128.4, 128.5, 128.7, 130.2, 131.2, 132.1, 136.1, 136.4, 141.0, 145.3, 152.2, 165.7.
\]

1,3-Dibromo-5,5-dimethylhydantoin (171 mg, 0.6 mmol) was dissolved in methylenechloride (1.25 ml) and cooled to 0 °C. 100 µl of Pyridinium poly(hydrogen fluoride) was added via polypropylene syringe, followed by the addition via cannula of dithiane 134 (112 mg, 0.2 mmol) dissolved in methylenechloride (0.5 ml). After 30 min, the deep red reaction mixture was diluted with methylenechloride (5 ml), filtered through a column (a 3-ml polypropylene syringe with a cotton plug) of basic alumina, and the column was washed with methylenechloride until no UV-absorbing material was eluding. The solvent was evaporated and the residue was chromatographically purified (gradient of 30% to 50% EtOAc in Hexane) to yield 36 mg (36%) of the gem-difluoro compound 135a and 41 mg (43%) of aldehyde 135b.
Compound **135a**: 

$^1$H-NMR (CDCl$_3$): $\delta$ 2.34 (s, 3H), 3.52 (t, $J = 5.7$ Hz, 2H), 4.55 (t, $J = 6$ Hz, 2H), 5.36 (s, 2H), 6.78 (t, $J = 54$ Hz, 1H), 7.00 (m, 1H), 7.26 - 7.40 (m, 5H), 7.37 (d, $J = 8.4$ Hz, 2H), 7.82 (d, $J = 8.1$ Hz, 2H), 8.03 (s, 1H), 8.16 (s, 1H).

$^{13}$C-NMR (CDCl$_3$): $\delta$ 21.8, 55.7, 59.7, 67.4, 116.3, 121.1, 125.7, 128.5, 128.8, 129.9, 130.5, 133.9, 136.5, 137.1, 138.2, 140.4, 145.9, 152.4, 165.4.

$^{19}$F-NMR (CDCl$_3$): $\delta$ 109.95 (d, $J = 54$ Hz).

Compound **135b**: 

$^1$H-NMR (CDCl$_3$): $\delta$ 2.31 (s, 3H), 3.52 (t, $J = 5.7$ Hz, 2H), 4.51 (t, $J = 6$ Hz, 2H), 5.34 (s, 2H), 7.26 - 7.40 (m, 5H), 7.37 (d, $J = 8.1$Hz, 2H), 7.79 (d, $J = 8.4$ Hz, 2H), 8.21 (d, $J = 8.7$, 1H), 8.35 (s, 1H), 8.38 (d, $J = 8.7$, 1H), 9.89 (s, 1H), 10.47 (s, 1H).

$^{13}$C-NMR (CDCl$_3$): $\delta$ 21.8, 55.4, 59.3, 67.3, 118.2, 120.9, 124.3, 128.3, 128.6, 128.7, 128.9, 130.2, 135.8, 136.5, 137.1, 138.0, 144.4, 152.3, 164.9, 194.6.

The gem-difluoro compound **135a** (41 mg, 82 μmol) was dissolved in ethanol. Catalytic amounts of Pd/C (10%) were added and the reaction vessel was three times evacuated and flushed with hydrogen. The solution was hydrogenated (at atmospheric pressure) for 1h after which it was filtered and concentrated to yield 34 mg (100%) of crude acid. The crude acid was dissolved in DMF and cooled to 0 °C. EDC (41 mg, 212 μmol) and HOBT (38 mg, 212 μmol) were added and stirring was continued for 30 min after which 2-pyridyldithioethylamine hydrochloride (30 mg, 159 μmol) dissolved in DMF (0.5 ml) was added via cannula. Stirring was continued overnight while the solution was allowed to slowly warm up. The reaction mixture was diluted with methylenechloride (10 ml), washed twice with water and brine, dried over MgSO$_4$, evaporated, and chromatographically purified (gradient of 30% to 75% EtOAc in Hexane) to yield 24 mg (50%) of the amide **136** as a film.
\(^1\)H-NMR (CD\(_2\)Cl\(_2\)): \(\delta\) 2.38 (s, 3H), 2.99 (t, \(J = 5.7\) Hz, 2H), 3.51 (t, \(J = 6\) Hz, 2H), 3.71 (dt, \(J = 5.7\) Hz, \(J = 5.6\) Hz, 2H), 4.51 (t, \(J = 6\) Hz, 2H), 5.36 (s, 2H), 6.69 (t, \(J = 54.6\) Hz, 1H), 6.97 (s, 1H), 7.14 (m, 1H), 7.37 (d, \(J = 8.4\) Hz, 2H), 7.50 (d, \(J = 8.1\) Hz) 7.60 (dt, \(J = 5.7\), \(J = 1.8\), 1H), 7.79 (d, \(J = 8.4\) Hz, 2H), 7.95 (s, 1H), 7.96 (d, \(J = 9.3\) Hz, 1H), 8.09 (d, \(J = 8.4\) Hz, 1H), 8.28 (br, 1H), 8.39 (d, \(J = 1.5\) Hz).

\(^{13}\)C-NMR (CD\(_2\)Cl\(_2\)): \(\delta\) 21.9, 37.9, 39.5, 55.8, 59.7, 113.0, 116.2, 119.3, 121.7, 121.9, 122.0, 127.6, 128.6, 130.5, 130.8, 137.2, 137.6, 138.8, 145.9, 150.3, 152.6, 159.6, 165.9.

\(^{19}\)F-NMR (CD\(_2\)Cl\(_2\)): \(\delta\) 112.45 (d, \(J = 55.4\) Hz)

Dithiopyridine 136 (20 mg, 34 \(\mu\)mol) was dissolved in methylenechloride and dithiothreitol (63 mg, 41 \(\mu\)mol) and catalytic amounts of triethylamine were added. The reaction mixture was stirred for 1h at r.t., concentrated and chromatographically purified (1% MeOH in methylenechloride) to yield 16 mg (99%) of C1 as a film.

\(^1\)H-NMR (CD\(_2\)Cl\(_2\)): \(\delta\) 1.48 (t, \(J = 8.6\) Hz, 1H), 2.37 (s, 3H), 2.76 (dt, \(J = 7.2\) Hz, \(J = 7.1\) Hz, 2H), 3.51 (t, \(J = 5.9\) Hz), 3.59 (dt, \(J = 6.4\) Hz, \(J = 6.3\) Hz), 4.50 (t, \(J = 5.8\) Hz, 2H), 6.64 (br, 1H), 6.67 (t, \(J = 54.5\) Hz, 1H), 6.93 (br, 1H), 7.37 (d, \(J = 8\) Hz, 2H), 7.80 (d, \(J = 8.1\) Hz, 2H), 7.85 (s, 1H), 8.07 (d \(J = 8.3\) Hz, 1H).

\(^{13}\)C-NMR (CD\(_2\)Cl\(_2\)): \(\delta\) 21.9, 25.1, 43.4, 55.8, 59.7, 116.2, 121.6, 127.5, 128.0, 128.6, 130.5, 130.7, 130.8, 138.1, 145.9, 150.3, 157.2, 166.0.

\(^{19}\)F-NMR (CD\(_2\)Cl\(_2\)): \(\delta\) 113.15 (d, \(J = 55.2\) Hz)
8.1.5. Synthesis of substrate S1

Pyridine (20.5 µl, 0.25 mmol) was added to a solution of diphosphene (19 µl, 0.15 mmol) and 2-(p-nitrophenyl)ethanol 109 (50 mg, 0.25 mmol) in THF (2 ml) at 0 °C. The solution was warmed up to r.t. and stirred for an additional 1h. The reaction mixture was diluted with methylenechloride (10 ml) and washed with saturated ammonium chloride solution and brine. The organic phase was dried over MgSO₄ and evaporated to yield 425 mg (81%) of the crude chloroformate. Amine 105 (73 mg, 0.25 mmol) was dissolved in THF (4 ml) and cooled to 0 °C. Pyridine (20.5 µl, 0.25 mmol) and above crude chloroformate dissolved in THF (1 ml) were added via cannula. Stirring was continued for 2h while the solution was allowed to slowly warm up. The reaction mixture was diluted with methylenechloride (20 ml) and washed with saturated ammonium chloride solution and brine. The organic phase was dried over MgSO₄, evaporated, and chromatographically purified (gradient of 30% to 50% EtOAc in Hexane) to yield 70 mg (55%) of the carbamate 110 as a yellow solid.

1H-NMR (CDCl₃): 8 2.87 (s, 6H), 3.03 (dt, J = 5.7 Hz, J = 5.6 Hz), 3.20 (m, 4H), 4.18 (t, J = 7 Hz, 2H), 5.27 (t, J = 5.6 Hz, 1H), 5.57 (t, J = 6 Hz, 1H), 7.16 (d, J = 7.5 Hz, 1H), 7.35 (d, J = 8.9 Hz, 2H), 7.52 (m, 2H), 8.09 (d, J = 9 Hz, 2H), 8.23 (m, 2H), 8.53 (d, J = 8.5 Hz, 1H).

13C-NMR (CDCl₃): 8 30.7, 41.0, 43.4, 45.6, 62.8, 115.5, 118.7, 123.4, 124.3, 126.6, 128.7, 129.7, 129.8, 130.1, 130.9, 134.5, 145.4, 146.4, 152.2, 156.6.
Compound **110** (65 mg, 126 μmol) was dissolved in methylenechloride (2 ml), *m*-CPBA (79.4 mg, 85%, 391 μmol) was added, and stirring was continued for 6h. The white suspension was diluted with methylenechloride (5 ml), and 1 ml of a saturated sodium sulfite solution was added (in order to reduce the amine oxide and quench excess *m*-CPBA). The layers were separated and the organic phase was washed with water and brine. The mixture was dried over MgSO₄, concentrated, and chromatographically purified (gradient of 30% to 70% EtOAc in Hexane) to yield 58 mg (91%) of substrate **S1** as a yellow solid.

**1H-NMR** (CDCl₃): δ 2.87 (s, 6H), 2.95 (dt, *J* = 5.7 Hz, *J* = 5.6 Hz), 3.11 (dt, *J* = 5.7 Hz, *J* = 5.6 Hz), 3.49 (t, *J* = 5.4 Hz, 2H), 4.38 (t, *J* = 5.7 Hz, 2H), 5.14 (t, *J* = 5.6 Hz, 1H), 5.45 (t, *J* = 6 Hz, 1H), 7.16 (d, *J* = 7.5 Hz, 1H), 7.52 (m, 2H), 8.08 (d, *J* = 8.4 Hz, 2H), 8.18 (t, *J* = 7.2 Hz, 2H), 8.35 (d, *J* = 8.7 Hz, 2H), 8.52 (d, *J* = 8.4 Hz, 1H).

**13C-NMR** (CDCl₃): δ 41.1, 43.0, 45.6, 55.7, 58.1, 115.5, 118.6, 123.4, 124.7, 128.8, 129.6, 129.8, 129.9, 130.0, 134.5, 136.2, 145.1, 151.2, 152.3, 155.8.

### 8.1.6. Synthesis of prodrug analog 25

Potassium hydride (88 mg, 2.2 mmol) was suspended in THF (5 ml) and alcohol **78** dissolved in THF (5 ml) was added via cannula. The mixture was stirred for 1h after which methyl iodide (156 μl, 2.05 mmol) was added neatly. After 2h, an additional equivalent of methyl iodide (77 μl) was added and the solution was stirred overnight. 10 ml of an aqueous ammonium chloride
buffer (saturated, pH 8.5) was added and the solution was extracted with methylenechloride. The combined extracts were dried over MgSO₄ and evaporated to afford 160 mg (63%) of crude dimethylated alcohol. The crude product was dissolved in ethyl acetate (10 ml) and catalytic amounts of 10% Pd/C were added. The resulting solution was stirred under hydrogen (1 atm) at r.t. After the hydrogen consumption stopped (8 h) the solution was filtered through celite (under inert atmosphere!) and evaporated to yield 141 mg (100%) of the amine. The air-sensitive amine was immediately dissolved in THF (15 ml) and triethylamine (176 μl, 1.26 mmol), trifluoroacetic anhydride (134 μl, 0.95 mmol), and catalytic amounts of DMAP were added. The solution was stirred at r.t. for 30 min after which it was evaporated and chromatographed on silica gel (gradient of 10% to 50% EtOAc in Hexane) to yield 128 mg (63%) of compound 79 as a white solid.

¹H-NMR (CDCl₃): δ 3.33 (s, 6H), 3.52 (m, 8H), 6.67 (d, J = 9.1 Hz, 2H), 7.34 (d, J = 9.1 Hz, 2H), 7.78 (br, 1H).

¹³C-NMR (CDCl₃): δ 51.2, 59.2, 70.2, 112.1, 122.7, 124.1, 146.6.
8.2. Biological methods

8.2.1. Preparation of carrier protein-hapten conjugates

Hapten-protein conjugation reactions were performed at room temperature with stirring in 5 ml covered flasks. Conjugates were exhaustively dialyzed against 0.2 M potassium phosphate buffer (Kpi, pH 7.2) at 4 °C. Exhaustive dialysis is defined as a minimum of 10⁶-fold dilution of the sample buffer.

Protein assays were performed according to the method of Bradford using the Bradford Assay solution (Bio-Rad) diluted 1/4 (v/v) with H₂O. Samples were diluted with the freshly prepared assay solution at appropriate concentrations and the OD was read at 595 nm. Pure BSA (Bovine Serum Albumin) and KLH (Keyhole Limpocet Hemocyanin) were used for the standard curve. Quantification of the hapten/protein ratios was determined through a comparison of the UV spectra of the protein-hapten conjugate, unconjugated protein and the free hapten.

R₁, R₂, C₁ conjugate

A solution of hapten R₁, R₂, or C₁ (4 mg) in DMF (100 μl) was added carefully to 100 μl of Kpi. This solution was added dropwise to a solution of 2 mg carrier protein (maleimide activated BSA or KLH; PIERCE) in 0.2 ml Kpi while swirling. After gently stirring for 2h, the solution was transferred into a dialysis tube (Spectra/Por 2, molecular cut-off 14,000) and dialyzed twice against 500 ml Kpi.

8.2.2. Immunizations

A solution containing 1 mg of hapten (R₁ or R₂)-KLH conjugate was diluted with PBS to a final volume of 2 ml. After transferring this solution into a prewarmed (37 °C) bottle of RIBI adjuvant (MPM and TDM emulsion), it was vortexed vigorously for 2-3 min to provide an emulsified antigen solution. This emulsion (100 ml) was injected subcutaneously to each side on the back of a Balb/c mouse using a 1 ml syringe with a 25G 5/8 needle. Two weeks (14 days) after the primary injection, a secondary injection was performed according to the above procedure. On day 21 after the first injection, the mouse was bled and the titer of the serum was estimated by Enzyme Linked ImmunoSorbent Assay (ELISA, vide infra). A third intraperitoneally injection into the abdominal cavity was performed about 5 weeks after the first injection using a 25G 5/8 gauge needle injecting 200 μl of above antigen solution. Three to four days after the last injection, the mouse was sacrificed (by CO₂ asphyxiation) and the spleen harvested to prepare hybridoma cells.
8.2.3. Hybridoma generation

All of the cell culture work including the fusion protocol was performed in a sterile laminar flow hood, using sterile instruments after sterilization of gloved hands with ethanol. Cells were incubated in a 37 °C sterile incubator containing 90% humidity and 5% CO₂ (for buffering of carbonate buffer in the cell culture media). Development of yellow color indicates the depletion of nutrients in the cell cultures (generation of acidic pH by metabolites indicated by phenol red in cell culture medium) and these cultures should be replenished immediately with the appropriate fresh medium (pre-warmed to 37 °C). Cells were never be left to stand at room temperature for longer than 15 minutes and sterile cell containers were only be opened to the atmosphere under a sterile laminar flow hood. Cell cultures were monitored daily and those that were observed to be infected were discarded immediately to contain the infection.

8.2.3.1. Fusion

All fusions were performed using the ClonaCell - HY Hybridoma Cloning Kit (StemCell Technologies, Inc.). Some slight modifications of this protocol were made in order to increase the fusion efficiency.

The kit supplied the following materials (a detailed list of ingredients could not be supplied since this information is propriety to StemCell Technologies, Inc.):

Medium A: Prefusion Medium and Hybridoma Expansion Medium (DME medium supplied with fetal bovine serum (FBS) and Gentamycin)
Medium B: Fusion Medium (serum free DME medium supplied with Gentamycin)
Medium C: Hybridoma Recovery Medium (DME medium supplied with FBS, Gentamycin and selected nutrients)
Medium D: Hybridoma Selection Medium (Methylcellulose based semi-solid medium supplied with FBS, Gentamycin, Hypoxanthine, Aminopterine, Thymine and selected nutrients)
Medium E: Hybridoma Growth Medium (DME medium supplied with FBS, Gentamycin, Hypoxanthine, Thymine, and selected nutrients)
Polyethylene Glycol: PEG for cell fusion diluted with DME medium (1/1)

Preparation of Myeloma cells.

A vial of frozen SP2/0 myeloma cells (ATCC) was removed from liquid nitrogen and immediately thawed in a 37 °C water bath. The cells were transferred into a 15 ml centrifuge tube containing 10 ml of prewarmed Medium A. After centrifuging the cells for 5 min (1100 rpm on bench centrifuge) the supernatant was aspirated and discarded. The cell pellet was loosened and
resuspended in 10 ml of Medium A. Again, the cells were centrifuged and the supernatant discarded. The pellet was loosened, suspended in 1 ml of Medium A and transferred into a T-75 flask containing 20 ml of Medium A. The cells should be grown at a cell density between 0.2 and \(1 \times 10^6\) cells/ml to obtain maximum viability (greater than 95%) and should be allowed to adapt to the medium for at least one week.

**Determination of Cell Viability.**

Cell densities were determined by trypan blue staining as follows. To a 20 ml aliquot of a thoroughly suspended cell sample in a small test tube was added 20 ml of trypan blue stain. The resulting suspension was thoroughly mixed. The cells were slowly pipetted into the sample groove of a clean hemacytometer until the observation window was completely covered. The hemacytometer was placed on an inverted phase microscope and the living cells (no blue stain in the interior of the cells) in the four counting grids were counted, divided by four and multiplied by \(10^4\). This number was then multiplied by 2, to account for the trypan blue dilution, to afford the number of cells/ml.

**Fusion Procedure.**

All procedures were performed in a laminar flow sterile hood and sterile technique was used throughout. All media were prewarmed to 37 °C.

1. An immunized mouse was sacrificed and the fur was washed with 95% ethanol. The chest cavity was opened with sterile instruments and the spleen was removed.
2. The spleen was placed in a sterile 50 ml conical tube containing 25 ml of prewarmed (37°C) medium B.
3. The spleen and the media were transferred to a 35 mm tissue culture dish. The spleen was washed by transferring it into a fresh tissue culture dish containing 10 ml of Medium B. Large pieces of fatty tissue were trimmed off. Holes were poked in the spleen with an 25G 5/8 gauge needle and medium was injected to wash out the splenocytes. The spleen was gently teased between two sterile glass slides to provide a spleenocyte suspension (approximately \(1 \times 10^8\) cells).
4. The cells were transferred into a 15 ml sterile centrifuge tube carefully leaving fatty tissue behind, and centrifuged for 5 min at 1000 rpm.
5. The supernatant was discarded and the cells were resuspended in 20 ml of Medium B.
6. The myeloma cells were counted and \(2 \times 10^7\) cells were transferred into centrifuge tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and the cells were resuspended in 20 ml of Medium B.
7. The spleen cells suspension from step 5 were added to the 50 ml centrifuge tube containing the myeloma cells. The cell suspension was centrifuged at 1000 rpm for 10 min. The supernatant was
discarded and the pellet washed twice with 40 ml of Medium B. (Note: It is important to remove all the serum adhering to the myeloma cells. Traces of serum will drastically reduce the fusion efficiency).

8. Excess supernatant above the pellet was removed with a Pasteur pipette. (Complete removal of the supernatant is essential to avoid dilution of PEG). The pellet was broken up by gently tapping the bottom of the tube.

9. 1 ml of PEG solution (prewarmed at 37°C) was added to the pellet using a 1 ml pipette, over a period of 1 min, continually stirring (gently) the cells. Stirring of the cells was continued for a further 1-2 min. Then, 1 ml of Medium B was added to the fusion mixture, with continuous stirring as before, over a period of 1 min. 3 ml of Medium B were added over a period of 3 min, with stirring continued. Then, 10 ml of Medium B were slowly added. The fusion mixture was incubated for 5 min in water bath at 37 °C.

11. 40 ml of Medium A (to stop the fusion) were slowly added. The cells were centrifuged at 1000 rpm for 7 min. The supernatant was discarded and the cells washed in 40 ml of the same medium (1000 rpm, 7 min) to ensure that all the PEG is removed. The supernatant was discarded.

12. The cell pellet was slowly resuspended in 10 ml of Medium C. The cell suspension was transferred to a 250 ml tissue culture flask containing 40 ml of medium C. (total volume = 50 ml). The cell viability was determined to be above 75%. The flask was incubated for 24 h at 37 °C.

12. On the next day Medium D was thawed in a 37 °C water bath and mixed by stirring with a 10 ml pipette. The cells in Medium C were poured into two 50 ml conical centrifuge tubes and centrifuged at 1000 rpm for 10 min. The supernatant from one tube was decanted, the pellet was broken up by tapping and resuspended in 0.5 ml of supernatant from the remaining tube. The supernatant from the remaining tube was discarded, the cells pooled and transferred to the thawed Medium D at 37°C.

13. The cell suspension was gently stirred with a 10 ml pipette and allowed to sit for 30 min in the incubator so the bubbles can rise to the top. 9.5 ml of Medium D suspension was plated out on each of ten 100 mm petri plates. The plates were tilted to level the mixture without introducing bubbles. The plates were incubated at 37 °C in a 5% CO₂ atmosphere.

14. 10-14 days later, the plates were examined for the presence of colonies clearly visible to the naked eye (a typical fusion will produce 1000 or more colonies over the ten plates). The isolated colonies (usually 500-1000 colonies are picked) were removed from the plates using a pipettor and sterile pipette tips (pipettor was set to 10 ml). Each clone was pipetted into an individual well of a 96-well tissue culture plate containing 200 ml of Medium E. The plates were incubated for 1-4 days without feeding. By the fourth day, each well had a high cell density and the medium began to turn yellow.
15. The hybridoma supernatants were tested using the ELISA and "Capture" CatELISA assay *(vide infra).*

16. The selected clones were expanded by gently pipetting the selected wells to resuspend the hybridomas. The cell suspension was transferred to the wells of a 24-well plate containing 1 ml of Medium E. When cells grew to a suitable density, half of them were frozen in liquid nitrogen and the rest was expanded in increasing volumes of Medium E.

17. In order to limit the consumption of expensive Medium E, stable cell lines were slowly weaned into Medium A (or alternatively into DME medium supplied with 10% FBS and 0.1% Gentamycine). This was accomplished by initially feeding the cells with a 1/1 (v/v) mixture of Medium E and Medium A. The ratio of Medium E was slowly decreased until only Medium A could be used. The cell viability was closely monitored to insure the well-being of the cell line.

*Cryogenic Storage of Cell Lines*

Each monoclonal cell line was frozen according to the following protocol. A healthy cell line (viability > 95%) was grown in 10 ml of Medium E in a T-15 tissue culture flask to a final density of 1 x 10^6 cells/ml. The cells were transferred into a 10 ml centrifuge tube, and centrifuged for 5 min at 1100 rpm. The supernatant was removed by aspiration and the pellet was resuspended in 1 ml of pre-warmed freezing solution (8% DMSO, 92% FBS). The resulting suspension was then aliquoted between four freezing vials (250 μl/vial) and placed on ice for 1 hour. The vials were then put in a styrofoam container leaving the outermost rows empty to insure gradual cooling and then were placed in a -78 °C freezer. Cell lines can be transferred to a liquid nitrogen storage container for long-term storage 24h after the initial freezing.

*8.2.3.2. Hybridoma selection*

*Enzyme Linked ImmunoSorbent Assay (ELISA)*

An ELISA assay was performed to identify positive hybridoma clones producing IgG molecules which specifically bind to the hapten. The standard ELISA protocol requires the following buffers:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating Buffer:</td>
<td>0.05 M NaCO₃ (pH 9.0)</td>
</tr>
<tr>
<td>Washing Buffer:</td>
<td>PBS: 0.8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 1.15 g</td>
</tr>
<tr>
<td></td>
<td>Na₂HPO₄ in 1 L dd H₂O</td>
</tr>
<tr>
<td>Blocking Buffer:</td>
<td>PBS containing 2% BSA</td>
</tr>
<tr>
<td>PBS-Tween:</td>
<td>PBS containing tween 20 (1:1000 dilution)</td>
</tr>
</tbody>
</table>
Substrate Solution: 0.1 M Sodium citrate (pH 5.0) containing 1,2-phenylenediamine (4 mg/10 ml) and 30% hydrogen peroxide (1:1000 dilution).

Each well of a 96-well polyvinyl assay plate (ELISA plate) was coated with 100 μl of the appropriate BSA-hapten conjugate (10 μg/ml) in Coating Buffer and incubated at 4 °C overnight or at room temperature for 2 hours. The solutions were discarded by gentle tapping, each well was filled with 200 μl of blocking buffer, and the plates were allowed to stand at room temperature for 1 hour. The plate was rinsed with PBS-Tween followed by three rinses with distilled water (Washing Protocol). The hybridoma supernatant from each well of the 96-well cell culture plates was added to the ELISA plates and incubated for 1 hour at r.t. The supernatant was removed, and the plates were washed according to the washing protocol. Goat anti-mouse IgG-HRP conjugate (PIERCE), diluted in washing buffer (1:5000 dilution), was added to each well (100 μl/well) and the plates were allowed to stand at r.t for 1 hour. The plates were washed again according to the washing protocol. Finally, each well was treated with 100 μl of substrate solution. The yellow color was allowed to develop for approximately 5 minutes, and the reaction was stopped upon addition of 20 μl of 1.0 M H2SO4. Positive clones were identified by measuring the absorbance at 405 nm using a microtiter plate reader or more simply by visual identification through comparison with the negative controls.

Serum Titration

Titration of antibodies in animal serum was carried out as follows. The BSA-hapten coated ELISA plate was prepared as described above, and to each well was added 100 μl of PBS. The serum was diluted 200-fold in PBS, and 100 μl was added to the first well (A1) of the plate. The resulting solution was serially diluted 2-fold from well A1 to well A8 (from 1:400 to 1:51,200 dilution), and the plate was incubated for 1 hour. The ELISA procedure was then applied as described above, and the absorbance at 405 nm was measured. A plot of the log of the serum dilution vs. absorbance provided a sigmoidal curve, and the titer was defined as the serum dilution at which the absorbance became comparable to that of the background.

"Capture" CatELISA

The newly developed "Capture" CatELISA assay was performed to identify positive hybridoma clones producing IgG molecules which specifically catalyze the elimination of substrate C1. Each well of four Hi-affinity protein binding plates (ELISA plate) was coated with 100 μl of the BSA-C1 conjugate (10 μg/ml) in PBS (Note: it is important to use a buffer of neutral pH since a higher pH would decompose compound C1 on the conjugate) and incubated at room temperature.
for 2 hours. After washing, blocking and again washing of the plates (identical to ELISA protocol) the supernatants of hybridomas, which tested positive in the previous ELISA assay, were added to the ELISA plates and incubated for either 1, 2, 8, or 48 h at 37 °C. The plate was washed first, according to the washing protocol, and then twice with 200 μl/well of a 0.1 M glycine-HCl solution (pH 2.7). After washing the plate according to the washing protocol, goat anti-mouse IgG-HRP conjugate (PIERCE), diluted in washing buffer (1:2000 dilution), was added to each well (100 μl/well) and the plates were allowed to stand at r.t for 1 hour. The plates were washed again according to the washing protocol. Finally, each well was treated with 100 μl of substrate solution. The yellow color was allowed to develop for approximately 10 min, and the reaction was stopped upon addition of 20 μl of 1.0 M H₂SO₄. Positive clones were identified by measuring the absorbance at 405 nm using a microtiter plate reader.

8.2.4. Ascites production from monoclonal hybridomas

In order to generate large quantities of monoclonal antibodies, cell lines were propagated in vivo in pristane primed mice. Balb/c mice (retired breeders) were intraperitoneally injected with pristane (0.5 ml per mouse) at least 14 days prior to the injection of hybridoma cell lines. Each monoclonal hybridoma cell line was individually prepared for injection into a mouse by growing the healthy cell line (viability > 95%) in 10 ml of Medium E in a T-15 tissue culture flask to a final density of 1 × 10⁶ cells/ml. A volume containing 1.5 × 10⁶ cells was transferred into a 10 ml conical tube and centrifuged for 5 minutes at 1100 rpm. The supernatant was removed by aspiration, the pellet was resuspended in 0.5 ml of sterile PBS prewarmed to 37 °C, and the suspension was injected intraperitoneally into a pristane primed mouse. Only one monoclonal hybridoma cell line was injected per mouse. Within one to two weeks, abdominal swelling was generally observed in the injected mice. Each mouse was "tapped" for the ascites fluid by draining the swelled abdominal area into a sterile tube using an 18-gauge needle. It is important that only the tip of the needle is inserted and penetration of underlying organs is avoided. Mice were tapped 1-3 times (depending on their health) prior to being sacrificed.

8.2.5. Purification of monoclonal antibodies

Monoclonal antibodies were purified from the ascites fluid according to the following protocol which limited the manipulations required to a minimum to ensure antibody activity. Freshly collected ascites fluid was centrifuged at 3000 rpm for 20 minutes at 4 °C to pellet red blood cells. The supernatant was collected and immediately frozen at -20 °C. After the tapping of each mouse was finished the ascites fluids stemming from the same clone were thawed and pooled. An equal volume of sodium phosphate buffer (20 mmol, pH 7.2) was added and the resulting solution was centrifuged at 3000 rpm for 20 minutes at 4 °C to remove any insoluble material. The
samples were filtered through a 0.22 μm low protein binding syringe filter (Millipore). A 5 ml HiTrap Protein G affinity column (Pharmacia Biotech) connected to a peristaltic pump was equilibrated with five column volumes (25 ml) of sodium phosphate buffer (20 mmol, pH 7.2) at a flow rate of 10 ml/min. The samples were applied onto the column and the column was washed with another six column volumes (30 ml) of the same buffer. The antibody was then eluted with 0.1 M glycine-HCl buffer (pH 2.7) at the same flow rate. 3 ml fractions were collected into 10 ml centrifuge tubes each containing 80 μl of Tris-buffer (1M, pH 9). The neutralization step ensures the activity of acid labile antibodies. The antibodies typically eluded between fraction four and six. Antibody concentrations were determined using the absorbance at 280 nm, an extinction coefficient of 1.35, and a molecular weight of 150,000. The positive fractions were pooled and directly dialyzed into assay buffer.

8.2.6. Cytotoxicity assays
LS174T human colon carcinoma cell line was cultivated in DMEM-medium supplemented with 10% FBS and 1% sodium pyruvate solution (100 mM) and no antibiotics.

Trypsinization and Plating of cells
Cell culture medium was replaced with fresh medium the day before the trypsinization. Medium was removed and the cells were incubated with 5 ml of Trypsin-EDTA solution (SIGMA) for 10 min. The cells were detached from the bottom of the flask by gently tapping the flask and the trypsinization was stopped by adding 9 ml of medium. The cells were centrifuged at 1000 rpm, resuspended in 10 ml of medium, and counted. A solution of $2.5 \times 10^{-6}$ cells in 50 ml was prepared and 100 μl of it was put into each well of four 96 well plates (i.e. 5000 cells per well). The plates were incubated for 24h.

Treatment of cells with drug versus prodrug
Prodrug 72 (0.66 mg, 2 μmol) was dissolved in 100 μl of acetone and diluted with 1.9 ml of cell culture medium to give a final volume of 2 ml. The resulting solution was further diluted with medium 1/10 (v/v) to give a final prodrug concentration of $1 \times 10^{-4}$ M. The supernatants of the first column of a 96 well plate (in which cells have been growing for 24h) was replaced with 200 μl of the prodrug solution. The prodrug solution was then serially diluted 2-fold from well A1 to well H1 (the supernatants from the other wells were not removed!). 100 μl of medium was added to each well to give a final volume of 200 μl in each well. The plate was incubated for exact 1h after which the prodrug containing solution was removed. The cells were washed with medium three times, 200 μl of medium was added to each well, and the plates were incubated for an additional six days.
At the same time parent drug 72 (0.54 mg, 2 μmol) was dissolved in 100 μl of hydrochloric acid (0.01 M) and diluted with 1.9 ml of growth medium. Above protocol was then carried out in an identical fashion with a different 96 well plate.

Staining of the cells

50 μl of cold trichloroacetic acid (50%) was added on top of the culture medium in each well (final concentration of trichloroacetic acid was 10%) and the plate was incubated at 4 °C for 1h. The plates were washed with water five times and allowed to dry. 100 μl of a solution of 0.4 % (w/v) sulforhodamine B (SIGMA) in 1 % (v/v) acetic acid was added and the plate was incubated for 30 min at r.t. The stain was removed, the plate was rinsed with acetic acid (1 %) four times, and the plates were allowed to dry. The bound dye was solubilized by the addition of 100 μl of Tris-buffer (10 mM, pH 10.5) to each well and the plate was shaken on a gyratory shaker for 5 min. The absorbance was measured at 490 nm using a microtiter plate reader and the OD was plotted versus the drug concentration. The IC₅₀ is defined as the drug concentration where the cell growth is 50 % of the growth of a control group of cells grown without drug (or at very low drug concentration).

8.2.7. Assays for catalysis

Assays for catalysis were performed at pH 7.0 (50 mM Tris-HCl, 100 mM NaCl). Antibodies were diluted to 6.25 μM (final volume 160 μl) with assay buffer and a solution of 1,2-dicyanobenzene (1 mM, 20 μl) as Internal Standard Calibrant (ISTD) was added. The mixture was preincubated at 37 °C for 30 min. The reaction was initiated by the addition of 20 μl of a 10-fold concentrated substrate stock solution in DMSO to give a final concentration of 20% DMSO (final antibody concentration was 5 μM). Stock solutions with appropriate dilutions were made at the beginning of the catalytic assays and were used throughout the assay for consistency purposes. The uncatalyzed rate was measured under the same conditions. Inhibiton experiments were carried out with 5 μM of antibody and at three concentrations of hapten (500, 750, and 1000 μM). Reactions were assayed at 37 °C for about 8h (less than 5% substrate depletion).

Initial velocities (ν) were determined by following the reactions at 248 nm (absorption maxima for N-dansyl-1,2 aminoethane 105) in a RAININ UV-D spectrophotometer (an absorption peak area of 1020 was calibrated to correspond to 1 μM of 105 release with a 20 μL sample volume). The reaction components were separated using a Waters Nova-Pak C₈ column (150 mm length × 3.9 mm i.d.) attached to a Rainin HPLC system. 18 % MeCN, 0.1% trifluoroacetic acid in ddH₂O was used as an eluent at 1.8 ml/min flow rate. After 5 min the gradient was increased to 30 % MeCN. The elution times of the observed species are as follows: 105 1.54 min, 1,2 dicyano-benzene 5.23 min, p-chlorophenyl vinyl sulfone 9.3 min, p-chloro
substrate S2 10.53 min. Typically spectra were recorded for 12 minutes. Integration of the peaks were performed using the Rainin Dynamax computer program run on an Apple Macintosh computer.

Rate constants ($k_{cat}$) were obtained by dividing $V_{max}$ by the concentration of antibody. Background rates ($k_{uncat}$) were determined in the absence of antibody under otherwise identical conditions. The Michaelis Menten parameters $k_{cat}$ and $K_m$ were obtained from Eadie-Hofstee plots\textsuperscript{111} of $v_{obs} - v_{uncat}$ (ordinate) versus $v_{obs} - v_{uncat}/[substrate]$ (abscissa), where $V_{max}$ is the y-intercept and $-K_m$ corresponds to the slope of the curve. Inhibition constants ($K_i$) were obtained from Dixon plot\textsuperscript{112} analyses of $1/v_{obs} - v_{uncat}$ (ordinate) versus total inhibitor concentration (abscissa) where $-K_i$ is obtained from the intercept of a vector drawn from $1/V_{max}$ or $1/k_{cat}$. The zero buffer rate ($k_0$) was determined by measuring the background rate at different buffer concentrations (50, 25, 10, and 5 mM) but constant substrate concentration (700 μM). A plot of buffer catalyzed rate (ordinate) versus buffer concentration (abscissa) is drawn and $k_0$ corresponds to the y-intercept. Kinetic plots were determined using KaleidaGraph program (SYNERGY Software) for Apple Macintosh computers.
References


(16) Suga, H.; Ersoy, O., Masamune, S. unpublished results.


(33) We have observed a similar phenomenon in our previous studies, see ref. 17b.

(34) This background rate is in accordance with a previously determined background rate for a similar p-nitroanilide substrate, see ref. 8a.

(35) J. Org. Chem. 1990, 55, 5867 and references cited therein


(45) During the preparation of this thesis a paper appeared advancing a similar idea (Tetrahedron 1998, 54, 4223-4242.) of using one protective group for several prodrugs. However, the authors could not yet show that their system will work and yield a prodrug activating antibody.


(59) The functionalities in the hapten structures were modeled after hapten O1 and O2.


(64) For carbamate hydrolysis see: (a) Vanrankanen, D. L.; Panomitros, D.; Schultz, P. G. Tetrahedron Lett. 1994, 35(23), 3873-3876. (b) see ref. 54. For amide hydrolysis see: ref 8.


(74) Bhongle, N. N.; Notter, R. H.; Turcotte, J. G. Synthetic Communications 1987, 17(9), 1071.


(80) Blanco, M.-J.; Masamune, S. unpublished results.


(83) The $K_1$ of antibody BH4 could get determined due the unavailability hapten R2.

(84) This assumption is in good agreement with earlier kinetic investigations of similar compounds: Crosby, J.; Stirling, C. J. M. J. Chem. Soc. B 1970, 679.

(85) Fleck, R.; Masamune, S. unpublished results.


(89) For general reviews on bispecific antibodies and antibody fragments see: (a) Fanger, M. W.; Morganelli, P. M.; Guyre. P. M.; Crit. Rev. Immunology 1992, 12, 101. (b) Hudson, P. Curr. Opin. Biotechnol. 1998, 9, 395. For diabodies see: ref. 53. For minibodies see: ref. 50.


(92) Muraro, R.; Kuroki, M.; Wunderlich, D. Cancer Res. 1988, 48, 4588-4596


37

[Chemical structure image]

-OTES

-NCO₂
hapten R1
Biographical Note

The author was born in Nürnberg, Germany on November 25, 1966. He received his high school diploma from the Emmy-Noether Gymnasium in Erlangen in 1985. After completing his civil service in 1986, he enrolled at the Friedrich-Alexander Universität in Erlangen to study chemistry. In 1992, he spent nine months in the laboratories of Dr. Arthur Pardee at the Dana-Farber Cancer Institute (Harvard Medical School) where he finished the requirements for the diploma thesis. He graduated in June 1993 with a diploma (master) in chemistry. After a brief period as a visiting scientist in the Masamune laboratories at MIT, the author became a graduate student at MIT in the spring of 1994. He joined the Masamune group, where he began to conduct research on catalytic antibodies. Upon completion of his doctoral work, the author will join the medicinal chemistry section of Boehringer Ingelheim in Danbury, Connecticut.